ANALYTICAL APPLICATION OF FUNCTIONALIZED CdS AND ZnS AS FLUORESCENCE LABEL FOR THE DETERMINATION OF PROTEINS

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Specially dedicated to my beloved Ganesha appa, mum, dad, brother, sister and nada.

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ABSTRACT

The quantitative analysis of protein is essential in biochemistry and clinical medicine. The most sensitive quantitation of protein at this present is generally based on fluorescence enhancement on organic dyes determination. However, this organic fluorophores often suffer from photobleaching and low signal intensity. In order to overcome such problems, the study was carried out to investigate the possibility of employing the luminescent particle functionalized CdS and ZnS for quantitative analysis of protein. CdS have been prepared and capped with mercaptoacetic acid (functionalized CdS) whereas ZnS was capped with cysteine (functionalized ZnS), which renders the particles water soluble and biocompatible. Fluorescence studies showed at excitation wavelength λ_{exc} = 233 nm, the maximum emission wavelength of functionalized CdS was at 350 nm whereas for functionalized ZnS was at 357 nm wavelength. Further, general optimization procedure such as the effect of pH, temperature, concentration, reaction time of the functionalized CdS and functionalized ZnS binding with BSA (Bovine Serum Albumin) was conducted. A positive correlation with R^2 = 0.9899 was obtained between CdS capped with mercaptoacetic acid binding with BSA meanwhile correlation between ZnS capped cysteine and BSA was 0.9805. The interferences of various metal ions and surfactant were subsequently performed in order to obtain the selectivity of the developed assay on the determination of BSA. The effect of surfactant such as ionic detergent sodium dodecyl sulfate (SDS), nonionic detergent Triton X-100 shows signification shift towards a shorter wavelength. Limit of detection for functionalized CdS binding with BSA was 0.14 ppm followed by limit of detection of functionalized ZnS binding with BSA was 0.09 ppm. This developed method was successfully applied to the several types of protein such as egg albumin, lysozyme and amylase. The developed novel assay is simple, inexpensive, rapid and sensitive.

ABSTRAK

Analisis kuantitatif bagi protein adalah salah satu bidang yang sangat penting dalam biokimia dan perubatan. Pada masa kini kaedah analisis kuantitatif bagi protein lazimnya berdasarkan kaedah penentuaan pengikatan secara pendarflor dengan bahan pewarna organik. Walau bagaimanapun kaedah ini didapati mempunyai kelemahan dari segi penyahlunturan warna bahan organik pewarna and kadar keamatan pendarflor yang rendah. Bagi mengatasi masalah ini, kajian mengenai penggunaan partikel berpendarflor seperti CdS dan ZnS telah dijalankan bagi menentukan kandungan protein. Dalam kajian ini, CdS telah diubahsuai dengan menggunakan asid mercaptoasetik manakala ZnS telah diubahsuai dengan asid amino sisteina bagi membolehkan partikel CdS dan ZnS bersifat larut dalam air dan mudah bertindakbalas dengan komponen biologi. Kajian pendarfluor menunjukkan pada puncak penyerapan λ_{ex}= 233 nm, puncak pemancaran telah diperolehi pada λ_{em}=350 nm bagi CdS dan λ_{em}=357 nm bagi ZnS. Kajian selanjutnya seperti pengoptimuman kesan pH, suhu, kepekatan dan masa tindakbalas bagi pengikatan protein dengan CdS dan ZnS telah dilakukan. Graf kalibrasi linear diperolehi bagi penentuan protein dengan CdS adalah R^2=0.9899 dan dengan ZnS adalah R^2=0.9805. Kesan gangguan seperti bahan bukan protein, ion serta surfaktan telah dilakukan bagi menentukan kejituan penentuan kuantitatif BSA. Kajian kesan surfaktan menunjukkan surfaktan ionik iaitu natrium dodesil sulfat (SDS) dan surfaktan bukan ionik iaitu Triton X-100 menunjukkan puncak pemancaran beranjak ke panjang gelombang yang lebih rendah. Had pengesanan bagi penentuan protein dengan CdS adalah 0.14 ppm manakala bagi ZnS adalah 0.09 ppm. Kaedah yang dibangunkan telah diaplikasikan bagi penentuaan pelbagai protein seperti albumin telur, lisozim dan amilase. Daripada hasil kajian yang telah dilakukan didapati bahawa kaedah penentuan protein yang dibangunkan adalah mudah, murah, pantas and sensitif berbanding dengan kaedah lain.

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BRB	-	Britton-Robinson buffer
BSA	-	Bovine Serum Albumin
CdS	-	Cadmium Sulfide
HSA	-	Human Serum Albumin
EDTA	-	ethylenediaminetetraacetic
INT	-	Intensity
LOD	-	Limit of detection
nm	-	nanometer
ppm	-	parts per million
RSD	-	Relative standard deviation
SDS	-	sodium dodecyl sulfate
UV-Vis	-	Ultraviolet visible
ZnS	-	Zinc Sulfide
σ	-	Standard deviation
λ_{exc}	-	Excitation wavelength
λ_{em}	-	Emission wavelength

CHAPTER 1

INTRODUCTION

1.1 Prelude

Proteins have long been recognized as biologically fundamental and medically important substance. International and industrial competition provided a healthy impetus to basic research and produced an explosive growth in fundamental understanding about protein molecules, which constituted the ‘stuff of life’ [1].

Proteins contributed to key body function, including blood clotting, fluid balance, production of hormones and enzymes, vision and cell growth and repairs. Day by day regulation and maintenance of the body requires thousands of different proteins, which is a much greater variety than carbohydrates and lipids in the body. Many of these proteins are very large and their protein molecular weights can exceed one million. In contrast, glucose has a molecular weight of only 180. Thus, from a biological standpoint, proteins deserved their name, from the Greek word *proteios*, means, “To come first” [2].

