Available online at www.sciencedirect.com
SCIENCE DIRECT®
Talanta 65 (2005) 206–210
Talanta
www.elsevier.com/locate/talanta

Development of functionalized terbium fluorescent nanoparticles for antibody labeling and time-resolved fluoroimmunoassay application

Zhiqiang Ye, Mingqian Tan, Guilan Wang, Jingli Yuan*

Department of Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116012, PR China

Received 1 March 2004; received in revised form 7 April 2004; accepted 15 June 2004
Available online 31 July 2004

Abstract

Silica-based functionalized terbium fluorescent nanoparticles were prepared, characterized and developed as a fluorescence probe for antibody labeling and time-resolved fluoroimmunoassay. The nanoparticles were prepared in a water-in-oil (W/O) microemulsion containing a strongly fluorescent Tb^{3+} chelate, *N*,*N*,*N*^{1},*N*^{1}-[2,6-bis(3′-aminomethyl-1′-pyrazolyl)phenylpyridine] tetrakis(acetate)-Tb^{3+} (BPTA-Tb^{3+}), Triton X-100, octanol, and cyclohexane by controlling copolymerization of tetraethyl orthosilicate (TEOS) and 3-[2-(2-aminoethylamino)-ethylamino]propyl-trimethoxysilane (AEPS) with ammonia water. The characterizations by transmission electron microscopy and fluorometric methods show that the nanoparticles are spherical and uniform in size, 45 ± 3 nm in diameter, strongly fluorescent with fluorescence quantum yield of 10% and a long fluorescence lifetime of 2.0 ms. The amino groups directly introduced to the nanoparticle's surface by using AEPS in the preparation made the surface modification and bioconjugation of the nanoparticles easier. The nanoparticle-labeled anti-human α-fetoprotein antibody was prepared and used for time-resolved fluoroimmunoassay of α-fetoprotein (AFP) in human serum samples. The assay response is linear from 0.10 ng ml^{-1} to about 100 ng ml^{-1} with the detection limit of 0.10 ng ml^{-1}. The coefficient variations (CVs) of the method are less than 9.0%, and the recoveries are in the range of 84–98% for human serum sample measurements.

Keywords: Functionalized terbium nanoparticles; Fluorescence; α-Fetoprotein; Time-resolved fluoroimmunoassay

1. Introduction

One of the most important developments of nanobiotechnology is the preparation of highly luminescent nanoparticles for sensitive biological detection applications recently. A variety of techniques have been developed to prepare different types of luminescent nanoparticles [1–8], some of them, such as semiconductor nanoparticles (quantum dots) [2–5], plasmon-resonant nanoparticles [6], and luminophore-doped silica nanoparticles [7,8], have been successfully used as luminescence probes for biological detections. Compared to conventional organic fluorescence probes, advantages of the nanometer-sized luminescence probes mainly include their higher photostability and stronger luminescence. The main problem of using nanometer-sized luminescence probes in biological detections is that the luminescence measurement is easily affected by the strong non-specific scattering lights, such as Tyndall, Rayleigh and Raman scatterings. This problem has limited their effective application for quantitative bioassays.

Time-resolved fluoroimmunoassay (TR-FIA) using lanthanide chelates as the fluorescence probes has been widely used for clinical diagnostics and biological researches [9–12]. Since the fluorescence lifetimes of lanthanide fluorescence probes are very long, the strong background noises from the coexisting biological compounds, the scattering lights, and the optical components in ultrasensitive bioassays can be effectively eliminated by time-resolved fluorescence measurement. Therefore, this technique is considerably favorable for bioassays using nanoparticles as the luminescence probe if only the nanoparticle probe has a luminescence lifetime long enough for time-resolved luminescence measurement. On the other hand, the TR-FIAs using fluorescent lanthanide chelate conjugated macromolecules, such as polyvinylamine [13–15], poly(Glu:Lys)-streptavidin conjugate [16], and polystyrene latex particles [17–21], as

* Corresponding author. Tel./fax: +86-411-83693509.
E-mail address: jingliyuan@yahoo.com.cn (J. Yuan).

