Talanta 80 (2009) 991–995

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Time-resolved fluorescence based DNA detection using novel europium ternary complex doped silica nanoparticles

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ARTICLE INFO

Article history:
Received 16 June 2009
Received in revised form 18 August 2009
Accepted 21 August 2009
Available online 28 August 2009

Keywords:
DNA detection
Time-resolved fluorescence
Eu(TTA)$_3$(5-NH$_2$-phen)
Nanoparticles

ABSTRACT

A two-probe tandem DNA hybridization assay including capture DNA$_1$, probe DNA$_2$, and target DNA$_3$ was prepared. The long-lived luminescent europium complex doped nanoparticles (NPs) were used as the biomarker. The complex included in the particle was Eu(TTA)$_3$(5-NH$_2$-phen)-IgG (ETN-IgG), the europium complex Eu(TTA)$_3$(5-NH$_2$-phen) linking an IgG molecule. Silica NPs containing ETN-IgG were prepared by the reverse microemulsion method, and were easy to label oligonucleotide for time-resolved fluorescence assays. The luminophores were well-protected from the environmental interference when they were doped inside the silica network. The sequences of *Staphylococcus aureus* and *Escherichia coli* genes were designed using software Primer Premier 5.0. Amino-modified capture DNA$_1$ was covalently immobilized on the common glass slides surface. The detection was done by monitoring the fluorescence intensity from the glass surface after the hybridization reaction with the NPs labeled probe DNA$_2$ and complementary target DNA$_3$. The sensing system presented short hybridization time, satisfactory stability, sensitivity, and selectivity. This approach was successfully employed for preliminary application in the detection of pure cultured *E. coli*, it might be an effective tool for pathogen DNA monitoring.

1. Introduction

Among many detection techniques of microbial pathogens, DNA hybridization method has been popularly developed for its accurate specificity and high selectivity [1–3]. Compared to the well developed electrochemical biosensors [4–6], there is increasing interest in the investigation of optical sensing systems. Fluorescence based detection, such as polymerase chain reaction (PCR) [7], fluorescence in situ hybridization (FISH) [8], and DNA chip technology [9] have become the standard techniques for quantifying extents of hybridization between surface-immobilized probes and fluorophore-labeled analytic targets in DNA microarrays.

A fluorescent dye is usually utilized to signal the hybridization. Many fluorescent dyes are well known as markers for nucleic acids, such as fluorescein [10], rhodamine [11] and cyanine [12] dyes. However, there is a main problem of how to eliminate background noises caused by autofluorescence from biological samples, the scattering light from solid substrates, and the luminescence from the optical components. For this purpose, more and more attention has been paid on the time-resolved fluorescent technique using lanthanide complexes as the fluorescence labels [13–15]. However, most reported time-resolved fluorescence DNA hybridization assay was based on fluorescence resonance energy transfer technology (FRET) [16–18]. It only occurs under very exacting conditions of overlapping of the emission peak of the donor and the excitation peak of the acceptor, the distance between the donor fluorophore and acceptor fluorophore, and the position of the fluorophores within the biomolecules, etc. It is difficult to find a suitable donor–acceptor system.

On the other hand, the demand of ultratrace gene analysis has driven nanomaterials toward biomedical fields and biotechnology. Recently, dye-doped silica NPs were extensively used in bioimaging and biochemical analysis due to its advantages like signal enhancement, photostability and surface modification for the immobilization of biomolecules [19–21]. To the best of the authors' knowledge, there are only a limited number of reports on the use of lanthanide complex doped NPs as biolabels [22–29]. Dye leakage can efficiently be reduced through covalent binding, and a special functional ligand is usually necessary. However, the synthesis of the chlorosulfonylated β-diketones such as 5-(4″-chlorosulfo-1′,1″-diphenyl-4′-yl)-1,1,1,2,2,3,3-heptafluoro-4,6-hexanedione

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doi:10.1016/j.talanta.2009.08.027

