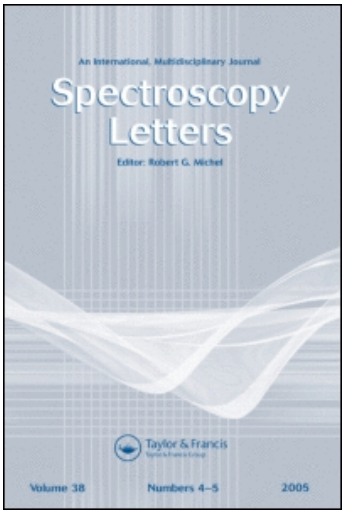

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Simple and Reliable Method for the Determination of Exocellular DNA in a Bacterial Culture by $Ru(phen)_2(dppz)^{2+}$

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Abstract: A novel fluorimetric method was developed for the selective determination of DNA under physiological conditions with the molecular "light-switch" complex $Ru(phen)_2(dppz)^{2+}$. Under optimal conditions, the fluorescence intensity was linear to the concentration of DNA. The linear range for calf thymus DNA (CT-DNA) was 0–2.0 μg/mL, and the limit of detection was 0.001 μg/mL. The proposed method was successfully applied to the determination of exocellular DNA in a bacterial culture.

Keywords: DNA, fluorescence, molecular "light-switch", $Ru(phen)_2(dppz)^{2+}$

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INTRODUCTION

In parallel with the boom in genetic engineering, public and scientific concern has grown with respect to the potential risks associated with unintentional and uncontrolled horizontal transfer of engineered DNA from genetically modified organisms (GMOs) to native organisms, thereby changing the properties of one or more of the species in the environment in an unwanted way.[1] Such concern is accentuated by the fact that exocellular DNA, liberated either by active excretion or by cell lysis, could persist in the environment for a considerable period of time and find a way eventually to enter and function in some bacterium in a process termed *natural genetic transformation*.[2,3] A great number of research projects have been conducted to monitor bacteria-produced exocellular DNA and to evaluate the potential risks of such "genetic pollution" in various environmental situations.[4–6]

Several methods have been employed to determine exocellular DNA liberated during growth of bacteria. Polymerase chain reaction (PCR) and genetic transformation are frequent choices among them because of their high sensitivity, but the procedures of both methods are complicated, time-consuming, and highly dependent on the specific DNA sequence.[7,8] The manipulation to determine DNA by ultraviolet absorbance is simple, but it has low sensitivity and is easily affected by other materials in physiological conditions. The fluorescence method has the merit of high sensitivity and high selectivity, and many fluorescence probes for DNA have been reported, such as ethidium bromide,[9] DAPI,[10] Hoechest 33258,[11] berberine,[12] ToTo and YoYo,[13] and so on. Among these fluorescence probes, the molecular "light-switch" is one of the most interesting probes.[14–16] These kinds of complexes are not photo luminescent in water but do emit in non-aqueous solvents or in the presence of DNA. They provide excellent candidates for DNA determination. Up to now, many light-switch complexes have been found: $Ru(bipy)_2(dppz)^{2+}$, $Ru(bipy)_2(dppx)^{2+}$, $Ru(phen)_2(dppz)^{2+}$, $Ru(phen)_2(dppx)^{2+}$ (bipy = 2,2′-bipyridine, phen = 1,10-phenanthroline, dppz = dipyrido[3,2-*a*:2′,3′-*c*]Phenazine, dppx = 7,8-dimethyl-dipyrido [3,2-*a*:2′,3′-*c*] Phenazine),[14–16] $Ru(bipy)_2(HNOIP)^{2+}$ (HNOIP = 2-(2-hydroxy- 5-nitrophenyl) imidazo[4,5-*f*]-1,10-phenanthroline),[17] $Ru(bipy)_2(PHEHAT)^{2+}$ (PHEHAT = 1,10-phenanthroline[5,6-*b*]1,4,5,8,9,12-hexazzatriphenylene),[18] $Ru(pztp)_2(phen)^{2+}$ (pztp = 3-(pyrazin-2-*yl*)*as*-triazino[5,6-*f*]-1,10-phenanthroline),[19] and $Os(phen)_2(dppz)^{2+}$.[20] The interaction mode between molecular light-switch complexes and DNA has been investigated.[21–24] This research laboratory has reported a method of quantitative analysis of DNA by use of some molecular light-switch complexes in basic medium.[25–27] Here we report the feasibility of the molecular light-switch $Ru(phen)_2(dppz)^{2+}$ in the determination of DNA in physiological condition, and it promises to be a simple, sensitive, and reliable method for the determination of exocellular DNA in a bacterial culture.

