THE ANALYST
FULL PAPER
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Fluorescence for the determination of protein with functionalized nano-ZnS

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Received 8th July 2002, Accepted 16th September 2002
First published as an Advance Article on the web 7th October 2002

ZnS nanoparticles have been prepared and modified with sodium thioglycolate. The functionalized nanoparticles are water-soluble. They were used as fluorescence probes in the determination of proteins, which was proved to be a simple, rapid and specific method. In comparison with single organic fluorophores, these nanoparticle probes are brighter, more stable against photobleaching, and do not suffer from blinking. Under optimum conditions, linear relationships were found between the enhanced intensity of fluorescence at 441 nm and the concentration of protein in the range 0.1–4.0 μg mL^{-1} for human serum albumin (HSA), 0.2–3.0 μg mL^{-1} for bovine serum albumin (BSA) and 0.1–4.5 μg mL^{-1} for γ-globulin (γ-G). The limits of detection were 0.015 μg mL^{-1} for HSA, 0.024 μg mL^{-1} and 0.017 μg mL^{-1} for BSA and γ-G, respectively. The method has been applied to the analysis of human serum samples collected from the hospital and the results were in good agreement with those reported by a hospital, indicating that the method presented here is not only sensitive and simple, but also reliable and suitable for practical application.

Introduction

The determination of protein is important in clinical medicine and laboratory practice. Currently the widely applied methods are those of the Bradford method,[1] the Lowry assay,[2] and the bromophenol blue method.[3] However, there are some limitations in these assays. For the Bradford method, there is non-linearity between the absorbance of the Coomassie brilliant blue G-250 dye–protein complex and the concentration of protein, and inconvenience in operation.[4] The Lowry method has low sensitivity, poor selectivity and is a complicated procedure. The bromophenol blue procedure can only be used for protein concentrations greater than 10 mg L^{-1}.[3] Therefore a great number of new methods have been put forward in recent years, such as those based on spectrophotometry, [5,6] light scattering,[7–9] calorimetry,[10,11] and capillary electrophoresis[12] to analyse and detect the proteins. However, no spectroscopic technique is more widely used in peptide and protein chemistry than fluorescence[13–16] which has achieved a considerable level of sophistication with characteristics of rapidity, good selectivity and high sensitivity. Since the intrinsic fluorescence of protein is very weak, most fluorescent protein probes are mainly based on organic dyes. However, the organic fluorophores tend to have narrow excitation spectra, and often exhibit broad emission bands with red tailing, which makes simultaneous quantitative evaluation of relative amounts of different probes present in the same sample difficult, due to spectral overlap.[17] Colloidal semiconductor nanoparticles have the potential to overcome problems encountered by small organic molecules in certain fluorescent tagging applications by combining the advantages of high photobleaching threshold, good chemical stability, and readily tunable spectral properties. The wide range of useful size-dependent excitation and emission wavelengths of colloidal semiconductor nanoparticles, their resistance to photobleaching, along with their high quantum yield in aqueous solutions make them attractive for labeling functionalized biomolecules for fluorescent tagging applications. Experiments involving labeling of metallic and/or magnetic nanoparticles having organic capping molecules on their surfaces have been reported.[18–20] It is still a challenge to synthesise water-soluble and biocompatible nanoparticles used as fluorescence probes. We have reported a class of nanometer-sized luminescent particles that allow for the ultrasensitive detection of the concentration of nucleic acids.[21] In this paper, we prepared the functionalized nanoparticles (ZnS)–SCH_2COONa. These fluorescent nanoparticles have a long fluorescence lifetime (3.95 μs) and high room temperature fluorescence quantum yields (0.227). These values are in accordance with those previously reported. The cited references report that fluorescence lifetime is at the microsecond level [22] and the room temperature fluorescence quantum yield is about 10–30%.[23] They are highly resistant to photobleaching and emit bright and steady fluorescence. The fluorescence of functionalized nano-ZnS can be enhanced by protein, and the extent of the fluorescence intensity enhancement is proportional to the concentration of protein. So a new determination method with high sensitivity for protein has been developed. This method is simple, rapid and specific.

Experimental

Apparatus

A Hitachi (Tokyo, Japan) F-4500 spectrofluorometer with a plotter unit and a quartz cell was used for fluorescence measurements. pH measurements were made with a pHS-3C pH meter (Dazhong Analytical Instruments Factory, Shanghai, China). Fluorescence quantum yields were calculated by comparison with a Rhodamin 6G dye solution emitting with a quantum yields close to 100%. Fluorescence lifetime was tested with a self-built spectrometer equipped with a LPX 105I laser (Germany). Transmission electron microscopy (TEM) images of the nanoparticles were acquired on a Hitachi H-600 transmission electron microscope. Colloidal solutions of the nanoparticles in water were dropped on to 50 Å thick carbon coated copper grids with the excess solution being immediately removed.