0039-9140/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2004.06.008

the fluorescence probes have shown that the sensitivity of TR-FIA can be remarkably improved since the fluorescence intensities of the macromolecule probes have been enhanced by attaching many fluorescent lanthanide molecules to the macromolecule probes.

Recently, we developed a method to prepare the BPTA-Tb^{3+} chelate-doped silica nanoparticles and established a highly sensitive TR-FIA method by using the Tb^{3+} chelate nanoparticle-labeled streptavidin [22], in which the advantages using silica-based lanthanide fluorescent nanoparticles as a fluorescence probe for time-resolved fluorescence bioassay have been discussed. However, the tedious surface activation procedure of the nanoparticles before the biolabeling was needed [22]. In the present work, the preparation method of BPTA-Tb^{3+} chelate-doped silica nanoparticles was further improved by using a copolymerization method between TEOS and AEPS in a water-in-oil microemulsion consisting of BPTA-Tb^{3+} chelate, Triton X-100, octanol, cyclohexane, water, and ammonia water. The new nanoparticles are spherical and uniform in size, strongly fluorescent having larger fluorescence quantum yield and a long fluorescence lifetime. Since amino groups have been directly introduced to the nanoparticle's surface by the copolymerization method, the nanoparticles are directly available for biolabeling without tedious surface activation procedure [7,22,23]. By using the nanoparticle-labeled anti-human AFP antibody, a simple TR-FIA method for determination of AFP in human serum samples was established to evaluate the utility of the fluorescent nanoparticles as a new type of fluorescence probe. The results reveal that the method is sensitive and adaptable with good precision and accuracy.

2. Experimental

2.1. Materials and instrumentation

The BPTA-Tb^{3+} chelate was synthesized by using a previous method [24]. Tetraethyl orthosilicate, AEPS, and Triton X-100 were purchased from Acros Organics. Mouse monoclonal and goat polyclonal anti-human AFP antibodies were purchased from OEM Concepts Co. and Nippon Bio-Test Laboratories Inc., respectively. The standard solutions of human AFP were prepared by diluting an α-1-fetoprotein solution (Dakopatts, Denmark) with 0.05 M Tris–HCl buffer of pH 7.8 containing 5% bovine serum albumin (BSA), 0.9% NaCl and 0.1% NaN_3. Human serum samples obtained from healthy people were stored at −20 °C before use. Unless otherwise stated, all chemical materials were purchased from commercial sources and used without further purification.

A JEOL model JEM-2000EX transmission electron microscope was used for measuring the shape and size of the nanoparticles. Fluorescence spectra and emission lifetime were measured on a Perkin Elmer LS 50B spectrofluorometer. UV–vis absorption spectra were measured on a Shanghai Tianmei UV7500 UV–vis spectrophotometer. The TR-FIA was carried out with a FluoroNunc 96-well microtiter plate as the solid-phase carrier and measured on a Perkin Elmer Victor 1420 multilabel counter with the conditions of excitation wavelength, 320 nm, emission wavelength, 545 nm, delay time, 0.2 ms, and window time, 0.4 ms.

2.2. Preparation of nanoparticles

A W/O microemulsion containing 2.38 g Triton X-100, 1.88 g octanol, 7.25 g cyclohexane, 200 μl TEOS and 1.1 ml aqueous solution of 160 mg BPTA-Tb^{3+} was mixed with a W/O microemulsion containing 2.38 g Triton X-100, 1.88 g octanol, 7.25 g cyclohexane and 200 μl concentrated ammonia water with vigorous stirring. After stirring for 5 h, 6 μl of AEPS was added, and the solution was stirred continuously for 24 h at room temperature. The nanoparticles were isolated by adding acetone, centrifuging, and washing with ethanol and water several times to remove any surfactant and unreacted materials. The obtained white Tb^{3+} chelate-doped silica fluorescent nanoparticles were dried in a desiccator for the following use.

