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Dye-binding protein assay using a long-wave-absorbing cyanine probe

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Abstract

A simple and fast protein assay that involves the binding of water-soluble sulfonate heptamethylene cyanine to protein is described. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 778 to 904 nm, and the increase in absorption at 904 nm is monitored. This assay is very reproducible, of good color stability for at least 80 min, and sensitive at the 100 ng/mL level of human serum albumin (HSA) when a spectrophotometer with near-infrared wavelength is used to measure absorbance. Few chemicals except ionic surfactants such as cetyltrimethylammonium bromide and sodium dodecyl sulfonate interfere with the assay. Purified proteins have different capacities to interact with the dye; under the experimental conditions, the linear ranges of bovine serum albumin (BSA), HSA and γ-IgG were 200–2000, 100–2400, and 200–3000 ng/mL, respectively. The relative standard deviation for the five replicate determinations of 1200 ng/mL BSA is 2.1%.

Keywords: Protein assay; SHMC; Spectrophotometry; Dye binding

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. Several colorimetric methods for quantitating proteins in solution, including the widely used biuret reaction [1] and Lowry assay [2], in addition to an assay that uses bicinchoninic acid [3] have been described. However, each of these methods requires more reagents and hydrolysis of protein. Other simpler colorimetric methods based on dye-binding techniques, such as the Bradford [4], bromophenol blue [5], eosin B [6], and bromocresol green [7] methods, have also been developed. All these methods rely on absorption-based measurement and are limited in sensitivity. Thus more sensitive methods such as colloidal gold assay [8] and fluorometric assays including CBQCA protein quantitation assay [9], NanoOrange [10] assay, fluoresamine [11] assay, OPA [12] assay and the reagents of albumin 670 [13] and albumin 580 [14] for protein assay have been introduced.

During the study of water-soluble cyanine in our laboratory, we discovered a sulfonated heptamethylene cyanine (SHMC)[1] that showed an obvious reaction with protein; thus a novel colorimetric method sensitive at nanogram levels for protein assay was developed. It is based on the observations that the SHMC showed a new absorption peak at 904 nm upon binding to protein and that the protein–dye complex exhibited a high absorbance leading to great sensitivity in the measurement of protein. Despite its sensitivity, the dye–protein complex of the method showed absorbance beyond 900 nm at the near-infrared region, where natural substances show no absorption; thus it can overcome the interference of strong absorption by long-wave-absorbing materials present in biological samples.

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[1] *Abbreviations used:* SHMC, sulfonate heptamethylene cyanine; BSA, bovine serum albumin; HSA, human serum albumin.

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Materials and methods

Reagents and materials

All chemicals were of analytical grade or the best grade commercially available unless otherwise mentioned. All aqueous solutions were prepaired in redistilled deionized water.

The standard solutions of bovine serum albumin (BSA; 856 μg/mL), human serum albumin (HSA; 836 μg/mL), ovalbumin (924 μg/mL) and γ-IgG (976 μg/mL) were prepared by dissolving BSA (Sigma Chemical Co.; essentially fatty acid free, fraction 5), HSA (Shanghai Chemical Reagents Co.), ovalbumin (Shanghai Chemical Reagents Co.), and γ-IgG (Serva Co.) in water, respectively, and were diluted to 20 μg/mL as working solutions. All solutions were stored at 0–4 °C.

A stock solution (1.0×10^{-3} mol/liter) of SHMC, synthesized according to [15], was prepared by dissolving it into methanol and further diluting to 1.0×10^{-4} mol/liter with ethanol as a working solution. These dye solutions were stored at 0–4 °C and the stock solution was thus stable at least for half of a year.

Series of HCl–KCl, glycine–HCl, and NaAc–HAc buffer systems were used for the pH adjustment.

Apparatus

All absorption spectrum recordings and absorbance measurements were performed on a GENESYS 5 Model spectrophotometer (Thermo Spectronic) equipped with 10-mm cells.

Protein assay

An appropriate volume of protein working solution or sample solution and 1.0 mL of glycine–HCl buffer solution (pH 2.6) were added to a 10-mL calibrated flask; this was diluted with water to the full volume and mixed thoroughly by inversion. Then 0.30 mL of SHMC working solution was added and the mixture was mixed thoroughly again. The mixture was then allowed to stand for 40 min at room temperature, and the absorbance of the solution was measured against the buffer solution (free of SHMC) or water at 904 nm. The concentration of protein was plotted against the corresponding absorbance, resulting in a standard curve used to determine the protein in unknown samples.