1.2 General Introduction to Proteins

The name of protein (Greek*, proteios*) was first used in 1838 by Mulder following a suggestion by Berzelius [3]. Mulder was among the first proposed a systematic study of the elemental composition of proteins. Most proteins were found to contain 50 to 55% carbon, 6 to 7% hydrogen, 20 to 23% oxygen and 12 to 19% nitrogen. Protein determinations were based on nitrogen (assuming an average content of 16%) came to be use for analysis of tissues and food samples. Sulfur (0.2 - 0.3%) was found to occur in proteins and phosphorus in some cases (as high as 3%). Trace elements identified in certain proteins (e.g. 0.34% iron in hemoglobin) permitted calculation of minimum molecular weights. These results give the first indication that proteins have large molecular weight compared to other organic substances known at that time [3].

During the late 1800s, amino acids were known as the basic building units of proteins. There are 20 amino acids occurred as components of most proteins. In 1902, Fischer proposed that proteins are long chains of amino acids joined together by amide bonds between the α-carboxyl group of the one amino acid and the α-amino group of another [3]. The water was eliminated between the α-carboxyl group of one amino acid and the amino group of another to produce an amide linkage [3, 4]. Proteins also can be identified as natural polymer of L-α-amino. Figure 1.1 shows the general structure of the α-amino acids.

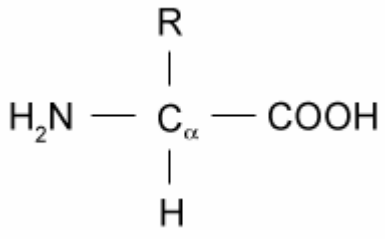

Figure 1.1: General structure of α-amino acids

1.2.1 Classification of Proteins

Proteins are classified in two additional ways as shape and composition. Based on their shape, proteins can be classified into two major groups that are fibrous protein and globular protein [1]. Fibrous proteins are insoluble and characteristically play a structural role in animals. Typical member of fibrous group are fibroin of silk, keratin of hair, nails and feather and elastin of elastic connective tissue. The proteins, which resemble the white of an egg closely, such as proteins of milk and blood plasma, are classified as globular proteins. This globular protein have approximately spherical dimension, which is in contrast with fibrous proteins. Globular proteins are characteristically soluble in aqueous solution [1].

On basis of composition, proteins are classified as simple proteins, conjugated proteins and derived proteins [2].

1.2.1.1 Simple Proteins

Simple proteins yield only amino acids on hydrolysis. Albumin, globulin, glutelins and protamines are classified as simple protein. Simple proteins usually are proteins of relatively low molecular weight.

1.2.1.2 Conjugated Proteins

Conjugated proteins contain an amino acid part combined with a non-protein material such as lipids, nucleic acids and carbohydrates. Some of the major conjugated proteins are phosphoproteins, lipoproteins, glycoproteins and chromoproteins.

1.2.1.3 Derived Proteins

Derived protein is protein that has been changed by physical agents such as heat and high hydrogen-ion concentration and by action of enzymes or chemical reagents. This protein can be divided into two groups by the amount of hydrolysis that can take place. These groups are classified as primary and secondary derivatives.

1.2.2 Proteins Structure

Proteins are extraordinarily complex molecules. There are four levels of the structural organization of proteins that have been distinguished, which are primary structure, secondary structure, tertiary structure and quaternary structure [6, 3]. The primary structure of proteins was related to the peptide bonds between the amino acids component and to the amino acid sequence in the molecule. Proteins have been defined as the linear sequence of amino acid residues making up the polypeptide chains of the molecule [3].

A polypeptide chain may involve in hydrogen bonding between amide nitrogen and carbonyl oxygen. These bonds may be formed between different areas of the same polypeptide chain or between adjacent chains. Based on this bond, secondary structure of proteins was established. The structure may be in two types helical structure and sheet structure. The helical structures are stabilized by intramolecular hydrogen bonds and the sheet structures by intermolecular hydrogen bonds [6, 7].

The tertiary structure of proteins was established when the chains are folded over into compact structures stabilized by hydrogen bonds, disulfide bridges and van der Waals forces. Further, proteins contained more than one polypeptide chain exhibit as an additional level of structural organization. Quaternary structure referred to the spatial arrangement of subunit and the nature of their contacts [8].

1.3 Bovine Serum Albumin

Serum albumin is one of the most widely studied proteins and is the most abundant protein in plasma with a typical concentration of 5 g/100 mL. Albumin generally regarded to mean serum albumin or plasma albumin. Albumins are the most abundant protein in the circulatory system and contribute 80 % to colloid osmotic blood pressure [9]. Albumins have been used as a model protein for diverse biophysical, biochemical and physicochemical studies.

Bovine serum albumin (BSA) is essentially a kind of plasma protein extracted from bovine (buffalo blood). BSA built by a single chain of 528 amino acid globular non-glycoprotein cross-linked with 17 cysteine residues (8 disulfide bonds and 1 free thiols) [10]. Table 1 shows amino acid composition of BSA [12, 13]. Albumins characterized by a low content of tryptophan and methionine and a high content of cysteine and the charged amino acids, aspartic and glutamic acids, lysine and arginine. The glycine and isoleucine content of BSA are lower than in the average protein [11]. (Table 1)

It has now been determined that serum albumin is chiefly responsible for the maintenance of blood pH [14]. Moreover, BSA has a wide range of physiological functions involving the binding, transport and delivery of fatty acids, porphyrins, tryptophan, tyrosine and steroids [10].

Table 1.1: Amino acid composition of BSA [12, 13]

Ala 48	Cys 35	Asp 41	Glu 58
Phe 30	Gly 17	His 16	Ile 15
Lys 60	Leu 65	Met 5	Asn 14
Pro 28	Gln 21	Arg 26	Ser 32
Thr 34	Val 38	Trp 3	Tyr 21

1.4 Functions of Proteins

Proteins play crucial roles in virtually all-biological process. Their significance and the remarkable scopes of their activity are exemplified in the following functions [1, 2, 8].

1.4.1 Enzymatic Catalysis

Most of the chemical reactions in biological systems are catalyzed by specific macromolecules called enzymes. Enzymes exhibited enormous catalytic power and usually reaction rates increased by at least a million fold. The striking fact is that nearly all known enzymes are proteins.