2.3. Preparation of the nanoparticle-labeled anti-human AFP antibody

The nanoparticles (2.0 mg) and 0.1 ml of 1% glutaraldehyde were added to a solution of 20 mg BSA in 1.0 ml of 0.1 M phosphate buffer of pH 7.0. After stirring at 4 °C for 24 h, the nanoparticles were centrifuged and washed with water. The nanoparticles were suspended in 1.0 ml of 0.1 M phosphate buffer of pH 7.0, and then 0.25 mg of goat polyclonal anti-human AFP antibody and 0.1 ml of 1% glutaraldehyde were added. The solution was stirred at 4 °C for 24 h again. Then 2.0 mg of $NaBH_4$ was added, and the suspension was incubated at room temperature for 2 h. The nanoparticle-labeled anti-AFP antibody was centrifuged, washed with phosphate buffer and water, and diluted with 0.05 M Tris–HCl buffer of pH 7.8 containing 0.2% BSA, 0.1% NaN_3 and 0.9% NaCl, and stored at 4 °C before use.

2.4. TR-FIA of AFP in human serium samples

After anti-human AFP monoclonal antibody (diluted to 10 μg ml^{-1} with 0.1 M carbonate buffer of pH 9.6) was coated on the wells (50 μl per well) of a 96-well microtiter plate by physical adsorption [25], 45 μl of AFP standard solution or human serum sample was added to each well. The plate was incubated at 37 °C for 1 h, and washed twice with 0.05 M Tris–HCl buffer of pH 7.8 containing 0.05% Tween 20, and once with 0.05 M Tris–HCl buffer of pH 7.8. Then 45 μl of the nanoparticle-labeled anti-AFP antibody was added to each well, and the plate was incubated at 37 °C for 1 h. The plate was washed four times with 0.05 M Tris–HCl buffer of pH 7.8 containing 0.05% Tween 20, and subjected to solid-phase time-resolved fluorometric measurement.

3. Results and discussion

3.1. Preparation of nanoparticles

The W/O microemulsion route is an easy and efficient method for the preparation of nanoparticles over other existing methodologies [26]. Generally, TEOS and AEPS undergo hydrolysis and copolymerization reactions simultaneously to form the monodisperse spherical nanoparticles. However, it was found that the nanoparticles prepared by this method have a wide size distribution. This phenomenon might be due to the faster hydrolysis of AEPS than that of TEOS, which forms a layer of amino groups on the surface of nanoparticles, and thus the further formation of Si–O–Si bond on the nanoparticle's surface is obstructed. In the present work, this problem was solved successfully by delaying the addition time of AEPS after 5 h hydrolysis of TEOS in W/O microemulsion. As shown in Fig. 1, the nanoparticles prepared by this method are spherical and very uniform in size distribution, 45 nm in diameter, with a standard deviation of less than 7% (±3 nm). The dark dots embedded inside the silica network that can be observed on the TEM image of higher solution (the inset TEM image in Fig. 1) show that the Tb^{3+} chelate molecules in the nanoparticles exist by physically interacting with the silica network. Free amino groups directly introduced to the nanoparticle's surface by using AEPS in the nanoparticle preparation made the surface modification and bioconjugation of the nanoparticles easier.

