Microchim. Acta 147, 81–86 (2004)
DOI 10.1007/s00604-004-0191-9

Microchimica Acta
Printed in Austria

Original Paper

Application of Functionalized Ag Nanoparticles for the Determination of Proteins at Nanogram Levels Using the Resonance Light Scattering Method

Bin Fang, Yingchun Gao, Maoguo Li, Guangfeng Wang, and **Yongxin Li***

College of Chemistry and Materials Science, Anhui Normal University, Wuhu 241000, People's Republic of China

Received July 20, 2003; accepted December 15, 2003; published online May 21, 2004

Abstract. A novel method for the determination of proteins in aqueous solutions has been developed based on the enhancement of resonance light scattering (RLS) of Ag nanoparticles in the presence of proteins. Factors including acidity of the media, concentration of Ag hydrosol, reaction time, temperature, and interference of non-protein substances were investigated. Under the optimal conditions, with the enhanced RLS signals at 452 nm, the linear ranges of calibration curves were 0–0.8 $\mu g\,mL^{-1}$ for bovine serum albumin (BSA), 0–1.2 $\mu g\,mL^{-1}$ for human serum albumin (HSA), and 0–2.5 $\mu g\,mL^{-1}$ for human γ-IgG (γ-IgG), respectively. The detection limits were 1.3 $ng\,mL^{-1}$ for BSA, 10 $ng\,mL^{-1}$ for HAS, and 5.7 $ng\,mL^{-1}$ for γ-IgG.

This method has been applied to the analysis of synthetic samples and real human serum samples, and the results were in good agreement with those reported by the hospital, indicating that the method presented here is not only sensitive and simple, but also reliable and suitable for practical applications.

Key words: Ag nanoparticles; proteins; resonance light scattering.

Determination of protein is essential in biochemistry and clinical medicine because it is often used as a reference for the measurements of other components in biological systems. The most frequently used methods are the Lowry [1], Coomassie brilliant blue (CBB) [2], bromophenol blue [3, 4], and bromocresol green [5] methods. However, they all have some limitations in terms of sensitivity, selectivity, stability and simplicity. Therefore, a number of assays have been reported in recent years, such as those based on spectrophotometric [6, 7], flurometric [8, 9], electrochemical [10] and chemiluminescence methods [11]. At present, the most sensitive quantitation of protein is based on their fluorescence enhancement effect on organic dyes. Yet the organic fluorophores often suffer from photobleaching, low signal intensities, and random on/off light emission (blinking) [12, 13].

Resonance light scattering (RLS) from both small and large molecules in the various states of matter has been extensively studied and applied for many years [14, 15]. Pasternack et al. developed a technique to detect the RLS by using a common spectrofluorimeter [16, 17], which simplifies the detection process (without turbidimeter) and makes this technique not only highly sensitive and selective but also simple. In this contribution, Huang has used the RLS method for analytical purposes to determine the quantities of nucleic acids and proteins [18, 19]. A method for the determination of proteins in aqueous solution has also been developed based on the enhancement effect of protein on RLS of organic dyes such as Acid

* Author for correspondence. E-mail: liyx72@yahoo.com

Green 25 [20], Acid Chrome Blue K [21], α, β, γ, δ-tetrakis (4-sulfophenyl) porphyrin ($TPPS_4$) [22], Arsenazo III [23, 24].

The study of nano-sized materials has attracted tremendous attention because of their potential use in microelectronics, catalysis, and chemical and biological sensors [25–27]. Nie et al. [28] have determined proteins with the functionalized ZnS-capped CdSe nanoparticles as a fluorescence probe. However, as far as we know, application of functionalized Ag nanoparticles as a new reagent to the RLS determination of proteins has not been reported yet. In this work, we synthesized a kind of silver nanoparticle stabilized by oleate and found that the interaction of proteins and Ag hydrosol results in strong enhancement of the RLS intensity. Based on this phenomenon, we developed a new method for the determination of proteins by the RLS technique. Compared with other RLS methods, this method is rapid, simple and very sensitive. In addition, functionalized Ag nanoparticles were easily synthesized, more stable and highly resistant to photobleaching. The present method was applied to the determination of total protein in real human serum samples with satisfactory results.