1.4.2 Transport and Storage

Specific proteins transport most of the small molecules and ions. For example, hemoglobin transports oxygen in erythrocytes, whereas myoglobin a related protein, transports oxygen in muscle. Iron is carried in the plasma of blood and transferred into the liver and stored as a complex with ferritin.

1.4.3 Immune Protection

Antibodies are highly specific proteins that recognize and combine with foreign substances such as viruses, bacteria and cells from other organisms. Without sufficient dietary protein, the immune system lacks the cells and other tools needed to function properly.

1.4.4 Generation and Transmission of Nerve Impulses

Receptor proteins mediated the response of nerve cells to specific stimuli. For example, rhodopsin is the light-sensitive protein in retinal rod cells. Receptor proteins triggered by specific small molecules, such as acetylcholine is responsible for transmitting nerve impulses at synapses (junctions between nerve cells).

1.4.5 Control of Growth and Differentiation

Control sequential expression of genetic information is essential for the orderly growth and differentiation of cells. In bacteria, repressor proteins are important control elements that silence specific segments of the DNA of the cell. In higher organism, growth factor proteins control growth and differentiation. Besides that, the activities of different cells in multicellular organisms are coordinated by hormone. Many of the hormones, such as insulin and thyroid stimulating hormone are proteins. Indeed, proteins served in all cells as sensors that control the flow of energy and matter.

1.4.6 Contribution to Acid Base Balance

The concentration of hydrogen ions in the bloodstream determines the acid-base balance (pH) of the blood. Proteins help to regulate the amount of free hydrogen ions by readily accepting or donating hydrogen ions. This regulation helps to keep the blood pH fairly constant and slightly alkaline (pH 7.35 to 7.45).

1.4.7 Providing Energy

Proteins supplies about 2% to 5% of the energy the body uses. Most cells use primarily carbohydrates and fats for energy. Proteins and carbohydrates contain the same amount of usable energy about 4 kcal/g. However, proteins are a very inefficient source of energy, due to the need for the metabolism and processing by liver and kidneys, prior to the utilization of this energy source.

1.5 The Basic Proteins Quantification Protocols

Numerous methods for determining the amount of proteins present in a sample have been devised over the years. The selection of analytical technique must depend on the definition of protein. Moreover, the criteria for choice of a protein assays are usually based on convenience, availability of protein for assays, presence or absence of interfering agents and accuracy. Several indirect ways to measure protein concentrations spectrophotometrically have been developed [15]. This method depended on:

i. Amount of protein
ii. Concentration of protein
iii. Presence of compound which may interfere with assay
iv. Specificity of assay
v. Ease and reliability, accuracy and precision of performing the assay

1.5.1 Colorimetric Method

Colorimetric assays for proteins are based on certain metal ions and dyes binding to protein in a specific mass ratio and upon binding become intensely colored. These reagents were reacted with protein samples to obtain an absorption band, which the intensity is linearly proportional to the protein concentration of a

solution in mass/volume units. The three most commonly used of these methods are: the Biuret assay, the Lowry assay and Bradford assay [16].

1.5.1.1 Biuret Method

The Biuret reaction is the most specific of the protein methods. The assay was developed following the observation that Biuret $NH_2CONHCONH_2$ reacts with an alkaline solution of copper sulphate to give a purple coloured complex [17, 18]. The intensity of the charge-transfer absorption band resulting from the copper-protein complex is linearly proportional to the mass of proteins present in solution. This method is a relatively insensitive method (detection limit ~ 0.5 to 80 mg/mL), but provides a fast and simple means of obtaining estimates of proteins concentration over a rather large range of concentration.

1.5.1.2 Lowry Method

Lowry method [19] is a widely used quantitative assay for determining proteins content in a solution. The method based on both Lowry and Biuret reactions, where the peptide bonds of proteins react with copper under alkaline conditions producing Cu^+, which reacts with the Folin reagent. Folin-Ciocalteau reaction is based on phosphomolybdotungstate reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids [16]. Besides that, the reactions resulted in a strong blue colour, due to presence of tyrosine and tryptophan in proteins. Most interfering substances removed by precipitating the proteins from solution prior to running the assay. The detection range of this method is 1 to 300 µg/mL. Advantages of this method are reliable method for proteins quantitation and little variation among different proteins. The disadvantages of this method are many interfering substances, slow reaction rate, instability of certain reagents and proteins irreversibly denatured.

1.5.1.3 Bradford Method

An assay originally described by Bradford [20] in 1976 has become the preferred method for quantifying proteins in many laboratories. This technique is simpler, faster and more sensitive than the Lowry method. This assay relies on the binding of the dye Coomassie Brilliant Blue G250 to proteins [20]. Under the pH conditions of the assay, the dye is present in its cationic form and does not absorb strongly at 595 nm. When the dye binds to a protein, there is a stabilization of the doubly protonated anionic form of the dye, which absorb in the 595 nm region [21]. The dye appeared to bind most readily to arginyl residues of proteins (but does not bind to the free amino acids) [21]. This specificity can lead to variation in the response of the assay to different proteins.

The technique of this assay is simple, faster (carried out in a single step) and more sensitive than the Lowry method. The method is capable of detecting as little as 0.5-50 μg/mL of proteins and the range of sensitivity is 20-200 μg/mL. One of the major advantages of this assay is insensitive to interferences from reagents that are commonly found in proteins solutions. Disadvantages of this method are the proteins used for this assays are irreversibly denatured [22].

1.5.2 Bicinhoninic Acid (BCA) Method

The bicinchoninic acid (BCA) assay was first described by Smith *et.al*. [23]. This developed method was a modification of the Lowry method. This method also depends on the conversion of Cu^{2+} to Cu^{+} under alkaline condition and the Cu^{+} detected by reaction with BCA. The two assays are similarly sensitive, but since BCA is stable under alkali conditions, this assay has the advantage that it can be carried out as a one-step process compared to the two steps needed in the Lowry assay. The reaction resulted in the development of an intense purple color with an absorbance maximum at 526 nm. Since the production of Cu^{+} in this assay is a function of proteins concentration and incubation time, the protein content of unknown samples can be determined by comparison with known protein standards.