3.2. Fluorescence characterization of nanoparticles

Time-resolved fluorescence spectra of pure BPTA-Tb^{3+} chelate and the nanoparticles in water are shown in Fig. 2. Both the Tb^{3+} chelate and the nanoparticles show the same excitation and emission maximum wavelengths at 324 and 543 nm, respectively. The emission patterns of pure BPTA-Tb^{3+} chelate and the nanoparticles are typical for the Tb^{3+} fluorescent compounds, and four sharp emission peaks at 489, 543, 582 and 620 nm correspond to the $^5D_4 \rightarrow {}^7F_6$ (489 nm), $^5D_4 \rightarrow {}^7F_5$ (543 nm), $^5D_4 \rightarrow {}^7F_4$ (582 nm), and $^5D_4 \rightarrow {}^7F_3$ (620 nm) transitions of Tb^{3+} ion [27]. The fluorescence lifetime of the nanoparticles in aqueous solution was measured to be 2.0 ms, which shows that the nanoparticles have enough of a long fluorescence lifetime for time-resolved fluorescence measurement.

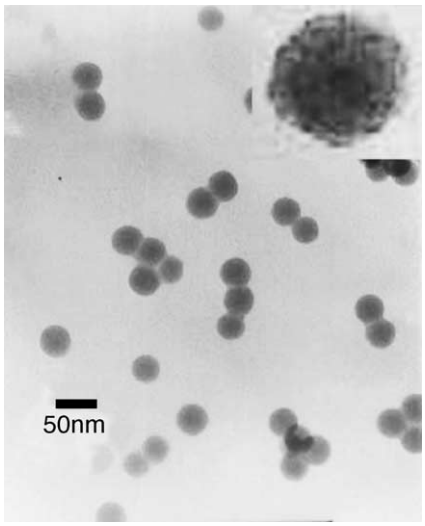

Fig. 1. TEM image of BPTA-Tb^{3+} chelate-doped silica nanoparticles at 100,000× magnification (inset showing a higher resolution image of one nanoparticle at 200,000× magnification).

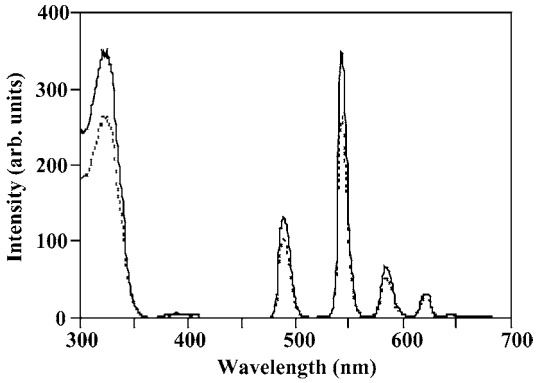

Fig. 2. Time-resolved fluorescence excitation and emission spectra of pure BPTA-Tb^{3+} chelate (1.1 μM, solid lines) and the nanoparticles (20 mg l^{-1}, dashed lines) in water.

The fluorescence quantum yield of the nanoparticles in 0.05 M borate buffer of pH 9.1 was measured to be 10.0% (the experimental uncertainty <15%) by using a reported method [28] and calculated using the equation of $\phi_1 = I_1A_2\phi_2/I_2A_1$ with a standard quantum yield [29] of $\phi_2 = 10.0\%$ for a Tb^{3+} chelate of *N*,*N*,N^1,N^1-[4′-phenyl-2,2′:6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo)tetrakis (acetate). In the equation, I_1 and I_2 are the fluorescence intensities of the nanoparticles and the standard, and A_1 and A_2 are the optical densities of the nanoparticles and the standard, respectively. The fluorescence quantum yield of the nanoparticles is remarkably lower than that of free BPTA-Tb^{3+} chelate ($\phi = 100\%$) [24]. This result can be explained as follows. As shown in Fig. 3, since pure silica also has absorption at excitation wavelength, and which is ineffective for the fluorescence emission, thus the fluorescence quantum yield of Tb^{3+} chelate in the nanoparticles is reduced due to the absorption of silica. Furthermore, the fluorescence quantum yield of Tb^{3+} chelate in the nanoparticles is also reduced by the interaction between the silica network and the Tb^{3+} chelate in the nanoparticles. The shorter fluorescence lifetime (2.0 ms) of the nanoparticles (the fluorescence lifetime of free BPTA-Tb^{3+} chelate in aqueous solution is 2.68 ms [24]) indicates that the emission of BPTA-Tb^{3+} chelate in the nanoparticles has been partly quenched by the interaction between the silica network and the Tb^{3+} chelate.