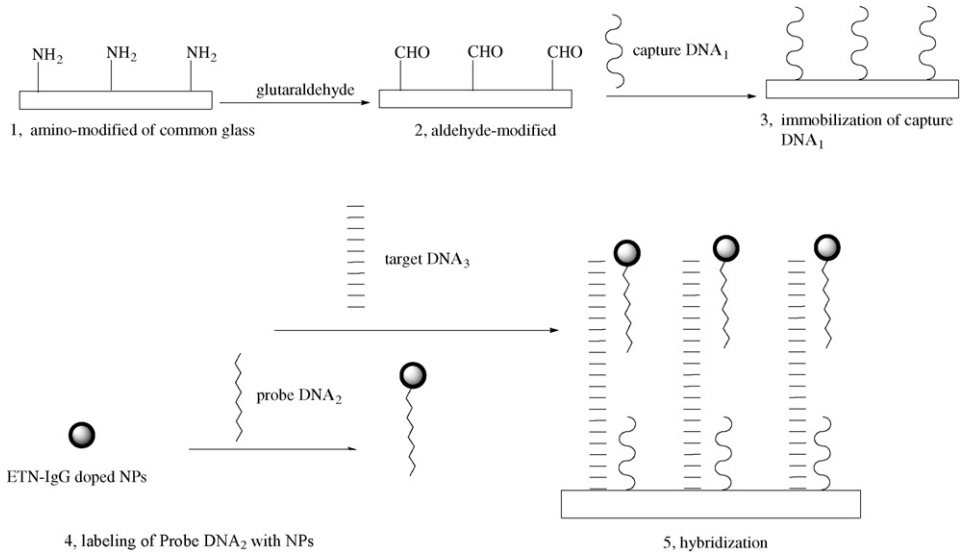

Fig. 1. The two-probe tandem DNA hybridization assay.

(CDHH), 4,4′-bis(1″,1″,1″,2″,2″,3″,3″-heptafluoro-4″,6″-hexanedion-6″-yl)chlorosulfo-*o*-terphenyl (BHHCT), and 4,4′-bis(1″,1″,1″-trifluoro-2″,4″-butanedion-4″-yl)chlorosulfo-*o*-terphenyl (BTBCT) was somewhat difficult. Furthermore, a number of reported biological detection systems were based on confocal fluorescence imaging technology to improve sensitivity [20,23,25,26,29–31]. The drawback is that confocal microscopes are expensive and require very precise mechanical alignment that can be difficult to maintain. DNA detection based on the measurement of time-resolved fluorescence intensity of a suitable labelling of lanthanide complex doped NPs is still an exciting area of research.

In this work, the authors synthesized a europium complex $Eu(TTA)_3(5\text{-}NH_2\text{-phen})$ with long fluorescence lifetime of 0.657 ms and intense time-resolved fluorescence intensity in simple steps. The amino substituent in the phenyl ring in phenanthroline allowed for facile conjugation to IgG. The ETN-IgG doped amino-modified silica NPs were prepared and allowed for facile conjugation to DNA. The DNA hybridization assay [20] was shown in Fig. 1. The combined sequences of capture DNA_1 and probe DNA_2 are complementary to that of the target DNA_3. The sequences of these DNA strands were designed using software Primer Premier 5.0, and commercially synthesized. The detection system showed satisfactory sensitivity, the detection limits could be as low as 4×10^{-10} mol l^{-1}. Additional applications will be used for detection in real samples of microbial pathogens.

2. Experimental

2.1. Instrumentation

All fluorescence measurements were conducted on a Perkin-Elmer LS-55 spectrofluorimeter with both excitation and emission slits set at 10 nm and controlled by a personal computer data processing unit. A modified home-made poly(tetrafluoroethylene) detection-cell and two arms of the bifurcated optical fiber were fixed in the detecting chamber of the spectrofluorimeter to carry the excitation and emission light. The excitation light was carried outside the spectrofluorimeter to the sensor fixed in the detection-cell through one arm of the fiber and the emission light was collected inside the spectrofluorimeter through the other. A foursquare glass plate (side length 12 mm, thickness 1.5 mm) covered with the hybrid of capture DNA_1, NPs labeled probe DNA_2, and target DNA_3 was fixed on the top of the flow chamber by the mounting screw nut. All measurements were performed at room temperature and atmospheric pressure.

