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Determination for *Enterobacter cloacae* based on a europium ternary complex labeled DNA probe

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ABSTRACT

The fast detection and accurate diagnosis of the prevalent pathogenic bacteria is very important for the treatment of disease. Nowadays, fluorescence techniques are important tools for diagnosis. A two-probe tandem DNA hybridization assay was designed for the detection of *Enterobacter cloacae* based on time-resolved fluorescence. In this work, the authors synthesized a novel europium ternary complex $Eu(TTA)_3(5\text{-}NH_2\text{-phen})$ with intense luminescence, high fluorescence quantum yield and long lifetime before. We developed a method based on this europium complex for the specific detection of original extracted DNA from *E. cloacae*. In the hybridization assay format, the reporter probe was labeled with $Eu(TTA)_3(5\text{-}NH_2\text{-phen})$ on the 5′-terminus, and the capture probe capture probe was covalent immobilized on the surface of the glutaraldehyde treated glass slides. The original extracted DNA of samples was directly used without any DNA purification and amplification. The detection was conducted by monitoring the fluorescence intensity from the glass surface after DNA hybridization. The detection limit of the DNA was 5×10^{-10} mol L^{-1}. The results of the present work proved that this new approach was easy to operate with high sensitivity and specificity. It could be conducted as a powerful tool for the detection of pathogen microorganisms in the environment.

1. Introduction

DNA hybridization detection techniques have dramatically impacted microbiology. It is widely applied in molecular biology, clinical diagnostics, bioengineering, environmental monitoring, and other fields [1–5]. Nowadays, various monitoring techniques based on fluorescence have been developed, for example, polymerase chain reaction (PCR) [6,7], DNA micro-arrays technology [8,9] fluorescence in situ hybridization (FISH) [10,11], and time-resolved fluorescent technique [12]. They are also the common employed techniques in research and the clinic diagnose of microorganisms. While PCR has gained widespread acceptance, it is quite susceptible to contamination, since even the smallest amount of contaminating DNA can be amplified [13]. The ease of use without the initial enrichment and the analysis rapidity has made the FISH a powerful tool for microorganism diagnostics. Signals from fluorescently labeled nucleic acid probes can be detected by fluorescence detection instrumentation. Along with the advantages, however, there are still drawbacks: the auto-fluorescence from microorganisms and samples, the decay of fluorescent intensity from the probes and the low signal intensity from insufficient probe penetration into the bacterial cell [14,15]. DNA microarrys based on fluorescence have become a useful tool of pathogen detection and characterization in natural environments for the high throughput analysis of nucleic acids due to their parallel detection capabilities. But the high cost and demanding conditions prohibit the widespread use of the technique [16,17].

Time-resolved fluorescent technique has developed to an attractive DNA detection method during the last decade. The fluorescent complexes were usually used as the labels. Among these fluorescent labels, lanthanide complexes have received much attention for DNA hybridization detection owning to its advantages of high sensitivity, low background and low detection limits [18–21]. However, the synthesis of most reported lanthanide complexes based on time-resolved fluorescence DNA hybridization were somewhat complicated, or the fluorescence intensity was weak. As we known, the importance of pathogenic bacterial infection in the world has got an increasingly concern. The fast and accurate detection of the prevalent pathogenic bacteria is important for the treatment of disease. It was desired to obtain a suitable labeling of lanthanide complex with high fluorescence intensity for DNA detection.

The authors synthesized a lanthanide europium complex of $Eu(TTA)_3(5\text{-}NH_2\text{-phen})$ using 2-thenoyltrifluoroacetonate(TTA) and 5-amino-1,10-phenanthrolin ($5\text{-}NH_2$-phen) as ligand reagents