Compared to the previous prepared silica-coated BPTA-Tb^{3+} chelate nanoparticles (the fluorescence lifetime and

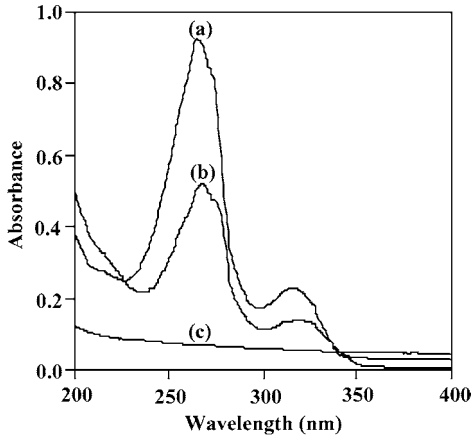

Fig. 3. UV–vis spectra of pure BPTA-Tb^{3+} chelate (a, 30 μM), BPTA-Tb^{3+} chelate-doped silica nanoparticles (b, 40 mg l^{-1}), and pure silica nanoparticles (c, 70 mg l^{-1}) in 0.05 M borate buffer of pH 9.1.

quantum yield are 1.52 ms and 5.3%, respectively) [22], the nanoparticles prepared by the present method have longer fluorescence lifetime and higher fluorescence quantum yield. Since the previous nanoparticles were prepared by the polymerization of TEOS, and the new nanoparticles by the copolymerization of TEOS and AEPS, the different preparation methods would result in the different structures of silica networks in two kinds of nanoparticles, thus the fluorescence quenching effect of the silica network-Tb^{3+} chelate interaction in two kinds of nanoparticles would be also different. The measurement results of fluorescence lifetime and quantum yield show that the fluorescence quenching effect of the silica network-Tb^{3+} chelate interaction in the new nanoparticles is weaker than that in the previous nanoparticles.

3.3. TR-FIA of AFP in human serum samples

After the copolymerization reaction of TEOS and AEPS, some amino groups have been introduced to the nanoparticle's surface. Those amino groups made the modification and the bioconjugation of the nanoparticles easier. As shown in Fig. 4, the nanoparticles were coupled to BSA firstly, and then the BSA-coated nanoparticles were conjugated to anti-AFP antibody by coupling the amino groups of the BSA-coated nanoparticle and antibody with glutaraldehyde [30]. Although the nanoparticles can be directly conjugated to antibody molecules, it was found that the nanoparticle-labeled antibody prepared by direct conjugation method was not useful for solid-phase TR-FIA. The reason is similar to the previous reported nanoparticle-labeled streptavidin [22].

The calibration curve of TR-FIA for AFP is shown in Fig. 5. The calibration curve shows a good straight line, which can be expressed as log(signal) = 0.893 log[AFP] + 3.649 ($r = 0.999$), in AFP concentration range of 0.10–10 ng ml^{-1}. The detection limit, defined as the concentration

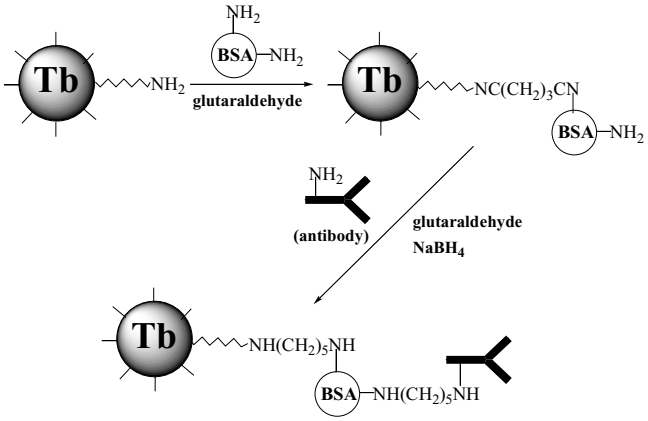

Fig. 4. Schematic representation of anti-AFP antibody immobilization process onto the nanoparticles.