2.2. Materials and reagents

Europium oxide was purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). 2-Thenoyltrifluoroacetone (TTA), a ligand reagent, was obtained from J&K Chemical Ltd. Human IgG was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). Tetraethyl orthosilicate (TEOS), Triton X-100, 3-aminopropyltrimethoxysilane (APTES), 2-(*N*-morpholino)ethanesulfonic acid (MES), *N*-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Acros organics. The oligonucleotides were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd (Shanghai, China). All other chemicals of analytical reagent grade were commercially obtained. PBS buffer (pH 7.2) was used during the oligonucleotide label and immobilization. Double-distilled water was used for the preparation of all aqueous solutions.

2.3. Synthesis of europium ternary complexes

The europium ternary complex $Eu(TTA)_3(5\text{-}NH_2\text{-phen})$ (ETN) using thenoyltrifluoroacetone (TTA) and 5-amino-1,10-phenanthroline ($5\text{-}NH_2$-phen) as the ligands was synthesized, the formula of the complex was shown in Fig. 2. The synthesis of $5\text{-}NH_2$-phen was according to the literature [32].

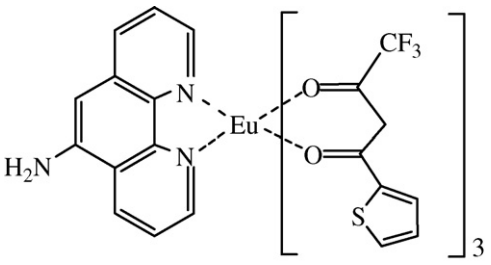

Fig. 2. Formula of complex $Eu(TTA)_3(5\text{-}NH_2\text{-phen})$.

The ternary europium complex was prepared by stirring an ethanol solution of the $EuCl_3$ (europium oxide was dissolved in hydrochloric acid to form europium chloride), TTA, and was adjusted to pH 6 by adding a sodium hydroxide solution, and the mixture was reacted for 40 min. Subsequently, another ethanol solution of 5-NH_2-phen was added, and the mixture was stirred for 5 h at 55–60 °C. A yellow precipitate formed upon cooling was filtered and recrystallized from a mixed solvent of methanol and chloroform (1:1, v/v), MS *m/z* 1032 (M^++H_2O). ^{1}H NMR (acetone) δ (ppm): 2.83 (6H, –CO–CH_2–), 6.02–7.28 (9H, -thiophene), 7.40 (2H, –NH_2), 8.01–11.11 (7H, -phenanthrene). The ternary complex was further characterized through IR and elemental analysis. The $\nu_{(C=O)}$ stretching vibration in the infrared spectrum at 1637 cm^{-1} of TTA shifts to 1626 cm^{-1} in the spectrum of ETN, and the $\nu_{(C=N)}$ stretching vibration at 1564 cm^{-1} of 5-NH_2-phen shifts to 1550 cm^{-1} of ETN. Elemental analysis: $EuC_{36}H_{24}N_3F_9S_3O_6$ (1013.66), Calcd. C, 42.62; H, 2.37; N, 4.14. Found C, 42.58; H, 2.31; N, 4.19.