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before. It has the long lifetime of 0.68 ms and a high fluorescence quantum yield of 0.62, for time resolved spectra: λ_{ex} = 375 nm, λ_{em} = 610 nm, delay time, 0.1 ms, gate time, 1.0 ms [23]. Compared to the common fluorescent dyes, the complex has the advantages of long-life fluorescence, high fluorescence quantum yield and label group. This new complex was utilized for the detection of *Escherichia coli* by us [22]. However, different bacteria requested diverse detecting conditions. The approach was not new, but the probe was new. It was not certain to get satisfactory results when we used the similar detection method to detect different bacteria. Thus, it was necessary to go further study when this detection method was used for other different bacteria. The Eu(TTA)$_3$(5-NH$_2$-phen) (ETN) labeled probes were employed for the specific detection of original extracted DNA from *Enterobacter cloacae* strain. An artificial sample containing *E. coli*, *E. cloacae*, *Staphylococcus aureus* and *Staphylococcus epidermidis* was prepared, and the concentration of *E. cloacae*, as low as 5×10^{-10} mol L^{-1}, can be detected. In the absence of the *E. cloacae*, the fluorescence intensity was almost as low as the background. The method was also studied with the detection of *E. cloacae* in the environmental sample. Satisfactory selectivity and high specificity was presented too. The novel method was verified easier to operate with no need of purification and enrichment procedures of nucleic acid. It is promised to be an important player in the future diagnosis of pathogen microorganisms.

2. Experimental

2.1. Materials and reagents

Genomic DNA isolation kit (for Bacteria) was purchased from Generay Biotech Co. Ltd. (Shanghai, China). Ammonium hydroxide (25–28 wt.%), ethanol, glutaraldehyde 50 wt.%), acetic acid and hydrogen chloride (HCl) were purchased from Hengxing Reagent Co. (Tianjin, China). The 3-aminopropyltrimethoxysilane (APTES) was purchased from Acros organics. The *E. coli*, *E. cloacae*, *S. aureus and S. epidermidis* were obtained from Microbial Culture Collection Center of Guangdong Institute of Microbiology (Guangdong, China). Phosphate buffered saline (PBS) buffer (pH 7.2) was used in the oligonucleotide labeling and immobilizing procedure. TE buffer (pH 8.0) consisted of 10 mM Tris–HCl and 1 mM EDTA. Sodium phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium citrate and sodium dodecyl sulfate (SDS) were from Kermel (Tianjin, China). All other chemicals obtained commercially were of analytical reagent grade and used without purification or treatment except otherwise stated. Double distilled water was used for the preparation of all aqueous solutions.

2.2. Instrumentation

A Perkin-Elmer LS-55 spectrofluorimeter controlled by a personal computer, two arms of the bifurcated optical fibers and a home-made poly (tetrafluoroethylene) detection cell constituted the detection system. A pair of the bifurcated optical fibers was fixed in the detecting chamber of the spectrofluorimeter. A home-made poly(tetrafluoroethylene) detection-cell matched with the optical fiber was fixed on the other end. A quadrate glass slide (side length 12 mm, thickness 1.5 mm) was immobilized in the center of the detection cell by the mounting screw nut. The excitation light was carried outside the spectrofluorimeter to the quadrate slide fixed in the detection-cell through one arm of the fiber and the emission light was collected inside the spectrofluorimeter through the other. All fluorescence measurements were carried out with both excitation and emission slits set at 10 nm. A Beckman DU-640 Nuclear protein analyzer was used in this experiment. All measurements were conducted at room temperature and atmospheric pressure.

Table 1
Sequence used to hybridizing.

Name	Sequence (5′–3′)	bps
DNA_1	CCCAC CACGC CGATT CTCAG CT$(T)_{10}$-NH_2	32
DNA_2	NH_2-$(T)_{10}$TGAGA ATAAA ACCCC GATGG CAAAC GTA	38
DNA_3	AGCTG AGAAT CGGCG TGGTG GGTAC GTTTG CCATC GGGGT TTTAT TCTCA	50

2.3. Oligonucleotide probes

A two-probe tandem DNA hybridization assay was conducted in our study. DNA oligonucleotide probes were designed using software Primer Premier 5.0. The capture probe DNA_1 and reporter probe DNA_2 were commercially synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). The sequences of these oligonucleotides are shown in Table 1. The sequences of the capture probe DNA_1 and reporter probe DNA_2 were complemented with the sequences of the specified target DNA_3. In order to reduce the steric hindrance, ten bases of 'T' were added on the 3′-end of capture probe and 5′-end of reporter probe.