The advantages of this assay are fewer interfering substances compared to Lowry assay and it is not affected by range of detergents and denaturing agents. A further advantage of this method is that it can be carried out as one-step process compared to the two steps needed in the Lowry method. However, the disadvantages of this method are some interferes with different proteins; colour is unstable with time and colour variations with different proteins. Besides, the presence of sulfhydryl agents [24] and lipids gives excessively high absorbance [25, 26], which interfere with the assay.

1.5.3 Kjeldahl Method

Johann Kjeldahl developed this Kjeldahl original method in 1883 [3]. The method has approval from the AOAC (Association of Official Analytical Chemists), formerly the Association of Official Agricultural Chemist [27]. The Kjeldahl methods were based on titration of ammonium ion produced by digestion of the protein with concentrated sulfuric acid [3]. This method is the legal basis for determining proteins (like N, $(NH_4)_2SO_4$, NH_4^+), which measure the total nitrogen, and converting this value to a protein equivalent. For proteins of unknown amino acid composition, quantitation was based on approximate 16% of nitrogen content in protein. The typical range for nitrogen content is about 12 to 18%, although a few types of proteins fall well outside this range (e.g. protamine has 30% nitrogen) [3].

The reagents for analysis need to be N-free so that there is no interference substances present. This procedure is not applicable to material containing N-N or N-O linkages. Interference of nucleic acid and other N-containing organic compounds can be removed by trichloroacetic acid (TCA) precipitation of proteins [28]. The advantages of this method are inexpensive and accurate. However, the disadvantages of this method are waste of time (normally takes 2 hours for digestion) and corrosive reagents [3].

1.5.4 UV Absorbance Method

The ultraviolet absorption method uses the UV spectrophotometer to measure the quantity of protein present. Spectroscopic investigations were initially pursued to take advantages of the absorption of peptide bonds in the far-UV portion of the spectrum (190-235 nm) [29] and aromatic amino acids in the near-UV (250-350 nm) [30]. The presence of proteins in a sample can be determined by measuring the amount of light absorbed at 280 nm (Abs_{280}). However, most proteins absorb in the UV region of 250-350 nm with maximum absorption at 280 nm. In this method, the absorption at UV range 280 nm wavelengths occur when the proteins have aromatic side chains such as tryptophan (Typ), tyrosine (Try) and phenylalanine (Phe). The range of sensitivity of this range is between 0.2 -2 mg/mL proteins [31]. Besides that, the peptide bonds of proteins absorb the far UV with maximum absorption at 205-235 nm. This absorption method is more sensitive compare to absorbance at 280 nm since there are many peptide bonds in proteins and the bonds are essentially constant for all proteins. At this UV range, a concentration of 0.01-0.05 mg/mL proteins can be determined.

The advantages of this method are this method is time saving because of the simple procedure and the sample is recoverable. It is also useful for estimation of proteins before using a more accurate method. Besides that, this method does not destroy the sample. However, this method also has some disadvantages including the interference from other chromophores such as detergent, nucleic acids and lipid droplets.

1.6 Recent Advances in Protein Analysis

The development of novel assays for proteins is a basic requisite in both clinical and laboratory tests. Therefore, a great number of assays have continuously been proposed in recent years such as based on spectrophotometric [32, 33], nuclear magnetic resonance (NMR) [34, 35], resonances capillary electrophoresis [36], fourier-transform infrared (FT-IR) [37] and Rayleigh light-scattering (RLS) [38].

Recent research in proteins determination had focus on spectrophotometric method. Zhong *et al.* [32] described the spectrophotometric determination of protein based on the binding interaction of protein, molybdenum(VI) and dibromohydroxyphenylfluorone (DBHPF). In this study, the DBHPF–Mo(VI) complex was applied as a new spectroscopic probe for proteins and a novel method for the determination of proteins were developed in the presence of Triton X-100. The assay was characterized with high sensitivity, long stability, good selectivity and simplicity. Besides, the binding of 2-hydroxy-3-nitro-9-fluorenone (HNF) (a new reagent with absorption antitumour activity) to human serum albumin (HSA) was investigated by Hong *et al*. [33]. This study was carried out by fluorescence spectroscopy combined with UV-Vis, circular dichroism (CD) and Fourier transform infrared (FT-IR) spectrophotometric techniques under simulative physiological conditions. Accordingly, the experimental results observed in this work indicated that the binding of HNF to HSA led to the conformational change of HSA.

Besides, nuclear magnetic resonance (NMR) spectroscopy is a powerful technique for the study of the structure, dynamics and folding of proteins in solution. Cui *et al.* [34] demonstrated that nuclear magnetic resonance (NMR) spectroscopy could be used as an alternative approach to study the competitive binding of two ligands ibuprofen (IBP) and salicylic acid (SAL) to protein (HSA) at the low-affinity binding sites. In this study, they utilized an excess of ligands, IBP and SAL, over the albumin in order to saturate the high-affinity binding sites and to ensure that the competitive binding of IBP and SAL to HSA. Here, the competitive binding was analyzed quantitatively using NMR based ^{1}H spin-lattice relaxation (R_1) measurements, NMR methods that can be use to study molten globule states of proteins have been described by Redfield [35]. This study has been illustrated using α-lactalbumin and apomyoglobin, which is two of the most widely studied molten globules. In this study, it was reported that the specific method used to study the molten globule state of a particular protein is determined by the quality of the NMR spectrum obtained. Accordingly, these NMR methods have provided detailed information about the specific residues involved in secondary structure within the molten globule.

Moreover, capillary electrophoresis is a powerful separation tool for proteins. Lee *et al*. [36] demonstrated a method for the analysis of picomolar concentration proteins using electrophoretically mediated microanalysis (EMMA) to label proteins on-column with a fluorogenic reagent. This labeling method was followed by capillary zone electrophoresis separation and post column detection based on laser-induced fluorescence. Accordingly, this method provides separation efficiency of 300 000 theoretical plates and compared to UV absorbance detection; the EMMA method provides 7 000 000-fold improvement in detection limit.