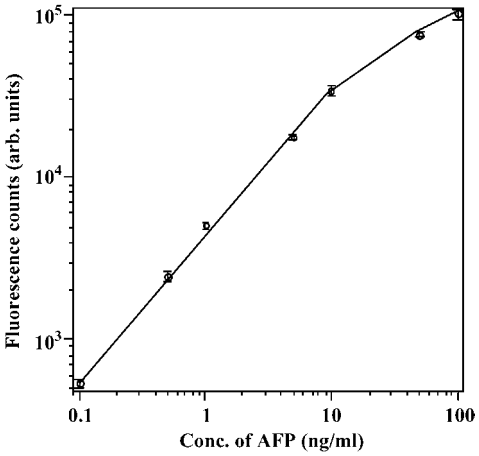

Fig. 5. Calibration curve of TR-FIA using the nanoparticle-labeled anti-AFP antibody for AFP in human serum samples.

corresponding to 3S.D. (standard deviation) of background signal, is 0.10 ng ml^{-1}. The upper limit of the calibration curve is ~100 ng ml^{-1}.

To evaluate the precision and accuracy of the assay, the CVs and recoveries of three standard solutions of AFP added to three serum samples containing different AFP concentrations were measured. As shown in Table 1, the CVs for

Table 1
Analytical precision and recovery of AFP added to human serum

Added (ng ml^{-1})	Found (ng ml^{-1})	CV (%, $n = 6$)	Recovery (%)
0.00	1.22	5.6	–
0.50	1.64	9.0	84.0
2.50	3.44	8.7	88.8
5.00	5.94	8.9	94.4
0.00	1.20	7.9	–
0.50	1.65	9.0	90.0
2.50	3.65	7.8	98.0
5.00	5.89	6.9	93.8
0.00	1.53	7.2	–
0.50	1.98	8.7	90.0
2.50	3.88	7.7	94.0
5.00	5.99	8.6	89.2

all assays are less than 9%, and recoveries are in the range of 84.0–98.0%. These results show that the present method has good precision and accuracy for serum sample measurements.

4. Conclusion

In the present work, novel fluorescent Tb^{3+} chelate-doped silica nanoparticles having surface amino groups were prepared, characterized, and used for TR-FIA of human AFP. As a new fluorescence reagent, the advantages of the nanoparticles including smaller size, high hydrophilicity and biocompatibility, strong fluorescence, long fluorescence lifetime, and direct availability for biolabeling, make them particularly useful as a fluorescence probe for time-resolved fluorescence bioassay. The results of TR-FIA for AFP using the nanoparticle-labeled antibody show that the method is simple and has enough sensitivity [31,32] for determination of AFP in human serum samples with good precision and accuracy. It can be expected that the new nanoparticle fluorescence probe would be widely useful for highly sensitive clinical analysis, time-resolved fluorescence bioimaging technology, and other biotechnology applications.

Acknowledgements

The present work was supported by the National Natural Science Foundation of China (No. 20175027) and Scientific Research Innovation Foundation of Chinese Academy of Sciences.