2.4. Glass slides surface modification with capture probe

The glass was cut into foursquare slides (12 × 12 mm), which were equivalent to the aperture size of detection cell. The glass slides surface was modified according to the literature [22–24]. The slides were immersed in 2.5% ammonium hydroxide for 24 h, and then washed with double-distilled water. Afterwards, the slides were dipped in a solution of 2.0% APTES in 95% ethanol and adjusted to the desired pH of 4.5 with glacial acetic acid. The slides were washed ultrasonically by ethanol and double-distilled water respectively after 30 min, and the reactive amino groups were introduced at the slides surface. Prior to the experiment, a solution of 2.5% glutaraldehyde in PBS buffer (pH 7.2) was prepared, and the amine-modified glass slides were immersed in it for 3 h. The slides were washed with PBS buffer (pH 7.2) and double-distilled water in turn (1 min/time). Finally, the glass slides were dried at room temperature.

30 μL of capture probe solution of *E. cloacae* (5 μg mL^{-1}) diluted with TE buffer was dropped onto the glutaraldehyde modified glass slides, which covered evenly on every slide surface. Then the slides were preserved at room temperature overnight to make sure the capture probe covalently immobilized on the glass surface. The glass slides were washed with 0.2% SDS solution and double-distilled water. After dried at room temperature, the slides were immersed in the glycin solution (0.02 mol L^{-1}) for 1 h to remove the unreacted aldehyde groups. Finally, the slides were washed respectively with 0.2% SDS solution and double-distilled water, eventually dried at room temperature.

2.5. Labeling of probe DNA_2

The fluorescent dye of Eu(TTA)$_3$(5-NH$_2$-phen) was used as the label. The complex was synthesized by our group before [23]. 1 mg of complex ETN was dispersed in 2.5% glutaraldehyde solution by ultrasonic vibration. The mixture was stirred at room temperature for 3 h, then removed from the solution by centrifugal (8000 rpm min^{-1}, 3 min) precipitation and washed with ethanol

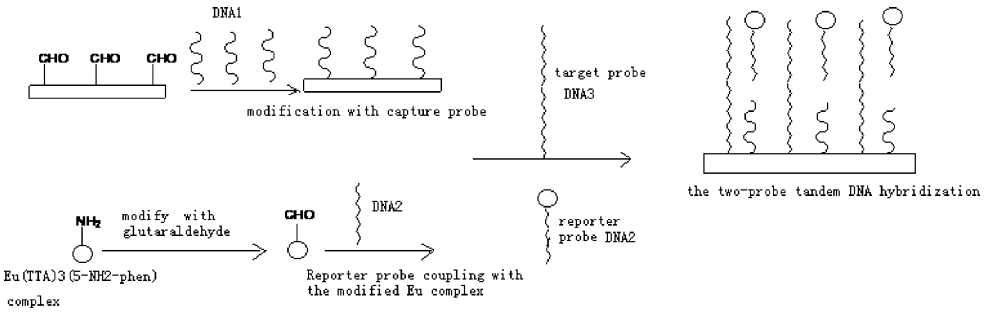

Fig. 1. The schematic diagram of DNA hybridization.

and double-distilled water twice, ultimately suspended in PBS buffer (pH 7.2).

The reporter probe was firstly diluted to a certain concentration by TE buffer (pH 8.0). The solution of reporter probe DNA_2 was added to the suspensions of glutaraldehyde-treated europium complex, and the stirring reaction lasted for 4 h. In order to eliminate the unbound oligonucleotides, the mixture was centrifuged (10,000 rpm min^{-1}, 3 min) by high speed refrigerated centrifuge and washed twice with PBS buffer (pH 7.2) and double-distilled water respectively. Finally, the DNA_2 modified europium complex was treated with 0.02 mol L^{-1} glycin solution for 1 h to inactivate the unreacted aldehyde groups, and resuspended in certain PBS buffer (pH 7.2) in reserve.