Fourier transform infrared (FT-IR) is a powerful technique for the study of hydrogen bonding and very useful for the structural characterization of proteins. The most important advantage of FT-IR spectroscopy for biological studies is that spectra of almost any biological system can be obtained in a wide variety of environments. There have been many studies carried out to investigate the interaction of proteins by FT-IR technique. Neault *et al.* [37] reported the FT-IR spectroscopic characterization on the interaction of *cis*-diamminedichloroplatinum (II) (cisplatin), an anticancer drug with human serum albumin. In this study, spectroscopic evidence regarding the drug-binding mode, drug binding constant and the protein secondary structure was provided.

In recent years, a new sensitive technique concerning resonance Rayleigh light-scattering (RRLS) technique has become a new attractive method for the determination of trace biomolecules such as protein. Based on that, Gang *et al.* [38] demonstrated the determination of proteins based on the interaction with carboxyarsenazo (CAA) by Rayleigh light scattering (RLS). It was found that the weak RLS of CAA enhanced greatly by the addition of proteins. Thus, the CAA assay based on RLS method is useful for routine analytical purposes. However, this technique traditionally regarded as suffering from the disadvantages of low signal levels and lack selectivity [38].

1.7 Fluorescence Method in Proteins Analysis

Sir G.G. Stokes described the phenomena of the fluorescence (absorption and emission) process in 1852. Sir G.G.Stokes suggested the name fluorescence is from the mineral fluorspar (lat *fluo*, to flow and *spar*, a rock), which produces a blue white fluorescence [39, 40]. Fluorescence occurs when a molecule absorbs photons from the UV-visible light spectrum (200-900 nm) and causing transition to a high-energy electronic state and then emits photons as it returns to its initial state, in less than 10^{-9} sec [39]. In fluorescence studies, one is generally concerned with two types of spectrum, the excitation spectrum and emission spectrum. The excitation spectrum is determined by measuring the emission intensity at a fixed wavelength while varying the excitation wavelength. The emission spectrum is determined by measuring the variation in emission intensity wavelength for the fixed excitation wavelength. The difference between the excitation and emission wavelengths is called as Stoke's shift.

Fluorescence has proven to be a versatile tool for a myriad of applications. It was a powerful technique for investigation of molecular interactions in analytical chemistry, biochemistry, cell biology, physiology, nephrology, cardiology, photochemistry and environmental science. In the last twenty years, fluorescence spectroscopy has evolved into a powerful tool for the studies of chemical, semiconductor, photochemical and biochemical species [40].

Fluorescence spectroscopy has become widely accepted as a modern method for the study of proteins structure, proteins biosynthesis and other biochemical problems. The characteristics of proteins fluorescence are dependent on structure of protein. The fluorescence of peptides is caused by the presence of the amino acids tyrosine, tryptophan and phenylalanine [41]. However, the fluorescence of phenylalanine only observed in the absences of tyrosine and tryptophan due the small quantum yield. Although, tyrosine is a weaker emitter than tryptophan, but it still contribute significantly to protein fluorescence because it is usually present in larger numbers. The fluorescence of tyrosine can be easily quenched by nearby tryptophan residues due to the energy transfer effect. Hence, generally only fluorescence of tryptophan was detected although in proteins where both tyrosine and tryptophan are present [41, 42]. Some proteins may also contain a fluorescent coenzyme such as

reduced nicotinamide-adenine dinucleotide, flavin-adenine dinucleotide or pyridoxal phosphate. Proteins in complex with other fluorescent molecules can be term as an extrinsic fluorescence [42].

Various studies of determining protein have previously been reported in the literature [43, 44, 45]. Cui *et al.* [43] demonstrated the interactions between 1-benzoyl-4-*p*-chlorphenyl thiosemicarbazide (BCPT) and bovine serum albumin (BSA) or human serum albumin (HSA) by fluorescence spectroscopy. Accordingly, the analysis of fluorescence spectrum and fluorescence intensity showed that BCPT has a strong ability to quench the intrinsic fluorescence of both bovine serum albumin and human serum albumin through a static quenching procedure.

Wiberg *et al.* [44] have developed a method for the simultaneous determination of albumin and immunoglobulin G (IgG1) with fluorescence spectroscopy. In this study, few parameters such as excitation and emission slit, detection voltage and scan rate were investigated. Accordingly, the proposed method is fast and required a minimum of sample pre-treatment and further development of the method might make the possibility to determine albumin and IgG directly in human serum. Besides, the study of the interactions of BSA and HSA with anionic, cationic and zwitterionic surfactant monitored by fluorescence spectroscopy was demonstrated by Gelamo *et al.* [45]. The result showed that the fluorescence of the interaction of BSA and the surfactant was quenched and the interaction of surfactants with HSA enhanced the fluorescence, which was an opposite effect compared to BSA.

1.7.1 Luminescent Organic Compounds Labeling Proteins

Labeling of biological molecules using fluorescent compounds is a common and very useful practice in biological science and biomedical science. The method of labeling proteins was introduced by Coon in 1941 when they demonstrated the use of antibody labeling with fluorescein [42]. This research tool has been proven useful in

immuno-chemistry, virology, bacteriology, parasitology, rickettsiology and mycology.

Fluorescent of small molecules such as organic dyes are used in both single and multiplex detection approaches. The changes in the fluorescence properties of dyes adsorbed or covalently attached to macromolecules may change the signal in the microenvironment of the dyes due to configurational changes in the larger molecule [40]. Reviews on the application of fluorescent compound covalently bounded with protein have been reported by several studies. Jiang *et al.* [46] reported the studies of the binding reaction and the effect of the energy transfer between terazosin and bovine serum albumin (BSA) by spectrofluorimetry. In this study, it was reported that the compound formed between terazosin and BSA quenched the fluorescence of BSA and the synchronous fluorescence technique was successfully applied to determine terazosin in blood serum and urine samples under physiological conditions.