Reagents

Stock standard solutions of HSA (Sigma), BSA (Sigma) and γ-G (Sigma) were prepared by directly dissolving HSA, BSA and γ-G in 0.5% NaCl at a final concentration of 100 μg mL^{-1} and stocked at 0–4 °C, respectively. Stock standard solutions of 0.1 mol L^{-1} $Zn(ClO_4)_2$ (Alfa) and 0.1 mol L^{-1} sodium hexametaphosphate (Riedel de Han) were obtained by dissolving 0.1 mol of each compound in 100 mL of water. All reagents were of analytical grade without further purification. Water used throughout was doubly deionized.

Procedures

The basic ZnS colloids were prepared as follows. The syntheses of the colloidal solutions were carried out in a 2 L three-necked round-bottomed flask with a pH electrode. 2 L of deionized water, 4.0 mL of 0.1 mol L^{-1} $Zn(ClO_4)_2$ and 4.0 mL of 0.1 mol L^{-1} sodium hexametaphosphate as the precursors and the stabilizer, respectively, were added into the flask. Then the pH was adjusted to 9.0 using 0.1 mol L^{-1} NaOH solution. Under vigorous stirring, 4.0 mL of $Na_2S.9H_2O$, was dropped slowly into the flask.

The prepared colloidal solutions are water-soluble, but they are unable to react with bio-macromolecules such as proteins. Therefore, the ZnS colloids were concentrated to 50 mL by rotary evaporation and transferred into a flask. Under vigorous stirring, 8.0 mL of 1.0 mol L^{-1} sodium thioglycolate solution was dropped slowly into the flask. The ZnS nanoparticles reacted with the sodium thioglycolate for 3 h. The mercapto group binds to a Zn atom, and the polar carboxylic acid group renders the nanoparticles water-soluble. The free carboxyl group is also available for coupling to various biomolecules such as proteins and peptides. Excess sodium thioglycolate was removed by repeated centrifugation.[20,21]

We use the functionalized nano-ZnS to detect the protein *via* changes in the relative fluorescence intensity of the system. To a 10 mL volumetric flask, 1.0 mL of buffer, a certain volume of colloids, and an appropriate volume of sample or protein working solution were added, the mixture was diluted to 10.0 mL with water, and then the fluorescence intensity was measured. The excitation wavelength was set at 288 nm and the emission wavelength at 441 nm.

Results and discussion

TEM images of nanoparticles

TEM images of ZnS nanoparticles and functionalized ZnS nanoparticles are shown in Fig. 1. The diameter of the ZnS nanoparticles is about 15 nm. The diameter of the functionalized nano-ZnS is larger by 2 nm than the starting ZnS nanoparticles.

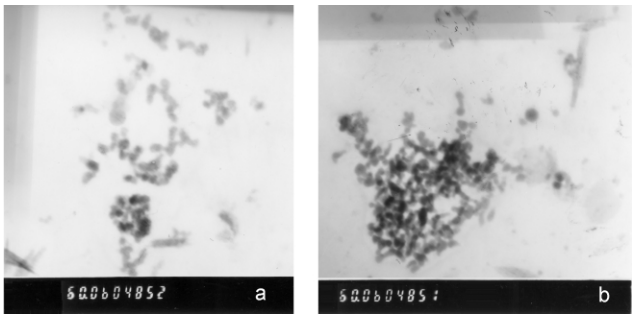

Fig. 1 (a) TEM image of ZnS colloidal solutions and (b) TEM image of functionalized ZnS colloidal solutions.

In addition, the TEM images showed that the solublization and cross-linking steps did not result in aggregation. The IR spectrum has a peak at $\nu_{C=O}$ = 1610 cm^{-1}, characteristic of the sodium thioglycolate capped onto the outer surface of the ZnS nanoparticles.

Effect of pH value

In order to determine the concentration of protein, functionalized ZnS nanoparticles were used as fluorescence probes. The effect of pH on the fluorescence intensity of the system was studied (Fig. 2). The maximum and constant fluorescence intensity occurred when the pH was 6.0. So a pH of 6.0 was recommended for use.