Experimental

Apparatus

All RLS measurements were performed using a Hitachi F-2500 Fluorescence Spectrophotometer (Tokyo, Japan) equipped with a plotter unit and a 1 cm quartz cell. The absorption spectra were recorded with a UV-Vis 3010 Spectrophotometer (Tokyo, Japan). The transmission electron microscopy (TEM) images of the nanoparticles were obtained by means of a Hitachi-600 transmission electron microscopy (Tokyo, Japan). The colloidal solution of the nanoparticles was dropped onto 50 Å thick carbon-coated copper grids with excess solution immediately wicked away. A pHS-3C pH meter (Shanghai China) was used for accurate adjustment of pH.

Reagents

Stock solutions of proteins ($100\,\mu g\,mL^{-1}$) were prepared dissolving commercial bovine serum albumin (BSA), human serum albumin (HSA), human γ-IgG (γ-IgG) (Sino-American Bio-Technology, Shanghai, China) in water, and storing at $0 \sim 4\,^{\circ}C$. More dilute solutions were prepared as required. Sliver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$), sodium oleate and other reagents were of analytical reagent grade without further purification. Freshly twice-quartz-distilled water was used in all experiments.

Preparation of Oleate-Stabilized Silver Nanoparticles

The oleate-stabilized silver nanoparticles were synthesized by $NaBH_4$ reduction [29]. The solution of $AgNO_3$ ($5 \times 10^{-4}\,mol\,L^{-1}$) was added dropwise into freshly prepared $NaBH_4$ ($2 \times 10^{-2}\,mol\,L^{-1}$) containing stabilizer, while vigorously stirring and keeping ice cold. Brown-yellowish colloidal solutions stabilized by oleate were obtained. Then they were taken out and exposed to room temperature.

General Procedure

Into a 10.0 mL volumetric flask were added 1.0 mL buffer, 1.0 mL Ag hydrosol, and a certain volume of standard solutions of proteins (or sample). The mixture was diluted to 10.0 mL with water and mixed thoroughly before the RLS intensity was measured by scanning synchronously with the same excitation and emission wavelengths ($\Delta\lambda = 0.0$ nm) through the wavelength range of $220 \sim 750$ nm. Based on these spectra, the intensity of RLS was measured with excitation and emission at 452 nm with a slit width of 2.5 nm for excitation and emission.

Results and Discussion

Characteristic of Ag Nanoparticles

Figure 1 shows the characteristic UV-vis absorption of the hydrosol of oleate-capped silver nanoparticles. The band has a peak at 414 nm and a full width at the half-maximum of 79 nm, characteristic of rather monodispersed small silver particles [30], while uncapped silver colloid exhibits absorption with a maximum at 390 nm [29]. The shift of the absorption band provided clear evidence of the adsorption of oleate on the silver particles [31]. The good symmetric absorption peaks with a nearly unchanged width imply that the size of the nanoparticles is very uniform. To detect the stability of the oleate-protected hydrosol of silver nanoparticles, we measured the absorption spectra of the