Besides, a new method based on fluorescence quenching, employing a red region fluorescent dye, tetra-substituted sulphonated aluminium phthalocyanine (AlS_4Pc) for the determination of albumin and globulin without separation was presented by Li *et al.* [47]. It was reported that, this experimental method was easy and time saving method and a red-region fluorescent dye with large Stoke's shift was employed to minimize the background signal of samples. Moreover, the fluorescent dye was easily synthesized and chemically stable which makes this method suitable for practical application.

Jiang and Li [48] have demonstrated the approach of a new fluorescence method for the determination of human serum albumin (HSA) by using doxycycline (DC)-europium (Eu^{3+}) as fluorescence probe. Accordingly, HSA remarkably enhanced the fluorescence intensity of the DC-Eu^{3+} complex and the enhanced fluorescence intensity of DC-Eu^{3+} is proportional to the concentration of HSA. Ercelen *et al*. [49] reported on highly specific and stochiometric binding of novel fluorescence probe FA, 2-(6-diethlaminobenzo[b]furan-2-yl)-3-hydroxychromone to bovine serum albumin (BSA).

Chun *et al.* [50] demonstrated Erythrosin B (EB) binding to proteins, which caused a decrease in the fluorescence maximum of EB. In this study, fast and simple fluorescence quenching method for the determination of proteins was developed. Accordingly, the method has advantages in aspect of high sensitivity, short reaction time, good reproducibility, stable fluorescence and very little interference developed simple, rapid and sensitive fluorescence method for determining microamount albumin. The same group demonstrated a simple, rapid and sensitive fluorescence method for determining microamount albumin [51]. According to the report, the fluorescence of 2, 5-di (orthoamino phenyl)-1, 3, 4-oxadiazole is enhanced greatly by the addition of albumin and little interferences on the assay were determined.

Besides, a new form of highly luminescent and photostable particles was generated by doping the fluorescent dye tris-(2′2-bipyridyl) dichlororuthenium(II) hexahydrate (Rubpy) inside silica material was successfully employed in various fluorescence labeling by Wei *et al.* [52]. This study demonstrated the potential to apply these newly developed fluorescent particles in various bio-detection systems. Perez-Ruiz *et al*. [53] demonstrated similar fluorescence quenching method based on binding of the dye. The study involved the quantitative analysis of protein based on the lower fluorescence of Rose Bengal dye binding to the proteins. This approach was satisfactorily applied to the determination of total proteins in different serum samples.

Shobini *et al.* [54] have published a comprehensive review of the interactions of 7-aminocoumarins with human serum albumin (HSA) by using fluorescence spectroscopic technique and modeling studies. In this study, a large change in fluorescence spectral parameters such as intensity, emission maxima and anisotropy for aminocoumarins was observed.

Although most of the reported protein assays are based on the binding of the dyes or organic compounds with protein has been proven the most sensitive assay and very useful practice in biological science and biomedical science, however this organic fluorophores have characteristics that limit their effectiveness for such applications. These limitations included narrow excitation bands and broad emission bands with red spectral tails, which can cause simultaneous evaluation of several

light-emitting probes problematic due to spectral overlap [55]. Moreover, the organic fluorophores often suffer from photobleaching, low signal intensities and random on/off light emission (blinking) [56]. Photobleaching were caused by sudden decomposition of the emitter, which the main factor limiting the maximum number of photons obtained from a fluorophores. Besides that, low signal intensities reduces the accuracy of the trace protein can be determined. Intermittent light emission causes problems in real-time studies of biomolecular dynamics such as protein folding, signal transduction and enzymatic catalysis [56].

Due to this, findings strongly suggested that colloidal semiconductor have the potential to overcome problems encountered by organic fluorophores in certain fluorescent labeling application by combining the advantages of high photobleaching threshold, good chemical stability and readily tunable spectral properties. Besides that, their resistance to photobleaching and high quantum yield in aqueous solution make them attractive for labeling functionalized biomolecules for fluorescent applications [55, 56, 57].

1.7.2 Luminescent Semiconductor Labeling Biomolecules.

Semiconductor particles are often composed of atoms from groups II-VI or III-V elements in the periodic table. Extensive research in the past 20 years has focused on the photophysics of nanostructures and their applications in microelectronics and optoelectronics [58, 59]. However, recent developments indicate that the first practical applications of semiconductor are occurring in biology and medicine [30, 31]. Semiconductor particles are considerable current interest, not only because of their unique size-dependent properties but also because of their dimensional similarities with biological macromolecules such as nucleic acids and proteins [60].

Recent advances in materials research have produced a new class of fluorescent labels by conjugating semiconductor with biorecognition molecules. These fluorophores will have key applications in biotechnology and bioengineering.

This review focuses on the biological applications of semi-conductor colloidal particles. Mattoussi *et al*. [60] described a novel and direct method for conjugating protein molecules to luminescent CdSe-ZnS core-shell for use as bioactive fluorescent probes in sensing, imaging, immunoassay and other diagnostics applications. In this study, the design and preparation of a semiconductor and protein conjugate based on E. coli Maltose binding protein was introduced and followed by functional characterization using luminescence method. Accordingly, the preparation of protein modified semiconductor dispersions with high quantum yield, little or no particle aggregation and retention of biological activity was achieved based on fluorescence method.

Gerion *et al.* [61] described the synthesis of water-soluble semiconductor particles, discussed and characterized their properties. The study involved the hydrophobic CdSe and ZnS were embedded in a siloxane shell and functionalized with thiols and amine groups. Accordingly, the introduction of functionalized groups into the siloxane surface would permit the conjugation of the semiconductor to biological entities**.**

Parak *et al.* [62] demonstrated a water-soluble and highly fluorescent silanized semiconductor nanocrystals were covalently attached to biological macromolecules with a variety of mild coupling chemistries. In this study, siloxane shells derivatized with thiols, amino and carboxyl functional groups was coupled to single or double stranded DNA. The author made a conclusion that, by using the strategies developed in this study, most biomolecules can be covalently coupled to semiconductor nanocrystals. Further, these nanocrystal-DNA conjugates promise to be a versatile tool for fluorescence imaging and probing of biological systems.

