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Near-Infrared Fluorometric Determination of Protein by Shifting the Ion-Association Equilibrium Between Cationic Heptamethylene Cyanine and Poly-glutamate

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Near-Infrared Fluorometric Determination of Protein by Shifting the Ion-Association Equilibrium Between Cationic Heptamethylene Cyanine and Poly-glutamate

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ABSTRACT

A new method based on near-infrared (near-IR) fluorescence recovery, employing a two-reagent system, which is composed of a cationic

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heptamethylene cyanine and poly-glutamate, is presented for the determination of protein. The fluorescence of cationic heptamethylene cyanine, with the maximum excitation and emission wavelengths at 800 and 815 nm, respectively, was quenched by poly-glutamate with a proper concentration, but recovered by adding proteins at pH 3.5. Under optimum conditions, the recovered fluorescence was in proportional to the concentration of proteins. The linear ranges of the calibration curves were 50–1000, 100–1500 and 100–1000 ng/mL with the detection limit of 37, 40, 43 ng/mL for BSA, HSA, and γ-IgG, respectively. The relative standard deviation ($n = 8$) was 1.7% for 400 ng/mL bovine serum albumin (BSA). The proposed method was applied to the determination of proteins in real serum samples with satisfactory results.

Key Words: Cyanine; Poly-glutamate; Protein; Ion association equilibrium; Fluorometry.

1. INTRODUCTION

Determination of protein is an important procedure in clinical testing, and laboratory practice in protein purification requires a rapid and sensitive method of protein assay. There are several methods for the direct determination of total protein. The simplest method is determination of the absorption at 280 nm; however, proteins have, by their very nature, poor UV absorption, and this method always suffers interference accompanying substances. The Kjeldahl method, which is the most accurate method of the total protein quantitation, is quite complex. Thus, other methods of greater simplicity and lower detection limits have been developed, including spectrophotometric methods such as the Biuret method,[1] lowry assay,[2] and Bradford method[3–5] and also spectrofluorometric methods such as the CBQCA protein quantitation assay,[6] NanoOrange assay,[7] fluoresamine assay,[8] OPA assay,[9] and the reagent of albumin 670.[10]

Nowadays, near-infrared (near-IR) probes are of more and more interest, since they have the characteristics of big molar absorptivity, high fluorescence quantum yield, and low background emission in the near-IR region, by which some useful analytical applications have been developed.[11–13] We have recently synthesized a cationic heptamethylene thiacyanine, 2-[4′-chloro-7′-(3″-ethyl-2″-benzothiazolinylidene)-3′, 5′-(1‴, 3‴-propanediyl)-1′, 3′, 5′-heptatriene-1′-yl]-3-ethylbenzothiazolium iodide (its molecular structure is shown in Fig. 1) according to the literature[14] and studied its interaction with the proteins. Unfortunately, unlike the sulfonated heptamethylene synthesized by us previously,[11] the cationic cyanine showed hardly interaction with protein and accordingly spectra change, which made it difficult to determine protein directly. In fact, a lot of dyes are not directly used for the protein

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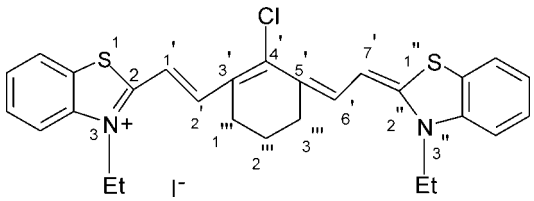

Figure 1. Structure of the near-IR cyanine used.

assay since there are no or too weak interactions between dyes and proteins to bring obviously spectral changes. In this case, it is a good strategy to employ a proper association equilibrium system for the indirect determination of proteins. Our experiment showed that, in the aqueous solution, the synthesized cationic cyanine emits intense fluorescence. However, in the presence of poly-glutamate, a negatively charged polymer under experimental conditions, the cyanine binds to the surface of poly-glutamate through electrostatic interaction, and accordingly the fluorescence emission is almost completely quenched. If protein is added, the cyanine becomes progressively replaced by the protein and then comes back to the solution, regains its initial fluorescence. Based on the above phenomenon, a near-IR fluorometric method for the determination of protein was developed. The merit of the method is that the determination was performed in near-IR region, a region of little biological interference. In addition, as revealed in the further investigation, the method has hardly shown protein specificity, which is very suitable for direct determination of the total protein in human serum samples.