2.6. Extraction and unlink of DNA

The strains of *E. coli*, *E. cloacae*, *S. aureus* and *S. epidermidis* were cultured at 30 °C for 48 h. Then four kinds of DNA were extracted according to the Genomic DNA Isolation Kit (for Bacteria). The OD260/OD280 of every DNA was in the range of 1.8–1.9 detected by Beckman DU-640 Nuclear protein analyzer, which was pure enough for further analysis [25]. All the extractions were preserved in −20 °C.

The DNA extractions were two helical chains, while only one of them was needed as template in hybridization. Therefore, double strands must be denatured. The DNA was heated to loosen the double-strand at 95 °C for 10 min, then immediately immersed in a mixture of ice and water for 5 min to prevent the renaturation. The single strand of target DNA_3 was finally obtained for hybridization.

2.7. Hybridization with target probe

Some absorbent cotton was put in hybridization box, and a solution of 3.0 × SSC (NaCl, sodium citrate) was added to make sure the surface was wet. Put the glass slides level in the box. The target probe was diluted to 5 μg mL^{-1} with double-distilled water. 5 μL of hybridization solution of 3.0× SSC was dropped onto the central surface of the capture probe modified glass slides. Then probe DNA_2 (10 μL) and target probe DNA_3 of single strand (10 μL) were pipetted to the slides almost at the same time. Closed the cover of the box and kept the box incubated at a designed temperature (47 °C) in a thermostat. A graphical diagram for illustrating the sensing strategy was shown in Fig. 1. Three types of washing buffers were prepared including the solution of 1× SSC + 0.03% SDS, 0.2× SSC, and 0.05× SSC [26]. All the assays were performed in triplicates. After hybridization at 47 °C for 9 h, the glass slides were washed with the washing buffers one by one, and then washed with double-distilled water to remove nonhybrids and undesired hybrids. The temperature was the same as hybridization temperature. For blank control, double-distilled water was used instead of the DNA sample unless otherwise stated. Fluorescence signals of the DNA_2 labeled europium complex left on the glass slides can be monitored by appropriate excitation.

3. Results and discussion

3.1. Optimal hybridization time

To determine the optimal hybridization time for *E. cloacae* probes, a series of hybridization experiments were done at different temperature from 30 °C to 57 °C. Every temperature was defined as one group, and the experiments were carried out in the same conditions except hybridization time. Time-resolved fluorescence intensity of different hybridization time was detected in each group. The concentration of the target probes was diluted with TE buffer to 5.0×10^{-7} mol L^{-1}. The tendency of each curve was similar of every group. For blank control, double-distilled water was used instead of the DNA sample. Take the case of the group of 47 °C for example, the points on the curve were the means of three experiments with the error bars, as shown in Fig. 2. The hybridization signal was increasing in the prior period of hybridization time less than 9 h, and then decreased slowly at the hybridization time longer than 9 h. What is more, the blank control signal was also the minimum at 9 h. Therefore, the optimal hybridization time was 9 h, and all of the following experiments were kept for 9 h.

3.2. Optimal hybridization temperature

The optimal hybridization temperature for *E. cloacae* probes was also investigated. Selecting a proper hybridization temperature can avoid nonspecific base pairing and reduce background value, following high specificity and efficiency [27,28]. The experiments were conducted at different temperatures for 9 h, and the target

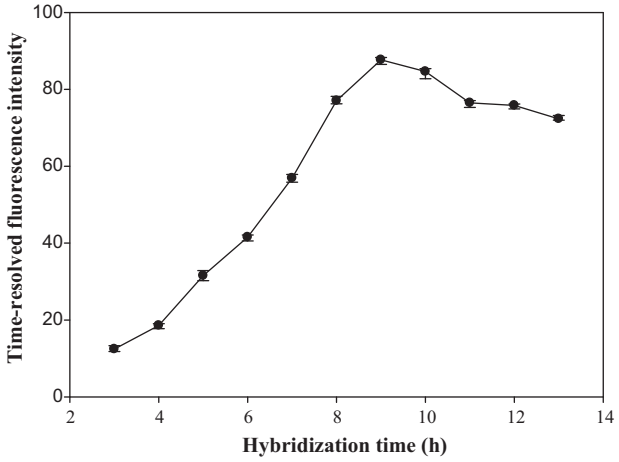

Fig. 2. Time-resolved fluorescence intensity for different hybridization time at 47 °C.