1.7.2.1 Cadmium Sulfide Labeling Biomolecules

Cadmium Sulfide (CdS) is an important semiconductor owing to its unique electronic, optical properties and its potential application in solar energy conversion, non-linear optical, photo electrochemical cells and heterogeneous photo catalysis

[63]. Cadmium sulfide occurs in nature as the mineral greenoktite. The physical properties are yellow to orange crystal. CdS occurs as two polymorphs, hexagonal alpha form and cubic beta form. It exhibits stable wurtzite structure at lower temperature and zinc blended type structure at the higher temperature [64]. CdS compound was widely used in pigments for paints, baking enamels is ceramics plastics. Other applications of this compound are in photovoltaic solar and cells (for converting solar energy to electrical energy), photoconductors (in xerography), thin film transistor and diodes, rectifiers, scintillation counters pyrotechnics and smoke detectors [64].

The synthesis of CdS has been tried by various methods such as the direct reaction of metals with sulfur powder under high temperature, the thermal decomposition of molecular precursors containing M-S bonds and chemical precipitation method involving the precipitation of metal ion with Na_2S as the source of S^{2-} ions [64]. The type of caping agents is a great importance in synthesis CdS since it affects the chemical as well as the physical properties of the semiconductor in term of stability and solubility [65, 66]. Most commonly used stabilizers are pyridine, alkyl amines and various thiols [66, 67, 68].

Recent research by several groups has linked colloidal CdS particles to bio-molecules. Mahtab *et al*. [68] have examined the adsorption of different DNA sequences to mercaptoethanol-capped CdS quantum dots as assay for nonspecific protein DNA interactions. In this study, the binding constant for mercaptoethanol-capped CdS with protein DNA interactions was established. Besides, the adsorption of calf thymus DNA to particles of Cd(II)-rich CdS has been examined by photoluminescence spectroscopy as a function of temperature. Moreover, Mathab *et al.* [69] also performed the adsorption of calf thymus DNA to Cd(II)-rich CdS particles by photoluminescence spectroscopy as a function of temperature. This study suggested that the driving force for adsorption is entropy, and the enthalpy contribution to DNA-surface binding is slightly unfavorable. Similar results also reported by Lakowicz *et al.* [70] who studied about time–resolved fluorescence spectra of cadmium-enriched particles (CdS-Cd^{2+}) and the effect of DNA oligomer binding.

Wang *et al.* [71] demonstrated a study of a novel composite particle prepared by an in situ polymerization method and applied as a protein fluorescence probe. In this study, the CdS has been prepared by polymerization with acrylic acid (AA) and the surface of the composite CdS particles was covered with abundant carboxylic groups (–COOH) to link proteins. Li and Du [72] have studied the synthesis CMCH (carboxymethly chitosan)-capped CdS particles. According to the report, this polysaccharide (carboxymethly chitosan)-capped CdS would have more affinity with other biomolecules such as protein and DNA molecules, thus making it a promising fluorescent bio-label.

1.7.2.2 Zinc Sulfide Labeling Biomolecules

Zinc sulfide (ZnS) is one of the II-VI semiconductor compounds, which have wide ranging applications in solar cells, infrared window materials, photodiode and cathode-ray tube [73], electro luminescent devices [74] and multiplayer dielectric filters [75]. ZnS occurs in two crystalline forms, one in the hexagonal system and other in cubic system. The minerals in hexagonal system are called as wurtzite whereas the cubic system known as sphalerite or zinc blended. Zinc sulfide is insoluble in water and the physical properties of ZnS are white to gray-white or pale yellow powder [64].

Considerable progress has been made in the synthesis of zinc sulfide powders. Irradiation method was extensively used to prepare the ZnS particles were introduced by Qiao *et al.* [76]. In this method, different sulfur sources such as sodium thiosulfate, thiourea and mercaptoethanol were used successfully to obtain ZnS. Due to the complexity and expensiveness of some of these methods, Wang *et al.* [77] developed a new solid-state method by which zinc sulfide particles can be obtained easily via solid-state chemical reaction of zinc acetate and thioacetamide at low temperature. However, all the methods described above are difficult to commercialize due to the high-cost and low-volume capacity. Finally, Kho *et al.* [78] proposed the most acceptable method for the ZnS preparation. This approach

described a simple, inexpensive and reproducible procedure for large-scale synthesis of highly stable of ZnS capped cysteine particles.

Several research groups have reported few reviews on the ZnS semiconductor linked biomolecules. Chan *et al.* [79] presented a highly luminescent semiconductor (zinc sulfide-capped cadmium selenide) have been covalently coupled to protein for ultra-sensitive biological detection. According to the report, this class of luminescent labels is 20 times as bright, 100 times as stable against photobleaching and one-third as wide in spectral line width compared with organic dyes such as rhodamine. Besides, these semiconductor conjugated with biomolecules are water-soluble and biocompatible.

Wang *et al.* [80] presented a more detailed study on application of ZnS water-soluble particles modified with sodium thioglycolate. In this study, the modified ZnS were used as fluorescence probes for the quantitation determination of proteins and was proved a simple, rapid and specific method. It has been concluded that these semiconductor particle probes are brighter, more stable against photobleaching and do not suffer from blinking compared with single organic fluorophores.

Li *et al.* [81] presented water-soluble cysteine capped ZnS particles as fluorescence probe for the determination of DNA. In this study, a synchronous fluorescence method has been developed for the rapid quantitative determination of DNA with ZnS capped cysteine.

1.8 Advantages and Disadvantages of Fluorescence in Proteins Analysis.

Fluorescence is a particularly important analytical technique because of its extreme sensitivity and good specificity.

1. First there are two wavelengths used in fluorescence analysis. Emitted light from each fluorescent color can be easily separated because each color has unique and narrow excitation spectra. Besides that, multiple fluorescent

colors within a single sample can be quantified by sequential measurement of emitted intensity using a set of excitation and emission wavelength pairs specific for each color.