EXPERIMENTAL

Reagents and Solutions

Double-distilled water was used to prepare all solutions. Unless stated, all the chemicals were of analytical reagent grade or better. The calf thymus DNA (CT-DNA) was purchased from HuaMei biochemical Co. (Luoyang, China). The solution of calf thymus DNA in 50 mM NaCl gave a ratio of UV absorbance at 260 nm and 280 nm of 1.8–1.9, indicating that the DNA was sufficiently free of protein. The concentration of CT-DNA was calculated according to the extinction coefficients at 260 nm ($\varepsilon_{260\,nm} = 6600\,L \cdot mol^{-1} \cdot cm^{-1}$). $Ru(phen)_2(dppz)(BF_4)_2 \cdot 2H_2O$ was synthesized according to Refs.[14–16] The stock solution of $Ru(phen)_2(dppz)^{2+}$ was prepared by dissolving 19.1 mg $Ru(phen)_2(dppz)(BF_4)_2 \cdot H_2O$ in 200 mL water.

Instrumentation

Fluorescence intensities were measured with a Perkin Elmer LS 55 luminescence spectrometer with a quartz cell (1 cm × 1 cm cross section). Ultraviolet-visible absorption spectra were recorded on a Shimadzu UV-1601 spectrophotometer using 1 cm pathlength cells. The pH was measured with a model pHS-3C meter (Shanghai Leici Equipment Factory, Shanghai, China).

Preparation of Samples Containing Bacterial-Released DNA

The bacterial species used in this work was *Bacillus subtilis*. A small amount of *Bacillus subtilis* was inoculated in a minimum of medium at 37°C overnight to be activated. Then, 0.50 mL of the sample was inoculated in 50 mL medium at 37°C. Samples were set at different times, and the absorbance at 600 nm that corresponded to the concentration of bacteria was determined. Samples were centrifuged at 12,000 rpm for $1 \sim 2$ min, and the upper phase was transferred to a new tube and purified by cellulose filter paper (aperture 0.3 μm). Then, the bacteria were thoroughly cleaned away, and the DNA in the solution could be determined. The components of the minimum medium (w/w) were 0.2% $(NH_4)_2SO_4$, 1.4% K_2HPO_4, 0.6% KH_2PO_4, 0.1% sodium citrate, 0.02% $Mg_2SO_4 \cdot 7H_2O$, and 0.5% glucose water solution.

Procedures

Samples containing appropriate concentrations of light-switch complexes and related DNA or samples containing released DNA, were made up to 10 mL in buffer solution (50 mM NaCl). Then, the fluorescence intensity or

the absorption spectra were measured. The fluorescence intensity was measured with the following setting of the spectrometer: excitation wavelength ($\lambda_{ex} = 393$ nm), excitation slit (EX = 10 nm), emission wavelength ($\lambda_{em} = 605$ nm), and emission slit (EM = 15 nm).

RESULTS AND DISCUSSION

Spectral Characteristics

The excitation and emission spectra in the absence and in the presence of CT-DNA were investigated (Fig. 1). The emission spectra of $Ru(phen)_2(dppz)^{2+}$ (Fig. 1b) and water (not shown in Fig. 1) are the same, which means that $Ru(phen)_2(dppz)^{2+}$ does not fluoresce in the absence of DNA and the emission spectrum is the background. But when CT-DNA was added, the fluorescence was enhanced dramatically. The emission peak occurs at 605 nm. There are two excitation peaks at the wavelengths of 393 nm and 444 nm, and the two peaks are almost equal in intensity. In the following studies, 393 nm was selected as the excitation wavelength considering the interference of the medium.