Effect of temperature and reaction time

The fluorescence intensity of the system dropped by increasing the temperature, and the system began to aggregate when the temperature reached 42 °C. However, increasing the temperature will increase the rate of the binding reaction of functionalized nano-ZnS and proteins. Therefore, room temperature (25 °C) was adopted in this work. The fluorescence intensity of the system can reach its maximum in 25 min and remain stable for 50 min. A 25 min incubation time was adopted in the study.

Effect of the concentration of functionalized colloidal solutions

The effect of the concentration of the functionalized colloidal solutions has also been investigated. With increasing the concentration of (ZnS)–SCH_2COONa nanoparticles, the fluorescence intensity of the system increased. However, the intensity increased very slightly after the concentration (represented by the concentration of ZnS existing in single molecules) of the functionalized ZnS colloidal solutions reached 2.5 × 10^{-4} mol L^{-1}, and it dropped slowly when the concentration of functionalized nano-ZnS reached 4.5 ×10^{-4} mol L^{-1} (see the results in Fig. 3). At last, a (ZnS)–SCH_2COONa concentration of 3.0 ×10^{-4} mol L^{-1} was recommended.

Calibration curve and sensitivity

The calibration graphs for HSA, BSA and γ-G were constructed by following the standard assay procedure. As shown in Table 1, the linear ranges for HSA, BSA and γ-G are 0.1–4.0 μg mL^{-1}, 0.2–3.0 μg mL^{-1} and 0.1–4.5 μg mL^{-1}, respectively, and the coefficients of correlation are above 0.998 (n = 6). The limits of detection are correspondingly 0.015, 0.024 and 0.017 μg mL^{-1}.

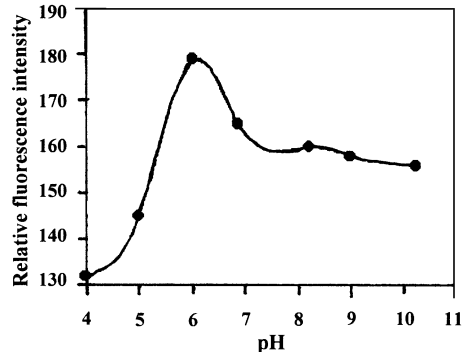

Fig. 2 Effect of pH on the fluorescence. Colloids, 3.0 × 10^{-4} mol L^{-1}.

Effect of ionic strength

The effect of NaCl content on this assay was examined at pH 6.0. The NaCl content did not affect the relative fluorescence intensity of colloids in the absence of protein. In the presence of protein, the relative fluorescence intensity remained constant with increasing salt concentration up to 6.0×10^{-3} mol L^{-1}; after that, the fluorescence intensity decreased slowly, as shown in Fig. 4. That is, low ionic strength has no effect on the assay. However, when the content of NaCl increases, the effect of the electrostatic shielding of charges, including the shielding of protein molecules from colloids and shielding of colloids from protein molecules, will reduce the binding of the colloids to protein and result in a decreased signal. The minor effect of NaCl content on the fluorescence intensity showed that the interaction of proteins and colloids was mainly a result of non-electrostatic binding (probably hydrogen bond binding).

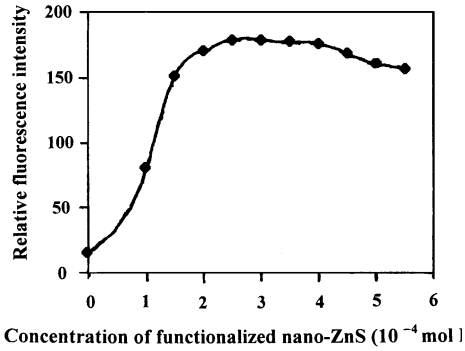

Fig. 3 Effects of the concentration of functionalized colloidal solutions on the relative fluorescence intensity, pH 6.0.

Table 1 Analytical parameters for the determination of proteins

Protein	Linear range/ μg mL^{-1}	Regression equation (C/μg mL^{-1})	LOD/μg mL^{-1}	r
HSA	0.1–4.0	$\Delta F = 0.50 + 17.94C$	0.015	0.9981
BSA	0.2–3.0	$\Delta F = 0.76 + 18.31C$	0.024	0.9983
γ-G	0.1–4.5	$\Delta F = 0.98 + 21.43C$	0.017	0.9988

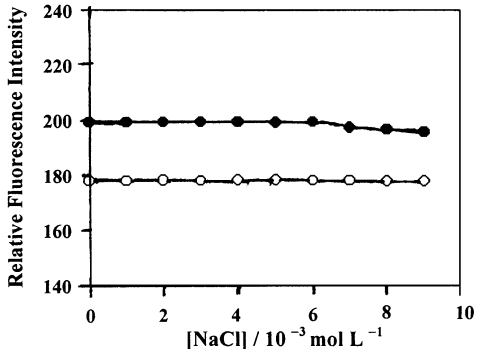

Fig. 4 Effect of NaCl solution on the relative fluorescence intensity in the presence of γ-G (filled circle) and in the absence of γ-G (open circle). Colloids: 3.0×10^{-4} mol L^{-1}; γ-G: 1.0 μg mL^{-1}.