2.4. Preparation of functional nanoparticles

It is reported that biomacromolecule conjugated organic dyes as the core material for the preparation of core–shell structure NPs can effectively improve the efficiency of dye-embedded and hydrophilicity [20]. The authors converted ETN to hydrophilic molecule by linking an IgG molecule to the ETN. The ETN-IgG doped NPs were successfully synthesized by the reverse microemulsion method [33]. A certain aqueous ETN-IgG solution was added in a microemulsion of 1.77 ml Triton X-100, 7.5 ml cyclohexane, 1.8 ml n-hexanol, and 340 μl water. The final luminophore concentration in the mixture was 1.2×10^{-3} mol l^{-1}. After stirring the microemulsion for 20 min, 0.1 ml of TEOS was added, the polymerization of TEOS was initiated by adding 0.06 ml of ammonium hydroxide. The reaction was allowed to continue for 24 h. Then 0.1 ml of silanization reagent APTES was introduced to the mixture, the reaction was allowed to continue for another 2 h. To release the NPs from the microemulsion and remove surfactant molecules, acetone and ethanol were used to break microemulsion and wash the NPs. After washing with ethanol and double-distilled water several times, the amino-modified ETN-IgG doped silica NPs were obtained and dried in a desiccator prior to modification.

2.5. Designing of oligonucleotide sequences

All the sequences were designed using Primer Premier 5.0 software, on the basis of the published specific nucleotide sequences of *Staphylococcus aureus* and *Escherichia coli* genes [34]. The sequences of these oligonucleotides and the hybridization conditions set for respective genes are shown in Table 1.

2.6. Glass slides surface modification with capture DNA_1

The glass slides surface was modified as described in the literature [35]. The glass slides were immersed in 25.0% ammonium hydroxide overnight then washed with double-distilled water. A solution of 2.0% APTES in 95% ethanol was prepared and adjusted to the desired pH of 4.5 with glacial acetic acid. The glass slides were soaked in this solution for 30 min then ultrasonically washed by ethanol and double-distilled water. Reactive amino groups were then introduced at the glass surface. The amine-modified glass slides were then immersed in the solution of 2.5% glutaraldehyde in PBS buffer stirring for 3 h, and washed with PBS buffer and double-distilled water (1 min/time), respectively, then dried at normal temperature.

The solution of capture DNA_1 was dropped onto the glutaraldehyde-treated glass slides, and was kept at room temperature overnight. The capture DNA_1 was then covalently immobilized on the glass surface. The DNA_1 modified glass slides were then soaked in a sodium cyanoborohydride solution for 30 min to block any remaining glutaraldehyde groups, and the carbon–nitrogen double bond was reduced. The final glass was dried at room temperature after washing with 0.2% SDS solution (sodium dodecyl sulfate) and double-distilled water.

2.7. Surface modification of NPs with probe DNA_2

Certain amine-modified silica NPs were suspended in PBS buffer by ultrasonication. Some 10% succinic anhydride DMF solution was added, the mixture was stirred for 3 h under the condition of nitrogen atmosphere. After centrifuging and washing with ethanol and double-distilled water, the NPs were dispersed in 0.1 mol l^{-1} MES buffer (pH 6.0). A certain probe DNA_2 dissolved in double-distilled water, EDC and NHS was added to the solution, and the reaction was continued for 3 h. The NPs were removed from the solution by centrifugal precipitation and then treated with 0.02 mol l^{-1} glycine solution for 1 h to remove the unreacted carboxyl groups. The final DNA_2 modified NPs were washed, and resuspended in PBS buffer for future usage.

2.8. Hybridization with target DNA_3

The target DNA_3 was first diluted in double-distilled water. The DNA_2 immobilized NPs and target DNA_3 were then added on the capture DNA_1 modified glass slides surface for hybridization under the condition of hybridization solution containing 5.0× SSC (NaCl, sodium citrate) and 0.1% SDS. Following hybridization, the glass slides were washed with 1× SSC + 0.03% SDS, 0.2× SSC, 0.05× SSC, and double-distilled water at room temperature in order to remove non-hybridized oligonucleotides. The detection of the DNA_3 was done by monitoring fluorescence signals of the DNA_2 labeled NP conjugates left on the glass slides surface with proper excitation.

3. Results and discussion

3.1. Nanoparticles characterization

ETN-IgG doped NPs were characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and fluorescence spectroscopy. As shown in Fig. 3, the average diameter of the NPs is about 55–65 nm. Europium ternary complex particles are visible as dark dots embedded inside the silica sphere as a result

Table 1
Sequences used to hybridizing.