2. EXPERIMENTAL

2.1. Apparatus

A Hitachi F-4500 spectrofluorometer (Tokyo, Japan) equipped with a R3896 photomultiplier and a 1 cm quartz cell was used for recording fluorescence spectra and making fluorescence measurements. The absorption spectrum was made on a Hitachi U-3010 spectrophotometer (Tokyo, Japan).

2.2. Reagents

The near-IR cyanine was synthesized in our laboratory according to the literature.[14] The cyanine was dissolved in *N*,*N*-dimethylformamide (DMF) to

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give a 1.5×10^{-3} mol/L stock solution and kept at 0–4°C. The stock solution was further diluted with DMF to a 7.5×10^{-5} mol/L working solution for use.

Bovine serum albumin (BSA), human serum albumin (HAS), and γ-IgG were purchased from Shanghai Biochemical Institute (Shanghai, China). The standard solutions were prepared by dissolving BSA, HAS, and γ-IgG in doubly distilled water as 100 μg/mL aqueous solutions and were stored at 0–4°C. Poly-glutamate (Sigma) was always prepared fresh by dissolving small amount of the sodium salt in water and its concentration was determined by measuring its absorbance at 205 nm using $\varepsilon = 3500\,M^{-1}\,cm^{-1}$, as reported in the literature[15] (poly-glutamate concentration was expressed in moles of Glutamate residues per liter). In this work, 3.0×10^{-4} mol/L poly-glutamate solution was used. A 0.1 mol/L HCl solution was used as buffer medium.

All reagents were of analytical-reagent grade and doubly distilled water was used throughout.

2.3. General Procedure

To a 10 mL volumetric tube transfer 0.1 mL of poly-glutamate solution, 0.06 mL of buffer solution, and protein solution of a series of concentrations or sample solution and dilute with water to the mark and mix thoroughly, then add 0.05 mL cyanine solution and mix once again, measure the fluorescence intensity of the samples (F) and the blank (F_0) (prepared in a similar manner without protein) with excitation and emission wavelength at 750 and 815 nm, respectively. It should be pointed out that, the fluorescence excitation and emission wavelengths of the cyanine in aqueous solution were 800 and 810 nm, respectively. To choose 750 nm as excitation wavelength is to reduce the possible interference from scattering light.

3. RESULTS AND DISCUSSION

3.1. Characteristics of Spectra

The cationic cyanine in DMF shows an intense monomer absorption peak at 810 nm and a weak shoulder-peak at about 720 nm (the spectra no shown). In aqueous solution, the monomer absorption greatly reduced and blue-shifted to 788 nm, at the same time, an intense absorption peak at 696 nm appeared (Fig. 2, curve 1). Cyanine is known as unique compounds to form aggregates of various compositions and structures.[16,17] The intense absorption at 696 nm formed in solution could be interpreted as a band of some H-aggregate form (I). In the presence of poly-glutamate, the band of 696 nm decreased and

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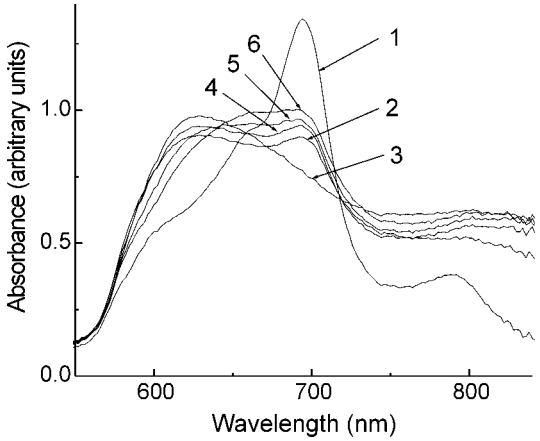

Figure 2. Absorption spectra of the cyanine in aqueous solution (curve 1) and in the presence of ploy-glutamate and BSA (curves 2–6). Ploy-glutamate concentration (mol/L): (2–6): 6×10^{-6}; (3): 7.5×10^{-6} mol/L. BSA concentration (ng/mL): (2 and 3): 0; (4): 100; (5): 500; (6): 1500. The cyanine concentration was 1.5×10^{-6} mol/L, pH 3.5.