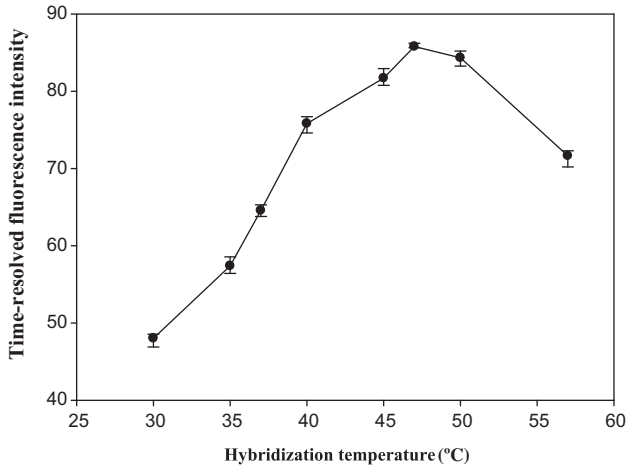

Fig. 3. Time-resolved fluorescence intensity for different hybridization temperature.

probes were diluted to a same concentration of 5.0×10^{-7} mol L^{-1}. For blank control, double-distilled water was used instead of the DNA sample. The result in Fig. 3 showed that, from 30 °C to 47 °C, the time-resolved fluorescence intensity was increased with the increasing temperature, and got the summit at 47 °C, then decreased slowly from 47 °C to 57 °C. Consequently, 47 °C was the optimal hybridization temperature, and all of the following hybridization experiments were carried out at 47 °C.

3.3. DNA hybridization detection

Time-resolved fluorescence intensity of different concentrations of *E. cloacae* were detected at λ_{ex} = 375 nm, λ_{em} = 610 nm after hybridization of 9 h at 47 °C. As shown in Fig. 4, the time-resolved fluorescence intensity was growing with the increasing concentration of the probe (fluorescence intensity from 7.7 of the blank group to 88.3 of the 5.0×10^{-7} mol L^{-1} group). There was a satisfactory linearity between the logarithm of the fluorescence intensity and the negative logarithm of the probe concentration with the correlation coefficient of 0.9933. The detection limit for *E. cloacae* was less than 5.0×10^{-10} mol L^{-1}. It can be proved as an excellent way to detect *E. cloacae* with high sensitivity.

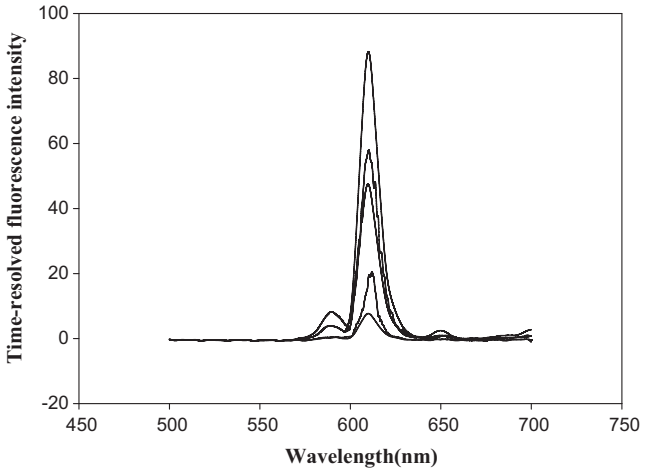

Fig. 4. Time-resolved fluorescence spectra for different concentrations of target probe of *Enterobacter cloacae* (from top to bottom: 5×10^{-7} mol L^{-1}, 5×10^{-8} mol L^{-1}, 5×10^{-9} mol L^{1}, 5×10^{-10} mol L^{-1}, 0 mol L^{1}, for time resolved spectra: λ_{ex} = 375 nm, λ_{em} = 610 nm, delay time, 0.1 ms, gate time, 1.0 ms).