Influence of foreign coexisting substances

As listed in Table 2, most of the examined coexisting substances have no marked interference on the assay. From the results, we can see that interference of arginine, lysine, glucose, citric acid and isoleucine is very weak. Of the tested metal ions, Mg(II), Zn(II), Fe(III), Fe(II) and Co(II) can be present at relatively higher concentrations, but Cu(II) and Ag(I) ions can only be present at relatively low concentrations. For the analysis of human serum, the interference of Cu(II) and Fe(III) can be conveniently minimized by further dilution. The allowed concentrations of these interfering substances, however, is still rather higher than that of protein, which means that this method has a high selectivity.

Mechanism of the fluorescence enhancement

Just as the CdSe–TOPO system,[24] excitation of polymer (TOPO) resulted in an electron being transferred onto the nanoparticle, which resulted in the fluorescence enhancement of the nanoparticle. In this study, the excitation peak of the functionalized nano-ZnS–proteins system at 288 nm coincided well with UV-vis absorption peak of proteins such as BSA and HSA at 284 nm. The excitation energy transferred from protein to the excitonic state of nanoparticles through the resonance energy transfer analogous to the classical Förster energy transfer.[25] The energy collected by nanoparticles from proteins resulted in the fluorescence enhancement.

Sample determination

The present method was applied to determine total protein in human serum samples. Table 3 shows the results, which are very close to those collected from hospital. The recovery and relative standard deviation are satisfactory. Therefore, the determination of protein by this method is reliable, sensitive, reproducible and practical.

Conclusions

In conclusion, the functionalized ZnS nanoparticles have a narrow, symmetric emission spectrum and are photo-chemically stable, and they also have a long fluorescence lifetime and high fluorescence quantum yields. A class of sensitive fluorescence probes has been established for which no small organic molecule equivalent exists. The functionalized nanoparticles

Table 2 Tests for the interference of coexisting substances[a]

Coexisting substance	Coexisting conc./μg mL^{-1}	Relative error (%)	Coexisting substance	Coexisting conc./μg mL^{-1}	Relative error (%)
Glysine	12.0	−4.2	Fe(II), SO_4^{2-}	16.0	−3.7
Arginine	12.0	−2.7	Zn(II), Cl^-	13.0	2.4
Citric acid	12.0	+1.8	Fe(III), Cl^-	16.0	−3.5
Isoleucine	12.0	−2.4	Cu(II), SO_4^{2-}	12.0	−4.2
Lysine	12.0	−2.3	Ca(II), Cl^-	8.0	−2.1
Glucose	12.0	−2.6	Mg(II), Cl^-	12.0	−2.8
Co(II), SO_4^{2-}	14.0	−3.1	Ag(I), Cl^-	7.2	−4.8

[a] γ-G 1.0 μg mL^{-1}; Functionalized ZnS 3.0×10^{-4} mol L^{-1}; pH 6.0.

Table 3 Analytical results for human serum samples[a]

Human serum sample	Content of protein: Clinical data/ mg mL^{-1}[b]	This method/ mg mL^{-1}, n = 6	Recovery (%, n = 6)	RSD (%)
1	72.2	72.9	98–106	2.0
2	73.1	72.3	96–102	1.6
3	74.5	73.7	97–103	1.5

[a] Functionalized ZnS 3.0 × 10^{-4} mol L^{-1}; pH 6.0. [b] Data from hospital.

have been used as probes for sensitive determination with the advantages of simplicity, rapidity and sensitivity. We envisage that the functionalized nanoparticles can be applied to the detection of nucleic acid sequences, immunoassays and clinical diagnostics.[26–28] Further studies in this field will open up the way to the application of nano-materials in analytical chemistry and analytical biochemistry.

Acknowledgements

This work (99JL079) was supported by the distinguished teacher program grant of National Education Commission of China and all the authors here express their deep thanks.

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