disappears with higher concentration of the ploy-glutamate (curves 2 and 3), following a new absorption band at 628 nm, which could be ascribed to the formation of a new cyanine aggregate (II) bound to poly-glutamate. When an amount of protein was introduced to the above system, a contrary spectral change was observed: the absorption at 696 nm increased whereas the absorption decreased at 628 nm (Fig. 2, curves 4–6), suggesting a displacement of the cationic cyanine bound to the poly-glutamate by protein. In fact, the two cyanine aggregates (I and II) are associated with the cyanine H-aggregates containing a different number of species.[18,19] Apart from clear absorption blue-shift from 696 to 628 nm, fluorescence quenching also gave the same proof of H-aggregates formation. In aqueous solution, the fluorescence of the cyanine quenched to some extent, compared with the fluorescence observed in DMF solution (spectra no shown), due to the formation of H-aggregate (I). In the presence of poly-glutamate, the fluorescence reduced further (Fig. 3, curves 1 and 2) since the cyanine monomer further aggregated and formed the H-aggregate (II). When successive aliquots of a protein solution were added, the fluorescence emission progressively recovered (Fig. 3, curves 2–8). The recovery of fluorescence suggests the cyanine aggregate (II) formed in the presence of poly-glutamate is destroyed due to the displacement of protein, which results in an increased amount of the aggregate (I) and monomer of cyanine in aqueous solution.

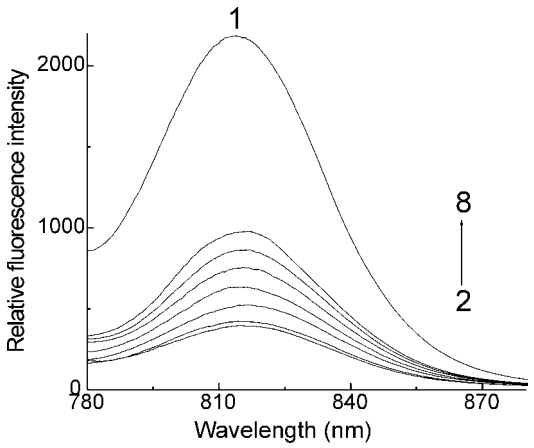

Figure 3. Fluorescence spectrum of the cyanine in buffer solution (curve 1) and in the presence of the ploy-glutamate and BSA (curves 2–8). The concentration of BSA (from 2 to 8, ng/mL): 0; 50; 100; 400; 800; 1000; 1500. Other conditions are the same as that described in the procedure.

3.2. Optimization of General Procedure

In this paper, we chose $F - F_0$ as parameter to investigate the influence of pH, cyanine, and poly-glutamate concentration on the extent of fluorescence recovery. And BSA was used for optimization of the general procedure.

3.2.1. Effect of pH

The interaction between proteins and poly-glutamate is mainly driven electrostatically. Therefore, in order to keep protein charges positive, it is necessary to control the reaction of protein with poly-glutamate to occur in acid or weak acid medium. We examined the influence of pH on the reaction over the pH range of 2.0–4.5 using 0.1 mol/L HCl as buffer system. The results indicated that the maximum fluorescence recovery was obtained pH 2.7–3.7 (Fig. 4). In this work, a pH 3.5 was chosen for further experiment.

3.2.2. Effect of Concentration of Cyanine and Poly-glutamate

It is expected that too low concentration of cyanine and poly-glutamate would lead to a limit fluorescence recovery since there are only a small amounts of cyanine completely replaced by protein from poly-glutamate matrix, which is

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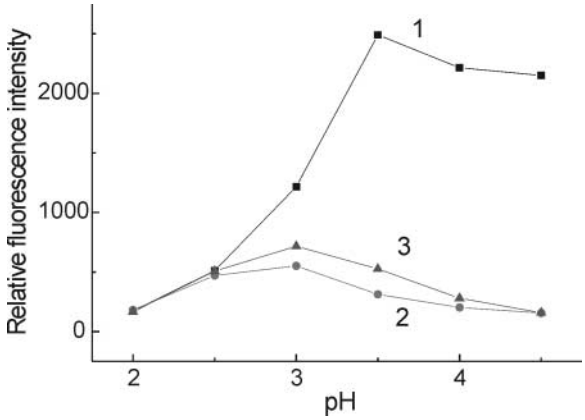