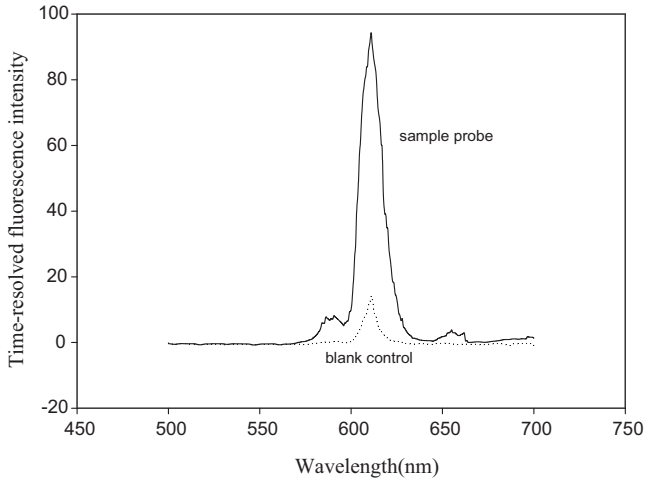

Fig. 5. Time-resolved fluorescence spectra for the target probe of *Enterobacter cloacae* in the environmental sample (the dashed line is that of the blank control and the active line is that of the sample probe, respectively).

3.4. Selectivity of the prepared detection method

The investigations were conducted by recording the time-resolved fluorescence intensity of hybridization when other DNA species existed, including *S. epidermidis*, *E. coli*, *S. aureus* and *E. cloacae*. They were defined respectively as group s1, s2, s3, s4. Four groups of different target probes were diluted to a same concentration of 5.0×10^{-7} mol L^{-1}, and an artificial sample of s1, s2, s3, s4 was prepared, which was regarded as sequence s5. The amounts of s1, s2, s3 and s4 were equal mixed. The hybridization of five groups was carried out simultaneously under similar optimal conditions. For blank control, double-distilled water was used instead of the DNA sample. The average fluorescence intensity of the five groups was 5.4, 11.2, 8.9, 83.8 and 85.6, respectively, which showed little nonspecific binding of different DNA sequences. The fluorescence intensity of s5 was a little higher than the fluorescence intensity of s4, because in this group, there were four different types of DNA species, the blank control of s5 was 9.2, which is a little higher than that of s4 (5.1). Little false positive signals were obtained from the other contrasted microorganisms. Another group of s1, s2, s3 was set with the absence of target probes, the following procedures were conducted homogeneously. The average value of the fluorescence intensity was as low as 7.3, which was close to the background. Therefore, the capability and selectivity of the detection method was good enough to detect *E. cloacae.*

3.5. Application for detection of E. cloacae in sewage

The following experiments were focused on the ability of the method to detect *E. cloacae* in complex environmental samples. Sewage (5 mL) was got from paddy field. Clarified solution was obtained after primary treatment of the sewage. The double-stranded DNA was extracted from clarified solution similarly with Genomic DNA extraction Kit (Generay Biotech Co., Ltd., Shanghai, China). The double-stranded DNA fragments were denatured to single-stranded DNA by heating at 95 °C for 10 min. All the probes were diluted to a same concentration of 5.0×10^{-7} mol L^{-1}. Detections of *E. cloacae* DNA were conducted under the optimal conditions by the hybridization. For blank control, double-distilled water was used instead of the DNA sample. As shown in Fig. 5, the average fluorescence intensity of environmental sample was 94.3, and the blank value was 14.2. The satisfactory result showed that this method can be used as a powerful tool for the detection of pathogen microorganisms.

4. Conclusion

A method of DNA detection adopting two-probe tandem DNA hybridization assay has been successfully developed in this work. The oligonucleotide sequences of *E. cloacae* genes were designed and the results were comparatively satisfied when the hybridization was conducted at 47 °C for 9 h. It can precisely distinguish target bacteria from the artificial sample, environmental sample and presented high sensitivity and good selectivity. The method can be developed for routine monitoring and offer a novel approach for *E. cloacae* pathogen detection.