Results and discussion

Reaction of SHMC and protein

SHMC (Fig. 1) is a sulfonated heptamethylene cyanine which is water soluble. In acidic medium, it exhibits

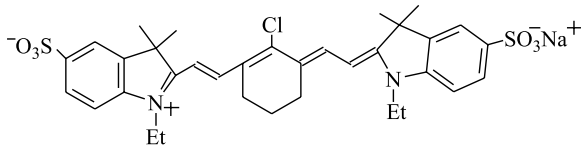

Fig. 1. Molecular structure of SHMC.

a green color with an absorption maximum at 778 nm and shows no absorbance at 904 nm; hence a buffer solution or water may serve as a blank in detection. The addition of protein causes a decrease in absorption maximum at 778 nm and shifting to 904 nm as a new peak; thus the absorption maximum is shifted by 126 nm. The absorption spectra are shown in Fig. 2.

Effect of pH

The binding reaction of protein with sulfonated dye occurs generally in acid or weak acid medium. In such a case, protein charges positively while dye charges negatively. Hence the comprehensive influence of pH on the binding reaction was examed over the pH range 1.0–4.0 with three buffer systems: HCl–KCl, glycine–HCl, and NaAc–HAc (Fig. 3).

Experimental results indicated that, at pH 1.6–3.2, the protein–SHMC complex gave larger absorbance at 904 nm, a pH 2.6 with glycine–HCl buffer medium was chosen. By considering the buffering capacity in a final 10-mL volume, 1.0 mL of buffer was recommended.

Stability of protein–SHMC complex

The time course of the reaction is very rapid, requiring 15 min to complete at room temperature with the absorbance at 904 nm reaching maximum. It becomes more stable when allowed to stand for another 25 min and then can remain constant for at least 80 min

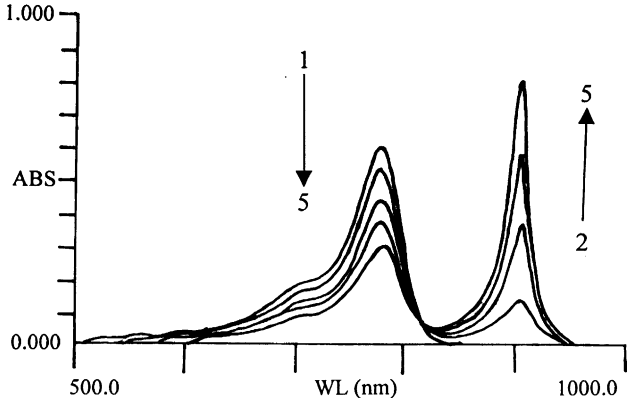

Fig. 2. Absorption spectra of SHMC and SHMC–BSA complex. [SHMC] = 3.0×10^{-6} mol/liter; [BSA] = (1) 0 ng/mL, (2) 400 ng/mL, (3) 800 ng/mL, (4) 1200 ng/mL, (5)1600 ng/mL; pH 2.6 (glycine–HCl buffer system).

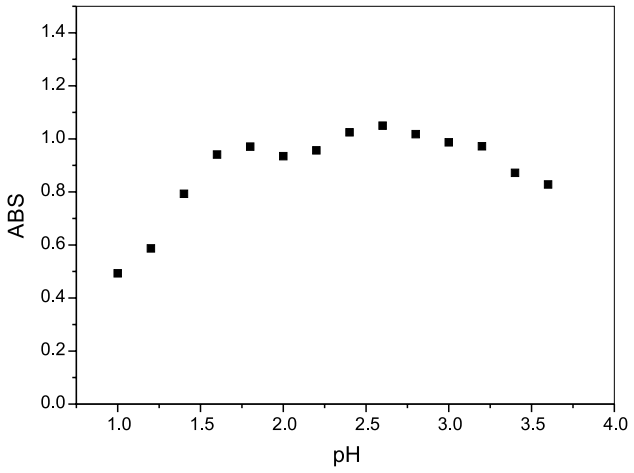

Fig. 3. Effect of pH on binding reaction. [SHMC] = 3.0×10^{-6} mol/liter; [BSA] = 2400 ng/mL; pH 1.0–1.8, HCl–KCl buffer system; pH 2.0–2.8, glycine–HCl buffer system, pH 3.0–4.0, HAc–NaAc buffer system.

(Fig. 4). Hence an incubation time of 40 min was recommended.