Figure 4. Effects of pH. 1, cyanine; 2, cyanine + poly-glutamate; 3, cyanine + poly-glutamate + BSA. The concentration of BSA is 200 ng/mL. Other conditions are the same as that described in the procedure. (*View this art in color at www.dekker.com.*)

unfavorable to enlarging linear range. On the other hand, too high concentration of cyanine and poly-glutamate would cause a low sensitivity, which may be ascribed to the difficulty to replace the cyanine from poly-glutamate matrix for low concentration of protein. In addition, it is also important to control the ratio of cyanine to poly-glutamate concentration. A high ratio would cause a high background signal and a low sensitivity of the method since only a small percentage of cyanine is in the aggregated form in this case, which results in a poor initial fluorescence quenching and thus a high background signal and low sensitivity. On the other hand, although low ratio of cyanine to poly-glutamate permits us to start the measurement with extremely low background fluorescence intensity, low ratio would increase the probability of direct interaction of protein with the polymer matrix due to the number of binding sites available on the matrix. As a result, the system could not give a ready response to the first few protein aliquots added until all binding sites on the polymer are saturated.[15] In addition, if the ratio is too low, it may cause a narrow linear range. Thus, this effect causes a difficulty in the quantitative determination of protein, especially in the low protein concentration range.

The effect of cyanine concentration was firstly tested (Fig. 5). From Fig. 5 it can be seen that, when the concentration of poly-glutamate keeps a constant, the fluorescence of cyanine, whether with protein or without protein, enhanced with the increasing concentration of cyanine, but the value of $F - F_0$ have not big increase. Therefore, the final concentration of cyanine was set at 3.7×10^{-7} mol/L, taking the lower background fluorescence into account.

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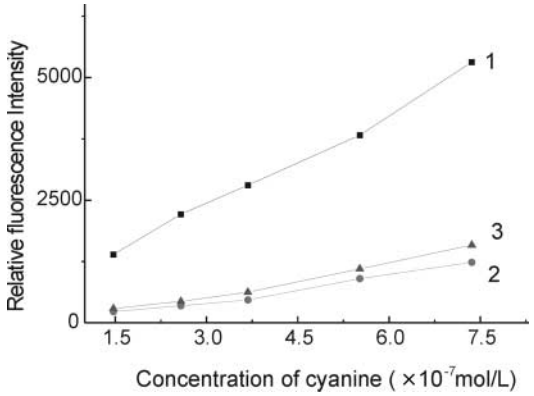

Figure 5. Effects of cyanine concentration. 1, cyanine; 2, cyanine + poly-glutamate; 3, cyanine + poly-glutamate + BSA. The concentration of BSA is 200 ng/mL. Other conditions are the same as that described in the procedure. (*View this art in color at www.dekker.com.*)

The effect of poly-glutamate concentration was shown in Fig. 6. At lower concentration of poly-glutamate, the fluorescence is not quenched completely; on the other hand, the fluorescence did not recovery clearly when the concentration of poly-glutamate is too high. Therefore, a appropriate concentration of 3.0×10^{-6} mol/L poly-glutamate was chosen in our experiment.

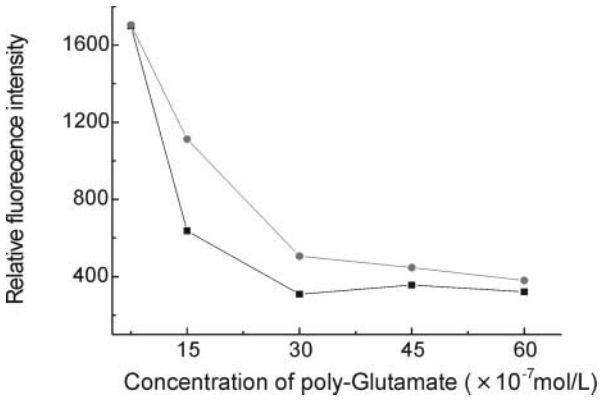

Figure 6. Effects of ploy-glutamate concentration on the fluorescence intensity of ploy-glutamate/cyanine system in the absence (bottom) and in the presence of BSA (top). The concentration of BSA is 200 ng/mL. Other conditions are the same as that described in the procedure. (*View this art in color at www.dekker.com.*)

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3.2.3. Effect of the Order of Adding Reagents

The effect of adding sequence of reagents on the fluorescence recovery was studied and the order of poly-glutamate, buffer, protein, and cyanine was proved to be a suitable one.

3.2.4. Incubation Time

The reaction of cyanine with ploy-glutamate is rapid, and furthermore, when protein added, the equilibrium of the system could also be built up rapidly according to our observation. Therefore, the measure of fluorescence intensity could be carried out at 5 min after mixing the sample solutions.