Name	Sequence (5′–3′)	Length (bp)	Hybridizing temperature
Staphylococcus aureus	DNA_1: CACTT TTTCT TAAAT GTTGT TC $(A)_{10}$-NH_2	22	38.3 °C
	DNA_2: NH_2-$(A)_{10}$ATTTT CTCTT TTTTC GCTT	19	
	DNA_3: GAACA ACATT TAAGA AAAAG TGAAG CACAA GCGAA AAAAG AGAAA AT	47	
E. coli	DNA_1: ACATT GACGC AGGTG ATCGG ACG$(A)_{10}$-NH_2	23	29.4 °C
	DNA_2: NH_2-$(A)_{10}$GTATC GGTGT GAGCG TCGCA GAA	23	
	DNA_3: CGTCC GATCA CCTGC GTCAA TGTAA TGTTC TGCGA CGCTC ACACC GATAC	50	

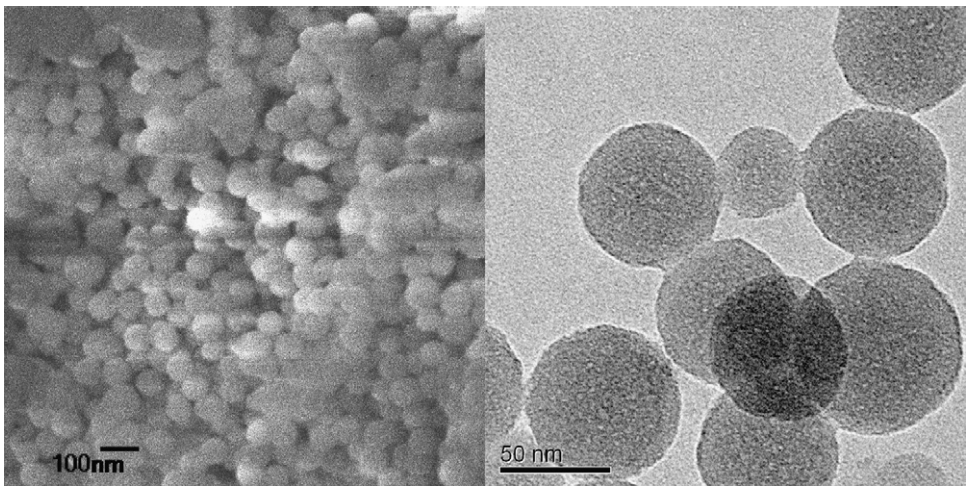

Fig. 3. SEM and TEM images of ETP-IgG NPs.

of the presence of the heavy metal europium atom from the TEM image. The time-resolved fluorescence spectra of the europium complex and its NPs were investigated (Fig. 4). Results showed that the bandwidth of excitation spectrum was compressed and the emission spectral maxima position shifts by 4 nm (from 615 to 611 nm) toward the shorter wavelength when compared to the complex ETN. This clearly confirmed that silica cell generally has no effect on the luminescence performance of europium complex.

The lifetime of ETN-IgG doped NPs was easily calculated with the spectrofluorimeter. As shown in Fig. 5, there is satisfactory linearity between the logarithm of the fluorescence intensity and delay time, and the lifetime is the negative reciprocal of the slope of 0.657 ms. The NPs gave an intense and long-lived signal which enables efficient elimination of background signal, and all measurements were carried out at a delay time of 0.1 ms.

3.2. DNA hybridization detection

It is desirable to use time-resolved fluorometry of lanthanide chelates-doped NPs in the area of nucleic acid hybridization assays as a means of improving the signal-to-noise ratio by increasing the signal intensity and decreasing the background associated with conventional fluorescence measurements. Time-resolved fluorescence intensity of different concentrations of target DNA_3 of *S. aureus* detected from glass surfaces was recorded at $\lambda_{ex} = 375$ nm, $\lambda_{em} = 611$ nm. As shown in Fig. 6, the time-resolved fluorescence intensity was increased with the increasing concentration of DNA_3 (from 11.55 of blank to 96.75 of 4×10^{-7} mol l^{-1}). The ETN-IgG doped NPs could be a super excellent labelling reagent for DNA detection with high sensitivity. The fitting spectra of *E. coli* detection were similar to that of *S. aureus*. The detection limit for *S. aureus* and *E. coli* was about 4×10^{-10} mol l^{-1}.