Effect of pH

The effects of different buffer solutions and pH on the emission of $Ru(phen)_2(dppz)^{2+}$–DNA were studied. In Tris-HCl buffer solution, in the

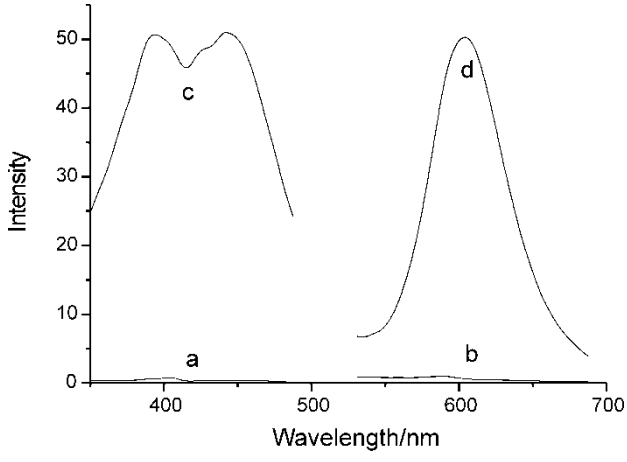

Figure 1. Excitation (a, c) and emission (b, d) spectra of $Ru(phen)_2(dppz)^{2+}$ in the absence (a, b) and in the presence (c, d) of CT-DNA. $Ru(phen)_2(dppz)^{2+}$, 1.0×10^{-6} mol/L; CT-DNA, 1.0 μg/mL.

pH range 7.0–9.5, the maximum emission of $Ru(phen)_2(dppz)^{2+}$–DNA occurs at the highest pH (9.5), but in the boric buffer solution (pH range 7.5–9.0) and phosphoric buffer solution (pH range 6.5–9.0), the maximum emission of $Ru(phen)_2(dppz)^{2+}$–DNA occurs at the lowest pH values (pH 7.5 and 6.5, respectively) (Fig. 2). The maximum emission of $Ru(phen)_2(dppz)^{2+}$–DNA occurs at pH 7.5 in boric buffer solution. Therefore, the boric buffer solution of pH 7.5 was used to control the pH in the subsequent experiments.

Effect of the Concentration of $Ru(phen)_2(dppz)^{2+}$

The influence of the concentration of $Ru(phen)_2(dppz)^{2+}$ was investigated. Figure 3 shows that the emission intensity increases with increasing concentration of $Ru(phen)_2(dppz)^{2+}$; when the concentration of $Ru(phen)_2(dppz)^{2+}$ was more than 1.7×10^{-6} mol/L, the fluorescence intensity reached a plateau, which corresponds to the bound ratio of about 3 base pairs per complex (according to the intersection of two fitted lines while the concentration of $Ru(phen)_2(dppz)^{2+}$ is lower than 0.7×10^{-6} mol/L and higher than 1.7×10^{-6} mol/L, respectively). A concentration of 2.0×10^{-6} mol/L was used in the subsequent experiments.