Effect of SHMC concentration on the standard curve

A range of concentrations of SHMC, such as 2.0×10^{-6}–1.0×10^{-5} mol/liter, can be used for protein determination, and the experiments showed that with the increase in the concentration of SHMC, the linear range was extended accordingly. In practical determinations, a suitable concentration of SHMC may be selected to meet the linear range which the concentration of protein in the samples requires. Considering the fact that the absorbance of protein–SHMC complex at 904 nm is always larger than 0.900 O.D unit caused by the use of 3.0×10^{-6} mol/liter of SHMC and 2000 ng/mL of BSA, a 3.0×10^{-6} mol/liter of SHMC was selected for the determination.

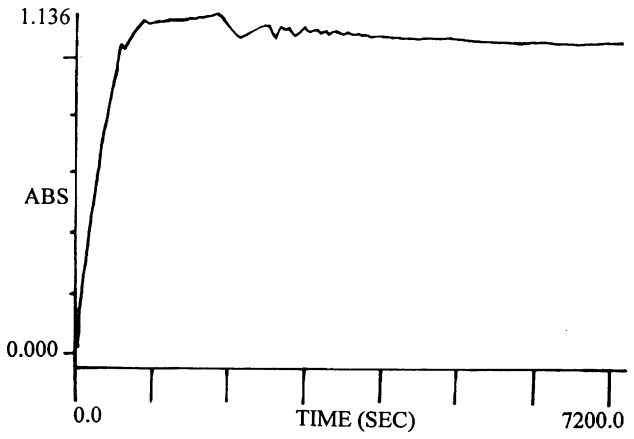

Fig. 4. Protein–dye complex formation rate and color stability. [SHMC] = 3.0×10^{-6} mol/liter; [BSA] = 2400 ng/mL; pH 2.6.

Sequence of addition of reagents

The experiments indicated that when protein and SHMC are mixed first, with water then added to the full volume, the system may become somewhat less stable than when the procedure that we recommended if followed. Adding SHMC last can keep the final volume of all tubes equal.

Effects of nonprotein substances

The effects of substances, including amino acids, metal ions, nucleic acids, and surfactants, on the assay were tested (Table 1). The results showed that the method could tolerate most of the interferences. Nucleic acids, such as CT DNA, could be tolerated when their relative concentrations are not beyond 10% of that of protein. Both cationic and anionic surfactants such as cetyltrimethylammonium bromide and sodium dodecyl sulfate at lower concentrations showed strong interferences

Table 1
Effects of foreign substances

Substance	Added (final concentration)	Recovery (%)
$MgCl_2$	1.0×10^{-2} mol/liter	88.4
KCl	4.0×10^{-3} mol/liter	98.1
$CaCl_2$	1.0×10^{-4} mol/liter	96.7
$CuSO_4$	2.0×10^{-5} mol/liter	93.1
$MnCl_2$	2.0×10^{-5} mol/liter	94.6
$ZnCl_2$	2.0×10^{-5} mol/liter	96.3
$FeCl_3$	1.0×10^{-5} mol/liter	90.5
$AlCl_3$	1.0×10^{-5} mol/liter	95.6
NaAc	1.0×10^{-3} mol/liter	96.6
Na_3PO_4	5.0×10^{-4} mol/liter	84.2
NaN_3	1.0×10^{-3} mol/liter	94.5
NaCl	2.0×10^{-2} mol/liter	98.1
Tris	4.0×10^{-4} mol/liter	98.5
$(NH_4)_2SO_4$	5.0×10^{-3} mol/liter	95.3
EDTA	1.0×10^{-4} mol/liter	102.3
Urea	5.0×10^{-5} mol/liter	95.7
Phenol	1.0×10^{-3} mol/liter	89.5
Glucose	1.0×10^{-5} mol/liter	98.5
Triton X-100	0.001%	105.6
Brij-35	0.001%	104.3
Tween 20	0.01%	111.8
Cetyltrimethyl-ammonium bromide	0.0003%	119.1
Sodium dodecyl sulfate	0.0001%	8.6
Sodium dodecyl sulfate	0.001%	2.8
Nucleic acid, CT DNA	60 ng/mL	96.9
Nucleic acid, CT DNA	120 ng/mL	94.5
Nucleic acid, CT DNA	240 ng/mL	82.8
Tyrosine	2.0×10^{-5} mol/liter	94.8
Tryptophen	2.0×10^{-5} mol/liter	93.9
Arginine	2.0×10^{-6} mol/liter	97.3
Phenylalanine	2.0×10^{-6} mol/liter	96.1
Alanine	2.0×10^{-6} mol/liter	98.9
Leucine	1.0×10^{-6} mol/liter	97.4

[SHMC] = 3.0×10^{-6} mol/liter; [BSA] = 1200 ng/mL.