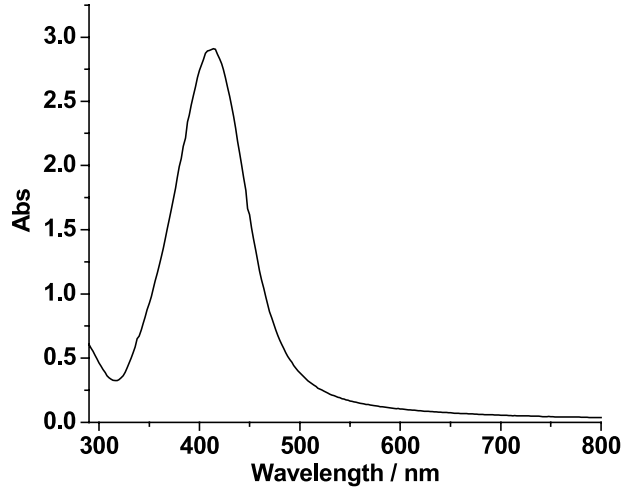

Fig. 1. UV-vis spectrum of the silver nanoparticles capped by oleate

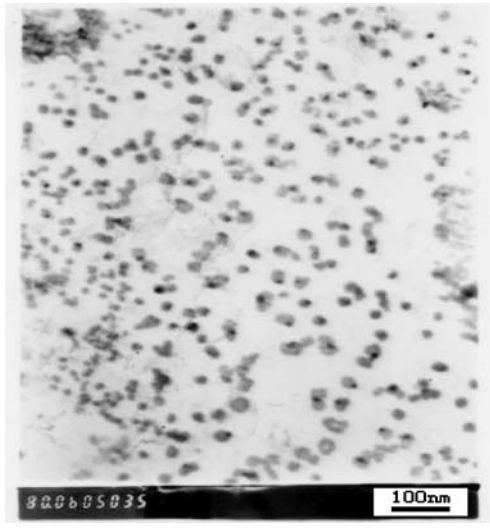

Fig. 2. Transmission electron microscopy of the silver nanoparticles capped by oleate

same hydrosol at different times. There is no obvious difference in the shape, position, and symmetry of the absorption peak during 30 days.

The nanoparticles were also examined by transmission electron microscopy, as shown in Fig. 2. Calculation showed that the mean diameter (D) of the small silver particles is 21.6 nm and that the relative standard deviation (σ) is 2.64% (both figures deriving from an average of 200 particles). The polydispersity defined as the ratio σ/D is 0.122.

Reaction and Spectral Characteristics

The spectra of the Ag hydrosol and the complex under optimum conditions are shown in Fig. 3. It can be seen from Fig. 3 that the light scattering of Ag hydrosol is comparatively small in the scanning region. However, when micro protein is present, the obviously enhanced light scattering can be observed at 289, 413, 452, and 495 nm, and the enhancement at 452 nm reaches the maximum. So for subsequent work, the measurement wavelength was set to 452 nm. In addition, due to the high scattering signal of colloidal silver, all the data in the spectra was obtained keeping the scale of the spectrofluorometer at the lowest level. Thus, the scattering light reading was stable, and the background signal was generally negligible.

Optimization of the General Procedures

The effect of pH on RLS enhancement of the system was studied in the range of 1∼10 (Fig. 4). Although each protein featured different degrees of enhancement, the maximum and constant RLS intensities occurred when pH was 5.0. When the pH was lower than 5.0, the intensity of RLS decreased rapidly, because under the strong acid conditions the Ag nanoparticles can not exist stably. When the pH was higher than 5.0, the intensity of RLS was also decreased. This is because under alkaline conditions the oleate adsorbed on the Ag nanoparticles can transform into $-\mathrm{COO}^-$, which can decrease the binding of the Ag nanoparticles and proteins. So an HAC-NaAC buffer of pH 5.0 was recommended for use.

The effect of the concentration of Ag hydrosol was also studied in the range of $2.5\times10^{-6}\sim$

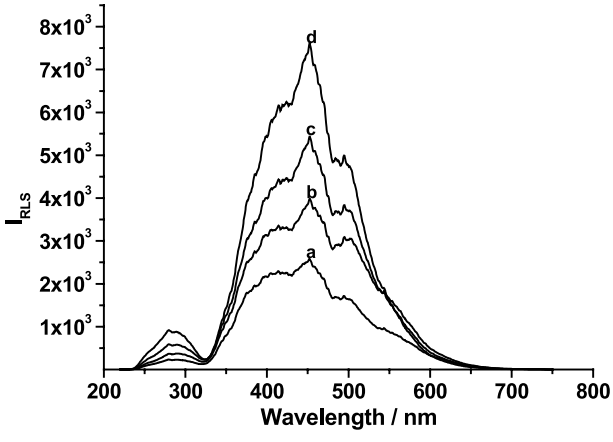

Fig. 3. Resonance light scattering spectra for Ag hydrosol with BSA. (*a*) In the absence of BSA, (*b*, *c*, *d*) in the presence of BSA 0.25, 0.5, 0.75 μg mL^{-1}. (Under the optimized conditions)