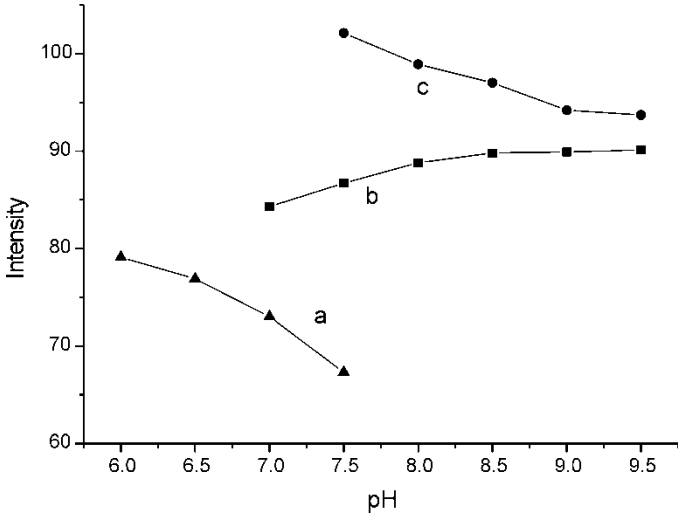

Figure 2. Effect of pH in different buffers on the fluorescence intensity of $Ru(phen)_2(dppz)^{2+}$ in the presence of DNA. (a) Phosphoric buffer, (b) Tris buffer, (c) boric buffer.

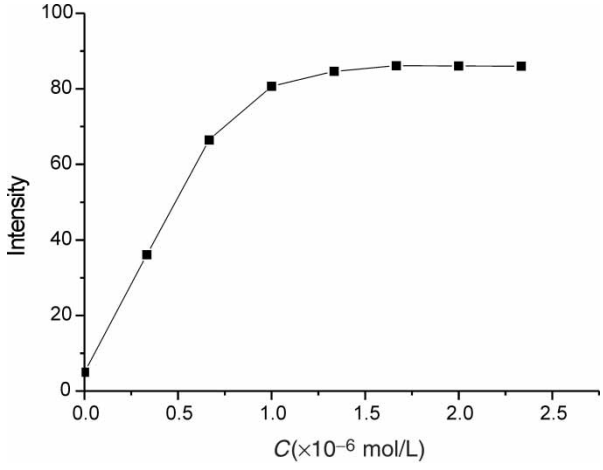

Figure 3. Effect of the concentration of $Ru(phen)_2(dppz)^{2+}$ on the fluorescence intensity of $Ru(phen)_2(dppz)^{2+}$–DNA. (2 μg/mL CT-DNA).

Interference of Coexisting Substance

The interference of coexisting substances such as protein, bases, yeast RNA, and metal ions was investigated. The results indicated that 10-fold excess of yeast RNA, A, G, C, T (w/w), 5-fold excess of BSA (w/w), 50-fold excess of Na^+, Mg^{2+}, and $H_2PO_4^-$ had no interference with the determination of DNA.

Limit of Detection, Linear Range, and Precision

The correlation between I_F (relative fluorescence intensity that is read from the spectrometer directly without correction) and the concentration of DNA was found to be $I_F = 2.55 + 43.0\ c$ (where the unit for c is μg/mL), with a correlation coefficient $r = 0.9998$. The linear range for CT-DNA was 0–2000 ng/mL, and the limit of detection was 0.001 μg/mL (3σ). The precision at 1.0 μg/mL was 0.31%. All these value were obtained with the general procedure.

Unlike the absorbance, the fluorescence intensity is always relative intensity, which was dramatically affected by many factors like lamp condition, excitation slit, emission slit, the sensitivity of the detector, and so forth. So when the proposed method was applied to determine DNA concentration under different conditions, a new equation correlating I_F and DNA concentration must be established under certain conditions.

Sample Analysis and DNA Recovery

$Ru(phen)_2(dppz)^{2+}$ could intercalate into the base pairs of double-strand DNA and result in fluorescence recovery. The bacteria-released DNA were also estimated as double strand and could be detected by the proposed method. Because $Ru(phen)_2(dppz)^{2+}$ does not emit in absence of DNA, the fluorescence intensities of the samples in the absence and presence of $Ru(phen)_2(dppz)^{2+}$ were determined to eliminate the interference of the minimum medium, and the fluorescence intensities of samples in the absence of $Ru(phen)_2(dppz)^{2+}$ were subtracted as background. The concentrations of DNA in samples were calculated by the adjusted formula $\Delta I_F = 43.0\ c$ (where the unit for c is $\mu g/mL$). The analytical results of the released DNA of bacteria at different times are shown in Fig. 4b. To detect the applicability of the proposed method, the addition and recovery of CT-DNA in samples were also studied. That is, a certain amount of CT-DNA was added into the sample, and the added CT-DNA was detected. The ratio of detected and added CT-DNA denotes the interference of the foreign substance in the sample and the accuracy of the determination. The recoveries of CT-DNA in bacterial culture show good reliability of the proposed method (Table 1).