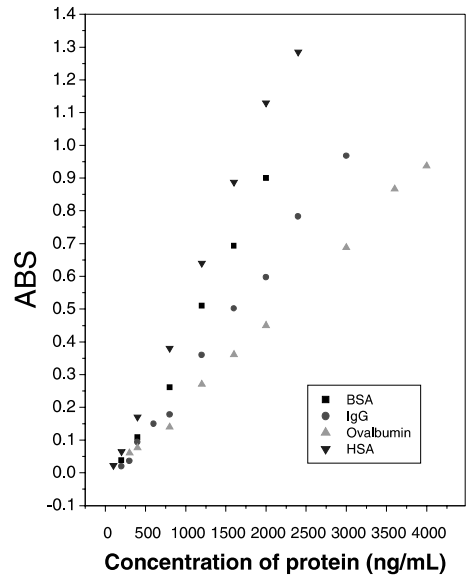

Fig. 5. Protein–dye binding response pattern for various proteins.

in the method, especially SDS. Although nonionic surfactants, such as Triton X-100, Brij-35, and Tween 20 at moderate concentrations, also showed some interference in the assay, considering the fact that such effects were on the assay of 1200 ng/mL of BSA, a concentration far lower than that in real samples, we can treat these nonionic surfactants as having no interference in the practical assay.

Standard curves and analysis of serum samples

The standard curves were constructed under optimal conditions, and the human serum samples were tested without other preliminary treatment except dilution. The protein–dye binding response pattern of four proteins are shown in Fig. 5 and the parameters of standard curves are given in Table 2.

Because the present method showed protein specificity, as revealed in the investigation, it is suitable for determination of the total protein in complex samples containing different types of proteins, e.g., serum samples. Hence the present method was used to determine the total protein in human serum samples. Standard human serum, which was used to construct the calibration curve, was obtained by mixing 20 normal serum samples, followed by determination of the total content of protein using the micro-Kjeldahl method. The construction of the calibration curve and the analysis of the serum samples were then performed according to the procedures described above. The human serum samples were totally diluted 50,000-fold in the determination in a final 10-mL solution and the results were compared with those obtained with a clinical method (biuret method). The analytical results are listed in Table 3.

We can see that the results obtained with the present method were in agreement with those obtained with the biuret method. In general, all the results are satisfactory and thus indicate that this method has the potential for practical application to the determination of microamounts of protein.

Conclusion

A novel protein assay sensitive at the nanogram-level and based on the binding interaction of protein with SHMC is described. The operation is very simple, fast, and reliable, and the method is tolerant of the existence of most nonprotein substances. In addition to its simplicity and rapidity, it has the following advantages. First, it shows extreme sensitivity in a spectrophotometric assay, e.g., with 2.0 μg/mL of BSA, producing an absorbance increase of 0.910 O.D unit, compared with 5.0 μg/mL of BSA, giving an absorbance increase of 0.275 O.D unit by Coomassie brilliant blue G-250 [4]. Second, we can

Table 2
Parameters of standard curves

Protein	Regression equations (C: ng/mL)	Linear range (ng/mL)	Coefficienct
BSA	$A = -0.085 + 4.874 \times 10^{-4}\,C$	200–2000	0.998
HAS	$A = -0.048 + 5.706 \times 10^{-4}\,C$	100–2400	0.999
γ-IgG	$A = -0.060 + 3.427 \times 10^{-4}\,C$	200–3000	0.998
Ovalbumin	$A = -0.027 + 2.425 \times 10^{-4}\,C$	300–4000	0.999

Table 3
Analytical results of serum samples

This method	Protein found[a] (mg/mL)	RSD (%; $n = 3$)	Recovery (%)	Clinical values[b] (mg/mL)
Sample 1	65.6	2.3	91.3	70.3
Sample 2	71.8	3.4	95.1	68.2
Sample 3	68.8	1.9	92.6	72.6

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

measure the absorbance in the near-infrared region, where natural products show no absorption, thus overcoming the interference of long-wavelength absorption by some materials present in biological samples. Third, it shows a good linear relationship of absorbance vs protein concentration, where absorbance increases much more greatly with moderate increase in protein concentration. Fourth, it shows no absorbance at 904 nm in the absence of protein; hence a buffer solution or water may serve as a blank in detection. The shortcoming, as with the Bradford method, is that different proteins have different capacities to interact with the dye used and could lead to erroneously high or low absolute values if BSA is used as a standard. This problem can be overcome, however, by using the same protein in the standards as the protein being measured. Moreover, if greater sensitivity is needed, the present method is an appropriate replacement for other routine methods such as the Bradford or Lowry method.

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