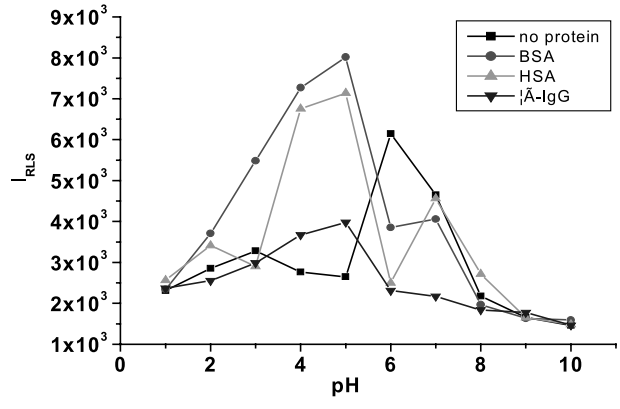

Fig. 4. Effect of pH on the enhancement of resonance light scattering in the presence of various proteins. All the proteins were 1.0 μg mL^{-1}, the concentration of Ag hydrosol was 2.5×10^{-5} mol L^{-1}

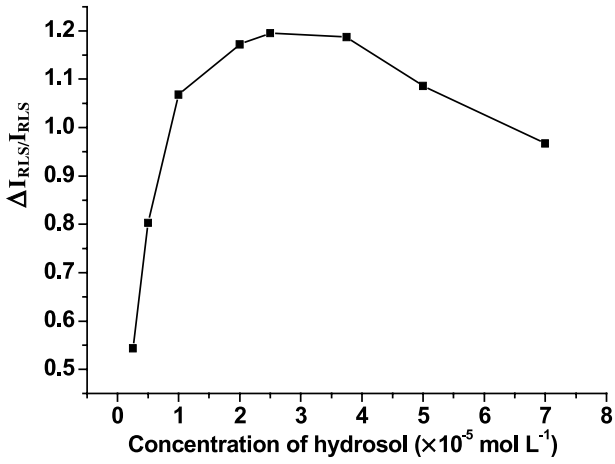

Fig. 5. Effect of concentration of Ag hydrosol on $\Delta I_{RLS}/I_{RLS}$. Concentration of BSA was 0.5 μg mL^{-1}, pH = 5.0

1×10^{-4} mol L^{-1} (Fig. 5). The experimental results indicated that the complex and constant RLS intensities were obtained when the concentration of Ag hydrosol was 2.5×10^{-5} mol L^{-1}. With the increase in concentration of Ag hydrosol, the relative intensity of RLS (ΔI_{RLS}) also increases until it reaches the maximum and stable value when the concentration of Ag hydrosol exceeds 2.0×10^{-5} mol L^{-1}. We found that the sensitivity was relatively low, and the stability of the complex decreased when the concentration of Ag hydrosol was too high. Thus, we used an Ag hydrosol concentration of 2.5×10^{-5} mol L^{-1} in this study.

In order to study the interaction of Ag hydrosol and the proteins, we studied the influence of ionic strength on RLS intensity. It was found that the intensity of RLS remained constant with increasing (NaCl) concentration. This phenomenon suggests that the interaction between Ag hydrosol and the proteins is non-electrostatic binding [32].

It was found that the reaction between Ag hydrosol and proteins occurs rapidly (within 5 min) at room temperature, and the signal is stable for 1 hour. Then the intensity of RLS decreases with increasing time. Thus, the experiment was completed in 1 h. The influence of temperature on RLS intensity was also investigated. The results showed that the temperature had little influence on the intensity of RLS. In this study the temperature was 25 °C.