3.2.5. Effect of Ionic Strength

The effect of ionic strength on the assay was investigated by adding the strong electrolyte, sodium chloride. Too high ionic strength is unfavorable for the interaction between poly-glutamate and cyanine since the binding of cyanine or protein to poly-glutamate is driven mainly by electrostatic interactions. Our results indicated that the sensibility of the method decreased dramatically in a medium with a high concentration of NaCl. Thus, the final concentration of salt in the system should be controlled not higher than 20 mM.

3.3. Tolerance of Foreign Substances

The influence of various ions and amino acids on the determination of 200 ng/mL BSA was studied and the results are summarized in Table 1. It can be seen from Table 1 that most of the substances tested scarcely interfered with the determination. Mg^{2+}, Ca^{2+}, and Al^{3+} can be tolerated at somewhat low levels, but their final contents in solutions being determined is far below the tolerance listed since the dilution of samples is needed for biological fluids before determination.

3.4. Calibration Graphs and Analyses of Human Serum Samples

The calibration graphs for three proteins assay were constructed under optimum conditions (Fig. 7), and all the analytical parameters of this method are given in Table 2. From Fig. 7 and Table 2, it can be seen that the method has little protein specificity, which is very suitable for the direct determination of total protein contents in real samples.

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Table 1. Interference of foreign substances (BSA: 200 ng/mL).

Substance	Added (ng/mL)	Error (%)
Zn^{2+}	100	−0.75
Mg^{2+}	50	−4.5
Ca^{2+}	75	−4.0
Mn^{2+}	300	−4.5
Al^{3+}	13.5	5.5
Fe^{3+}	500	3.8
Glycine	3,000	5.0
Aminopropionic acid	2,000	4.5
Lyrosine	1,000	−0.44
Avginine	500	1.7
Proline	2,000	−1.3
Cysteine	2,000	0.24
Serine	2,000	1.9
Valine	1,000	1.1
Aspartate	1,000	−2.3

The real samples of human serum were obtained from the Second Hospital of Wuhu. The samples were tested without other pretreatment except a 20,000 dilution. The analytical results are listed in Table 3. From Table 3 it can be seen that the results obtained by the this method are in good agreement with the clinic results, suggesting that the proposed method has potential for practical application to the determination of micro amounts of protein.

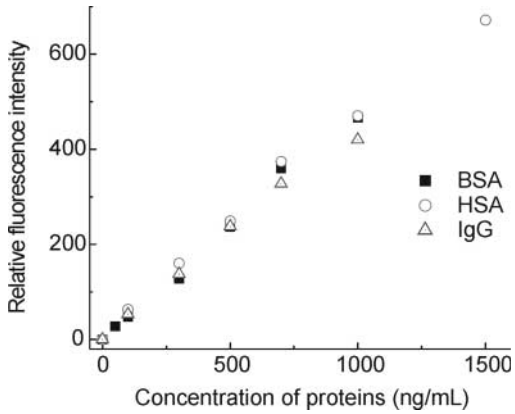

Figure 7. Calibration graphs for various proteins. (*View this art in color at www.dekker.com.*)

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Table 2. Analytical parameters of this method.

Protein	Regression equations (C: ng/mL)	Linear range (ng/mL)	Detection limit (ng/mL)	Coefficient
BSA	$\Delta I = -1.84077 + 0.48094C$	50–1000	37	0.9974
HSA	$\Delta I = 22.69865 + 0.44639C$	100–1500	40	0.9960
γ-IgG	$\Delta I = 13.67944 + 0.42802C$	100–1000	43	0.9931

Table 3. Analytical results of serum samples.

This method	Protein found[a] (mg/mL)	Recovery (%)	Clinical values[b] (mg/mL)
Sample 1	83.13	102	85.1
Sample 2	71.16	97	71.7
Sample 3	76.67	109	74.4

[a]Average of three measurements.
[b]Clinical values of hospital.

4. CONCLUSION

A cationic cyanine dye has been synthesized according to literature in our lab. The spectral characteristics of its various aggregates formed in aqueous solution with or without poly-glutamate and protein have been discussed. By means of a ternary system of cyanine-poly-glutamate-protein, a near-IR fluorescence enhancement method has been developed for the determination of the total proteins in human serum samples. Due to the unique nature of the cyanine, the method is expected to suffer little interference from the background fluorescence and the scattering light of the determination system. In addition to its rapidness and sensibility, the method also displayed scarce protein specificity, which greatly simplified the analysis procedure.

ACKNOWLEDGMENTS

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