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References

[1] H. Lü, Y.J. Zhao, J.M. Ma, W.Y. Li, Z.H. Lu, Colloids Surf. A: Physicochem. Eng. Aspects 175 (2000) 147–152.
[2] H. Cai, N.N. Zhu, Y. Jiang, P.G. He, Y.Z. Fang, Biosens. Bioelectron 18 (2003) 1311–1319.
[3] M.J. Heller, Annu. Rev. Biomed. Eng. 4 (2002) 129–153.
[4] G. Marrazza, I. Chianella, M. Mascini, Anal. Chim. Acta 387 (1999) 297–307.
[5] Q.T. Zhang, V. Subramanian, Biosens. Bioelectron 22 (2007) 3182–3187.
[6] J.D. Durtschi, J. Stevenson, W. Hymas, K.V. Voelkerding, Anal. Biochem. 361 (2007) 55–64.
[7] H. Volkmann, T. Schwartz, S. Kirchen, C. Stofer, U. Obst, Mol. Cell. Probes 21 (2007) 125–133.
[8] J.Y. Deng, X.E. Zhang, Y. Mang, Biosens. Bioelectron 19 (2004) 1277–1283.
[9] M. Barbaro, A. Bonfiglio, L. Raff, Sens. Actuators B 118 (2006) 41–46.
[10] R. Ekong, J. Wolf, Curr. Opin. Biotechnol. 9 (1998) 19–24.
[11] A.K. Raap, Mutat. Res. 400 (1998) 287–298.
[12] Y. Chen, Y.M. Chi, H.M. Wen, Z.H. Lu, Anal. Chem. 79 (2007) 960–965.
[13] K. Doi-Kawano, E. Nishimoto, Y. Kouzuma, D. Takahashi, S. Yamashita, M. Kimura, J. Fluoresc. 19 (2009) 631–639.
[14] J.R. Epstein, I. Biran, D.R. Walt, Anal. Chim. Acta 469 (2002) 3–36.
[15] J. Szollosi, S.J. Lockett, M. Balazs, F.M. Waldman, Cytometry 20 (1995) 356–361.
[16] G.J. Vora, C.E. Meador, D.A. Stenger, J.D. Andreadis, Appl. Environ. Microbiol. 70 (2004) 3047–3054.
[17] L. Bodrossy, A. Sessitsch, Curr. Opin. Microbiol. 7 (2004) 245–254.
[18] Y. Kitamura, T. Ihara, Y. Tsujimura, Inorg. Biochem. 102 (2008) 1921–1931.
[19] Y.F. Li, K.Z. Tang, Y. Tang, W.S. Liu, M.Y. Tan, Spectrochim. Acta A 71 (2008) 1153–1157.
[20] G.L. Wang, J.L. Yuan, X.D. Hai, K. Matsumoto, Talanta 70 (2006) 133–138.
[21] M. Liu, W.B. Yuan, Q. Zhang, L. Yan, R.D. Yang, Spectrochim. Acta A 70 (2008) 1114–1119.
[22] M. Ruan, C.G. Niu, P.Z. Qin, G.M. Zeng, Z.H. Yang, H. He, J. Huang, Anal. Chim. Acta 664 (2010) 95–99.
[23] P.Z. Qin, C.G. Niu, G.M. Zeng, M. Ruan, Talanta 80 (2009) 991–995.
[24] P.Z. Qin, C.G. Niu, G.M. Zeng, M. Ruan, X.Y. Wang, Analyst 135 (2010) 2144–2149.
[25] C. Yeates, M.R. Gillings, A.D. Davison, N. Altavilla, D.A. Veal, Biol. Proced. Online 1 (1998) 40–47.
[26] T. Sakata, M. Kamahori, Y. Miyahara, Mater. Sci. Eng. C 24 (2004) 827–832.
[27] S.C. Tao, Y. Li, Y.H. Liu, X.M. Ma, J. Cheng, Biotechniques 34 (2003) 1260–1262.
[28] B.E. Lang, F.P. Schwarz, Biophys. Chem. 131 (2007) 96–104.