3.3. Dye leaching and stability experiment

In order to investigate the dye leakage from the NPs, appropriate NPs were ultrasonically exposed to an aqueous environment. The time-resolved fluorescence intensity at emission wavelength 611 nm was recorded after washing for 10 times. A mean value of 105.3 ($n = 10$) and a relative standard deviation of 3.18% were obtained. The ETP-IgG NPs displayed little dye leaching. Furthermore, the time-resolved fluorescence intensity has little decrease (from 125 to 121) under continuous lighting for 5 h.

3.4. Capability and selectivity of the prepared detection system

The experiments were carried out by recording the time-resolved fluorescence intensity of hybridization with different

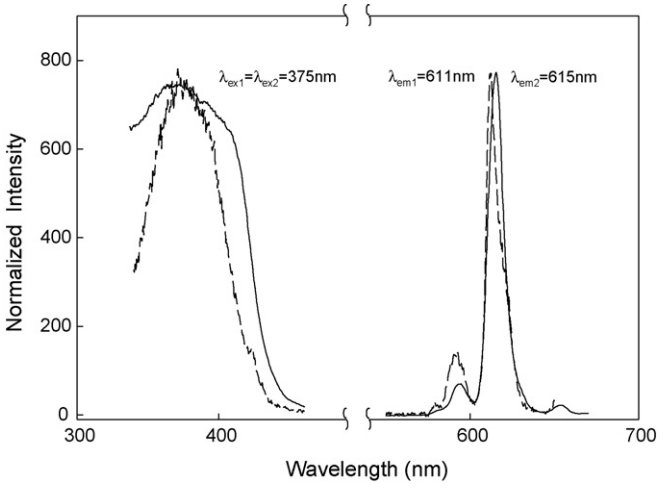

Fig. 4. Time-resolved fluorescence spectra for the ETN (—) and ETN-IgG doped NPs (- - -). For time-resolved spectra: delay time, 0.1 ms, gate time, 1.0 ms.

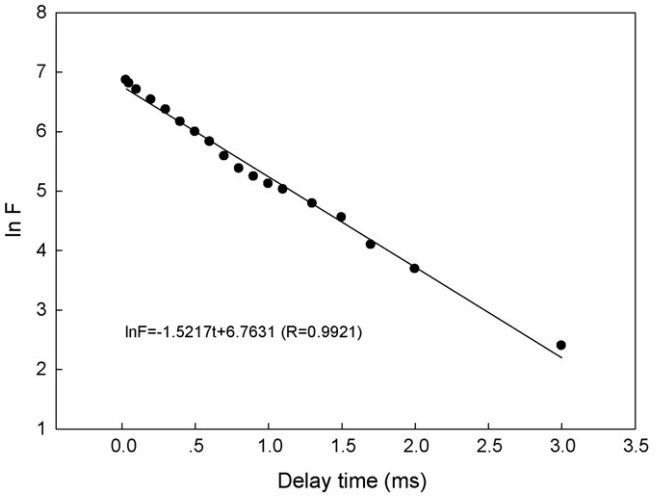

Fig. 5. The natural log of the intensity, ln *F*, as a function of the delay time.