Interference of Coexisting Foreign Substances

The possible interference of various ions and amino acids was checked at 1.0 μg mL^{-1} of BSA under the conditions of the general procedure. The results of the interference study are summarized in Table 1. Of the metal ions tested, Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} can be allowed at relatively higher concentrations, whereas Ag^{+}, Pb^{2+}, Hg^{2+} and Fe^{3+} ions are tolerated at relatively lower concentrations. The allowed concentration of these interference ions is still larger than those in biological fluids. So, no special preparations were made before sample determination.

Table 1. Effects of non-protein substances on the determination of BSA

No.	Substances	Concentration (μg mL^{-1})	Change in I_{RLS}(%)
1	Cr^{3+}	10	0.7
2	Fe^{3+}	10	−3.0
3	Ca^{2+}	20	0.05
4	Ba^{2+}	20	5.2
5	Cd^{2+}	10	1.5
6	Zn^{2+}	5	1.3
7	Mg^{2+}	10	−1.8
8	Co^{2+}	10	0.8
9	Mn^{2+}	10	−0.9
10	Hg^{2+}	2	−0.8
11	Pb^{2+}	2	1.9
12	Ag^{+}	10	8.6
13	Br^{-}	20	2.7
14	F^{-}	20	1.0
15	L-Ser	40	−0.2
16	DL-Ala	40	2.8
17	L-Leu	40	0.3
18	Gly	40	1.5
19	L-Lys	40	3.4
20	L-Cys	40	0.3
21	L-Arg	40	−2.8
22	L-Pro	40	−2.8
23	L-Val	40	−0.8
24	L-Phe	40	3.2
25	DL-Asp	40	1.2

Concentration of BSA was 1.0 μg mL^{-1}, concentration of Ag hydrosol was 2.5×10^{-5} mol L^{-1}, HAc-NaAc buffer pH = 5.0. Each result was the average of five measurements.

Calibration Curve and Sensitivity

Under the optimized experimental conditions described above, the relation between ΔI_{RLS} and the concentration of protein was obtained. The results are shown in Table 2. They indicate that the limit of detection is lower than that of coomassie brilliant blue protein assay. So it is very clear that this method is sensitive.

The limit of detection is calculated using the following formula

$$C_L = kS_{b1}/S$$

Table 2. Analytical parameters for the determination of different proteins

Protein	Linear range ($\mu g\,mL^{-1}$)	Linear regression equation ($\mu g\,mL^{-1}$)	Determination limit ($ng\,mL^{-1}$)	Correlation coefficient
BSA	0–0.8	$\Delta I_{RLS} = -14 + 8029c$	1.3	0.9908
HSA	0–1.2	$\Delta I_{RLS} = 8.6 + 1023c$	10.2	0.9957
γ-IgG	0–2.5	$\Delta I_{RLS} = 9.3 + 1840c$	5.7	0.9975

Concentration of Ag hydrosol was $2.5 \times 10^{-5}\,mol\,L^{-1}$ HAc-NaAc buffer pH = 5.0.

Table 3. Determination results for synthetic samples containing non-protein substances

Protein samples	Non-protein[a]	Found (n = 5,%)	Relative standard deviation (n = 5,%)	Recovery
$10.0\,\mu g\,mL^{-1}$ BSA	Ca^{2+}, Pb^{2+} Pro, Leu, Cys	$10.24 + 0.04$	0.02	98
$10.0\,\mu g\,mL^{-1}$ HSA	Ca^{2+}, Pb^{2+} Pro, Leu, Cys	$10.37 + 0.11$	0.08	97.5
$10.0\,\mu g\,mL^{-1}$ γ-IgG	Ca^{2+}, Pb^{2+} Pro, Leu, Cys	$9.97 + 0.17$	0.14	100.3

[a] Non-protein substances, $10\,\mu g\,mL^{-1}$ Ca^{2+}; $10\,\mu g\,mL^{-1}$ Pb^{2+}; $40\,\mu g\,mL^{-1}$ Pro, Leu, Cys. Concentration of Ag hydrosol was $2.5 \times 10^{-5}\,mol\,L^{-1}$. HAc-NaAc buffer pH = 5.0. Each result was the average of five measurements.

where C_L is the limit of detection; k is a constant related to the confidence level, using the suggestion of the IUPAC, $k = 3$; S_{b1} is the standard deviation of 8 blank measurements, and S is the slope of the calibration graph.