References

[1] S.Y. Chang, L. Liu, S.A. Asher, J. Am. Chem. Soc. 116 (1994) 6739.
[2] M. Bruchez Jr., M. Moronne, P. Gin, S. Weiss, A.P. Alivisatos, Science 281 (1998) 2013.
[3] W.C.W. Chan, S. Nie, Science 281 (1998) 2016.
[4] J.R. Taylor, M.M. Fang, S. Nie, Anal. Chem. 72 (2000) 1979.
[5] G.P. Mitchell, C.A. Mirkin, R.L. Letsinger, J. Am. Chem. Soc. 121 (1999) 8122.
[6] S. Schultz, D.R. Smith, J.J. Mock, D.A. Schultz, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 996.
[7] S. Santra, P. Zhang, K. Wang, R. Tapec, W. Tan, Anal. Chem. 73 (2001) 4988.
[8] W. Yang, C.G. Zhang, H.Y. Qu, H.H. Yang, J.G. Xu, Anal. Chim. Acta 503 (2004) 163.
[9] E. Soini, T. Lövgren, CRC Crit. Rev. Anal. Chem. 18 (1987) 105.
[10] E.F.G. Dickson, A. Pollak, E.P. Diamandis, Pharmac. Ther. 66 (1995) 207.
[11] I. Hemmilä, V.-M. Mukkala, Crit. Rev. Clin. Lab. Sci. 38 (2001) 441.
[12] K. Matsumoto, J. Yuan, in: A. Sigel, H. Sigel (Eds.), Metal Ions in Biological Systems, vol. 40, Marcel Dekker, New York, Basel, 2003, pp. 191–232.
[13] A. Scorilas, E.P. Diamandis, Clin. Biochem. 33 (2000) 345.
[14] A. Scorilas, A. Bjartell, H. Lilja, C. Moller, E.P. Diamandis, Clin. Chem. 46 (2000) 1450.
[15] L.-Y. Luo, E.P. Diamandis, Luminescence 15 (2000) 409.
[16] Q.-P. Qin, T. Lövgren, K. Pettersson, Anal. Chem. 73 (2001) 1521.
[17] H. Härmä, T. Soukka, T. Lövgren, Clin. Chem. 47 (2001) 561.
[18] T. Soukka, H. Härmä, J. Paukkunen, T. Lövgren, Anal. Chem. 73 (2001) 2254.
[19] T. Soukka, J. Paukkunen, H. Härmä, S. Lönnberg, H. Lindroos, T. Lövgren, Clin. Chem. 47 (2001) 1269.
[20] T. Soukka, K. Antonen, H. Härmä, A.-M. Pelkkikangas, P. Huhtinen, T. Lövgren, Clin. Chim. Acta 328 (2003) 45.
[21] T. Matsuya, S. Tashiro, N. Hoshino, N. Shibata, Y. Nagasaki, K. Kataoka, Anal. Chem. 75 (2003) 6124.
[22] Z. Ye, M. Tan, G. Wang, J. Yuan, Anal. Chem. 76 (2004) 513.
[23] M. Qhobosheane, S. Santra, P. Zhang, W. Tan, Analyst 126 (2001) 1274.
[24] J. Yuan, G. Wang, K. Majima, K. Matsumoto, Anal. Chem. 73 (2001) 1869.
[25] K. Matsumoto, J. Yuan, G. Wang, H. Kimura, Anal. Biochem. 276 (1999) 81.
[26] T. Li, J. Moon, A.A. Morrone, J.J. Mecholsky, D.R. Talham, J.H. Adair, Langmuir 15 (1999) 4328.
[27] V. Bekiari, G. Pistolis, P. Lianos, Chem. Mater. 11 (1999) 3189.
[28] L. Qu, X. Peng, J. Am. Chem. Soc. 124 (2002) 2049.
[29] M. Latva, H. Takalo, V.-M. Mukkala, C. Matachescu, J.C. Rodríguez-Ubis, J. Kankare, J. Luminesc. 75 (1997) 149.
[30] J. Yuan, G. Wang, H. Kimura, K. Matsumoto, Anal. Biochem. 254 (1997) 283.
[31] M.A. Chan, A.C. Bellem, E.P. Diamandis, Clin. Chem. 33 (1987) 2000.
[32] H. Arakawa, T. Ikegami, M. Maeda, A. Tsuji, J. Biolumin. Chemilumin. 8 (1993) 135.