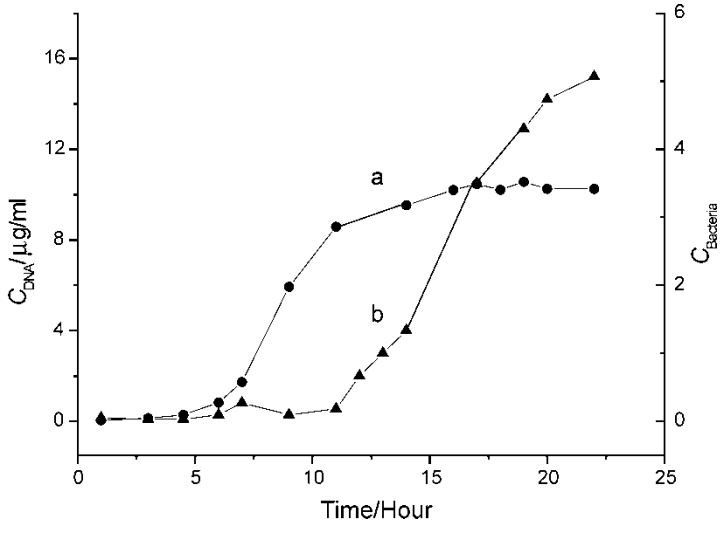

Figure 4. Growth curves of *Bacillus subtilis* (a) and the changes of DNA concentration (b) with the growing of bacteria in minimum medium.

Table 1. Recovery of CT-DNA in bacterial culture

	CT-DNA (μg/mL)		
Sample	Added	Found	Recovery (%)
1	0.10	0.0967	96.7
2	0.20	0.2030	101.5
3	0.67	0.6499	97.0
4	0.67	0.6767	101.0
5	0.67	0.6686	99.8

CT-DNA, calf thymus DNA.

Growth Curve of Bacteria and Relationship of Bacteria and DNA

The values of relative concentration of bacteria were determined according to the absorption at 600 nm. The samples were diluted while the absorbance was higher than 1.2. The growth of bacteria is shown in Fig. 4a.

Figures 4a and 4b show that the concentration of DNA corresponded to the growth of bacteria. The growth of *Bacillus subtilis* can be divided into four phases; that is, lag phase, exponential phase (log phase), stationary phase and decline phase. In the first 4 hours (lag phase), the concentration of bacteria is quite low, and there is almost no DNA released by it. From 4 hours to about 7 hours (beginning of log phase), along with the growth of bacteria, the DNA released by it and the concentration of DNA increases slightly. From 7 hours to about 14 hours (exponential phase), the concentration of DNA increases greatly with the growth of bacteria, and within this period, at about 9 hours, an excretion summit of DNA appeared, which is in accordance with the report in a reference.[8] After 14 hours (stationary phase and decline phase), some bacteria die and some cell walls are destroyed, much DNA is released, and the concentration of DNA is enhanced dramatically.

CONCLUSIONS

The fluorescence intensity of the molecular light-switch complexes were estimated to increase 10,000 times when intercalated into the base pairs of DNA.[14] Though the apparent enhancement factor did not reach the estimated ones due to the background related to the spectrometer under certain conditions, the characteristic of having no detectable fluorescence in water and high selectivity make $Ru(phen)_2(dppz)^{2+}$ more flexible for DNA determination than other probes. Good recoveries of CT-DNA, in the minimum medium containing bacteria-released DNA, show that it is a

sensitive, selective, and accurate fluorescence probe for the determination of DNA. Molecular light-switch complexes could be widely used as probes for exocellular DNA determination in various situations.

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