Sample Determination

Synthetic samples for BSA, HSA or γ-IgG containing metal ions and amino acids were analyzed. As can be seen from Table 3, the contents of BSA, HSA or γ-IgG synthetic samples can be determined with good reproducibility.

The present method was also applied to quantify protein in real human serum samples. Human serum samples, obtained from the Second Remin Hospital of Wuhu, were stored at $0 \sim 4\,^{\circ}C$ and diluted 1000-fold with distilled water without pretreatment prior to determination. Under the optimized experimental conditions, a new linear equation was obtained: $\Delta I_{RLS} = 7.9 + 8756c$. The results are displayed in Table 4. It is clear that the results are in good agreement with the data obtained from the hospital. Therefore, determination of proteins using this method is reliable, sensitive and practical.

Mechanism of the Reaction

The enhancement of RLS is closely connected with the diameter of the nanoparticles, so the TEM image of the complex of the Ag nanoparticles and the proteins was obtained (Fig. 6). From these figures it can be seen that the aggregate size of Ag hydrosol in the presence of protein is much larger than that in the absence of protein. This research shows that the enhanced resonance light scattering stems from particles of much larger size than that of the Ag nanoparticles and proteins. It suggests that the proteins bind to the Ag nanoparticles. Under weak acid conditions (pH = 5.0), the oleate adsorbed on the Ag nanoparticles can transform into –COOH, which can bond with the remainder $-NH_2$ in proteins.

Table 4. Total content of proteins in human serum samples

Sample	Content of protein ($mg\,mL^{-1}$)		Recovery (%) (n = 5)	R.S.D (%)
	The clinical data[a]	This method		
1	75.3	$75.8 + 0.2$	100.6	−0.12
2	75.5	$76.4 + 0.4$	101.2	−0.32
3	79.1	$79.7 + 0.8$	100.7	−0.83

Concentration of Ag hydrosol was $2.5 \times 10^{-5}\,mol\,L^{-1}$. HAc-NaAc buffer pH = 5.0. Each result was the average of five measurements.
[a] Data acquired from the hospital.

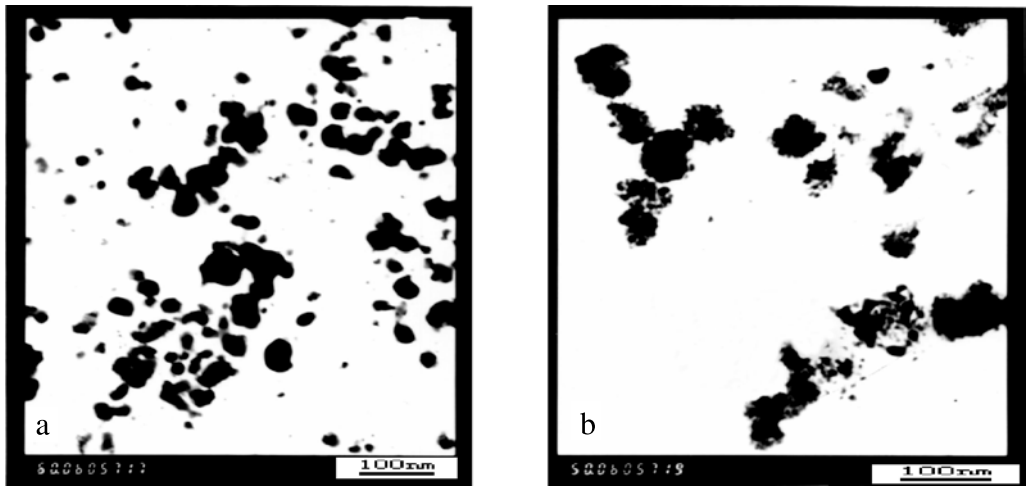

Fig. 6. Transmission electron microscopy of the silver nanoparticles in the presence of proteins. (a) BSA 0.5 μg mL^{-1} (b) HSA 0.5 μg mL^{-1}

Conclusion

Based on the enhancement of RLS intensity by binding of the proteins and Ag hydrosol, a new method is proposed for determination. The present method has been applied to the determination of protein in human serum samples, and the results are in good agreement with those obtained by hospital physicians. Compared with other methods, the method has the advantages of high sensitivity and simplicity.