2. Second advantage of fluorescence is low signal to noise ratio because the emitted light enters the emission monochromator, which is positioned at 90^{o} angels from the excitation light path to eliminate background signal and minimize noise due to stray light.

3. The third advantage is that fluorescent methods have a greater range of linearity. Due to this, the sensitivity of fluorescence is approximately 1000 times greater than absorption spectrometric methods.

1.8.1 Disadvantages

1. Fluorescence is particularly sensitive to contaminating substances. Thus, for example, the cuvette may be contaminated by fluorescence material.

2. The fluorescence reading is not stable due to variety of reasons below.

   i. Fogging of the cuvette when the contents are much colder than the ambient temperature.
   ii. Bubbles forming in the solution.
   iii. Drops of liquid on the external faces of the cuvette.

1.9 Research Background

The development of novel assays for proteins is a basic requisite in both clinical and laboratory tests. The analysis of protein can be used as a reference for measurements of other components in biological fluids and clinical diagnosis. The most frequently used approaches for the determination of protein is the ultraviolet and visible absorption spectroscopy, Lowry method [19], dye binding method like Bradford [20], Bromocresol green procedures [82] and Bromophenol blue [83]. However, they all have some limitation in terms of sensitivity, selectivity, stability and simplicity. Disadvantages of the Lowry method include low sensitivity, poor selectivity and complexity [19]. The Bradford method is also inconvenient in operation and application due to the requirement for calibration and to the nonlinearity between the absorbance of the Coomassie Brilliant Blue G-250 (CBB G-250) dye-protein complex and the concentration of protein [20]. The bromocresol green method is insensitive and susceptible to interference by turbidity [82] and the Bromophenol blue method can be used only for protein concentrations greater than 10 mg L^{-1} [83]. Therefore, a number of assays have been continuously reported in recent years such as those based on spectrophotometric [32, 33], nuclear magnetic resonance (NMR) [34, 35], capillary electrophoresis [36], Fourier transform infrared (FT-IR) [37] and resonance Rayleigh light-scattering (RRLS) [38].

Findings strongly suggested that, the most sensitive quantitation of protein at this present is generally based on their fluorescence enhancement effect on organic dyes. Fluorescent small molecules (organic dyes) are used in multiplex detection approaches that had achieved a considerable level of sophistication with character of rapidity, good selectivity and high sensitivity. However, the organic fluorophores often suffer from photo bleaching, low signal intensities, random on/off light emission (blinking) and narrow excitation [55, 56].

Colloidal semiconductor particles have the potential to overcome problems encountered by organic small molecules in certain fluorescent labeling applications by combining the advantages of high photobleaching threshold, good chemical stability and readily tunable spectral properties [55]. These colloidal particles are very resistance to photobleaching and have a high quantum yield in aqueous solution

make them attractive for labeling functionalized biomolecules for fluorescent probe [55]. In this study, the new direction for the synthesis of CdS capped with mercapto acetic acid and ZnS capped with cysteine was employed for the binding with protein. This functionalized CdS and ZnS will be covalently link to protein (BSA) for further studies.

1.10 Research Objective and Scope of Study

Fluorescence method is one of the most sensitive methods frequently used in the determination of protein. The interest in the use of fluorescence technique of determination involves the development of methods that are faster, more efficient, accurate, simple and more sensitive for the determination of protein. Recent advances in fluorescence studies have produced a new class of fluorescent labels by binding semiconductor with biological compounds.

The objective of this study is to:

i. Synthesize the CdS capped with mercaptoacetic acid and ZnS capped with cysteine.
ii. Develop the method for Bovine serum albumin binding with functionalized CdS and functionalized ZnS.
iii. Apply the developed method in the quantitative analysis of protein

In this study, functionalized CdS and functionalized ZnS binding with protein (BSA) was characterized with fluorescence technique. The binding fluorescence properties of functionalized CdS and ZnS with BSA were optimized for the quantitative analysis of protein. The effect of interfering substances and surfactant binding with functionalized colloidal and BSA was performed. In order to employ the developed method, different type of protein such as albumin, lysozyme and amylase detection was carried out.

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The fluorescence spectra of l-cysteine-coated ZnS QDs and its fluorescence titration with histidine were recorded at optimum experimental conditions. The fluorescence of l-cysteien-coated ZnS QDs is significantly decreased with increasing the concentration of histidine Fig.6. Fig.6: Effect of histidine concentration on the fluorescence intensity of l-cysteine-capped ZnS QDs.Â Here we demonstrated the potential application of luminescent QDs to develop new fluorescence sensors for the detection of amino acids .It is possible that changing the modifier of surface of QDs could make the method more effective. A good linear relationship was observed when using a modified Sternâ€“Volmer plot. The detection limits l 0.05Ã—10-7 mol.L-1. The competitive assay using CdSe/ZnS QDs as fluorescence-labeled probe showed good sensitivity, steady and fast response, and excellent anti-interference ability compared to conventional fluorescence-quenching methods. Finally, the feasibility of the proposed methodology was successfully applied for detection of triazophos in real samples.Â 3 Department of Pharmacology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. 4 Department of Medical Pharmacology, Medical Faculty, Ataturk University, Erzurum, Turkey. In the present study we have developed a direct competitive CdSe/ZnS quantum dot (QD) fluorescence assay based on micro-array-imprinted membranes for the determination of triazophos in cabbage and apple. C-reactive protein (CRP), as an acute phase protein from liver cells, has been used clinically to monitor infection and autoimmune disorders [35, 36]. Especially, high-sensitivity C-reactive protein (hs-CRP), much higher sensitivity than CRP, has been regarded as an independent biomarker for cardiovascular disease (CVD) in clinic [37, 38]. This FLISA approach employs a novel water-soluble QDs fluorescent probe by using CdSe as core and ZnS as shell, instead of enzyme-linked antibody, and the process is shown in Scheme 1. The high quality CdSe/ZnS core/shell QDs were synthesized according to th