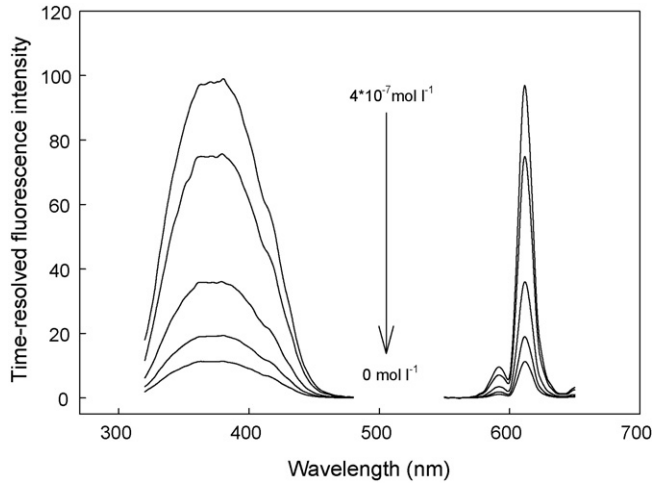

Fig. 6. Time-resolved fluorescence spectra for different concentrations of target DNA_3 of *Staphylococcus aureus* (from top to bottom: 4×10^{-7} mol l^{-1}, 4×10^{-8} mol l^{-1}, 4×10^{-9} mol l^{-1}, 4×10^{-10} mol l^{-1}, 0 mol l^{-1}), for time-resolved spectra: λ_{ex} = 375 nm, λ_{em} = 611 nm, delay time, 0.1 ms, gate time, 1.0 ms.

sequences of target DNA_3 and a mixture sample with other DNA species existed. Take *S. aureus* for example. In order to investigate the availability of this assay, the authors employed three random target sequences: s_1, 5′GAA GAA CAT TTA AGA AAA AGT GAA GCA CAA GCG AAA AAA GAG AAA AT3′; s_2, 5′AAG CCA TGA AGC GGC TTA TGA TTC TTA CCG CCC ACT TGA CGA AAG CG3′; s_3, 5′AAG GGA CGA TGA AAG CCG ATG AAG CCT CCT TTA CCT GGA CCG ATA AC3′. Five samples of different target DNA in same concentration including s_0 (the pure target DNA_3 of *S. aureus*), s_1, s_2, s_3, and a mixture sample of s_0, s_1, s_2, s_3 (same concentration of s_0 with first sample) were prepared. The results showed that fluorescence intensities of the five samples were 75.88, 15.22, 14.93, 19.92 and 80.67, showing little non-specific binding of random DNA sequences.

3.5. Optimal hybridization time

The optimal hybridization time for this assay was also investigated. The result showed that, in the initial stage of hybridization time less than 2.5 h, the hybridization signal increased, and then reached a steady state at a hybridization time longer than 2.5 h. On the basis of this, all the experiments were carried out for the hybridization time of 2.5 h.

3.6. Preliminary analytical application

Detections of DNA extracted from pure cultured *S. aureus* and *E. coli* were studied with the proposed hybridization assay. Strains of *S. aureus* and *E. coli* purchased from Guangdong Institute of Microbiology were cultured, and the double-stranded DNA was extracted using Genomic DNA extraction Kit (Generay Biotech Co., Ltd., Shanghai, China). The double-stranded DNA fragments were denatured to single-stranded DNA by heating at 90 °C for 15 min before use. Targets DNA of *S. aureus* and *E. coli* in a same concentration of about 2×10^{-7} mol l^{-1} were prepared. Satisfactory fluorescence intensities of 56.43 and 48.71 obtained showed that the method might have potential values for pathogen detection.

4. Conclusion

This work reported an approach of DNA detection using a lanthanide europium complex ETN-IgG doped NPs based two-probe tandem DNA hybridization assay. The oligonucleotide sequences of *S. aureus* and *E. coli* genes were designed. The NPs exhibited extremely long lifetime and excellent signaling ability of a trace amount of target DNA. The selectivity and capability of the prepared detection system were also investigated. The authors believe the method has enormous promise for applications to pathogen detection.

Acknowledgements

This work was financially supported by the National 863 High Technology Research Foundation of China (2006AA06Z407), the Hunan Provincial Natural Science Foundation of China (06JJ20062), the National Natural Science Foundation of China (50808070, 50608029) and the Changjiang Scholars and Innovative Research Team in University (PCSIRT).

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