Acknowledgements. The authors gratefully acknowledge the financial support provided by the Science and Technology Foundation of the Ministry of Education of China (NO. 00117).

References

[1] Lowry O H, Rosebrough N J, Farr A L, Randall R J (1957) J Biol Chem 193: 265–275
[2] Bradford M M (1976) Anal Biochem 72: 248–254
[3] Flores R (1978) Anal Biochem 88: 605–613
[4] Jung K, Nickel E, Pergande M (1990) Clin Chim Acta 187: 163–172
[5] Doumas B T, Watson W A, Biggs H G (1971) Clin Chim Acta 31: 87–96
[6] Guo Z X, Hao Y M, Cong X, Shen H X (2000) Anal Chim Acta 31: 225–233
[7] Zhu K, Li K A, Tong S Y (1996) Anal Lett 29: 575–585
[8] Li D H, Yang H H, Zhen H, Gang Y, Zhu Q Z, Xu J G (1999) Anal Chim Acta 401: 185–189
[9] Nakamura J, Igarashi S (1996) Anal Lett 29: 981
[10] Zhang H M, Zhu Z W, Li N Q (1999) Fresenius' J Anal Chem 363: 408–412
[11] Tsakagoshi K, Okumura Y, Akasaka H, Nakajima R, Hara T (1996) Anal Sci 12: 525–528
[12] Bruchez M, Moronne M Jr, Gin P, Weiss S, Alivisatos A P (1998) Science 281: 2013–2016
[13] Mattoussi H, Manro J, Goldman E R, Anderson G P, Sundar V C, Mikula F V, Bawendi M G (2000) J Am Chem Soc 122: 12142–12150
[14] Cao Q E, Ding Z T, Fang R B, Zhao X (2001) Analyst 126: 1444–1448
[15] Huang C Z, Li Y F, Feng P (2001) Anal Chim Acta 443: 73–80
[16] Pasternack R F, Collings P J (1995) Science 269: 935–940
[17] Pasternack R F, Bustmante C, Collings P J, Giannetto A, Gibbs E J (1993) J Am Chem Soc 115: 5393–5399
[18] Huang C Z, Li K A, Tong S Y (1996) Anal Chem 68: 2259–2263
[19] Huang C Z, Li K A, Tong S Y (1997) Anal Chem 69: 514–520
[20] Ma C Q, Li K A, Tong S Y (1997) Anal Chim Acta 338: 255–260
[21] Ma C Q, Li K A, Tong S Y (1997) Analyst 122: 361–364
[22] Huang C Z, Li Y F, Feng P (2001) Anal Chim Acta 443: 73–80
[23] Tao G, Li K A, Tong S Y (1998) Anal Lett 31: 10–21
[24] Jia R P, Dong L J, Li Q F, Chen X G, Hu Z D (2002) Talanta 57: 693–700
[25] Tian Y, Tewton T, Kotov N A, Guldi D M, Fendler J H (1996) J Phys Chem B 100: 8927–8939
[26] Hines M A, Guyot-Sionnest P J H (1996) J Phys Chem B 100: 468–479
[27] Wang L Y, Zhou Y Y, Wang L, Zhu C Q, Li Y X, Gao F (2002) Anal Chim Acta 4669: 87–92
[28] Chan W C W, Nie S (1998) Science 281: 2016–2018
[29] Wang W, Efrima S, Regev O (1998) Langmuir 14: 602–608
[30] Creighton J A, Eaton D G (1991) J Chem Soc Faraday Trans 87: 3881–3891
[31] Kerker M (1985) J Colloid interface Sci 105: 297–302
[32] Li Q F, Liu S H, Zhang H Y, Hu Z D (2001) Anal Lett 34: 1133–1142