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Determination of DNA by Rayleigh light scattering enhancement of molecular "light switches"

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Base on the enhancement of Rayleigh light scattering signals of molecular "light switches" by DNA under acidic condition, a sensitive and convenient method for DNA determination was proposed. The experiments indicated that, under optimum conditions, good linear relationships were obtained between the Rayleigh light scattering intensity and the concentration of nucleic acids. The detect limits of calf thymus DNA (ctDNA) were 13.0 ng ml^{-1}, 4.2 ng ml^{-1}, 51.5 ng ml^{-1} and 3.0 ng ml^{-1} with four "light switches", respectively. Plasmid DNA extracted from *Bacillus subtilis* were determined by the proposed method with satisfactory results, and the recovery rates of calf thymus DNA were in the range of 94.6–110.7%.

1. Introduction

The qualitative and quantitative analysis of nucleic acids, especially the micro-determination of nucleic acids, is becoming more and more important in many biological studies. Generally, the conventional spectrophotometric method based on ultraviolet absorption at 260 nm is performed routinely in the laboratory for the determination of DNA. However, this method has been limited severely by low sensitivity and serious interferences.[1,2] Fluorescence methods have merits of high sensitivity and high selectivity. Based on the fluorescence enhancement by DNA, methods using ethidium bromide,[3] DAPI,[4] Hoechest33258,[5] berberine,[6] ToTo, YoYo[7] and some molecular "light switches" $Ru(phen)_2dppz^{2+}$,[8] $Ru(phen)_2dppx^{2+}$,[9] $Ru(bpy)_2dppz^{2+}$,[10] $Ru(bpy)_2dppx^{2+}$[11] *etc.* have been reported (bpy = 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).

In common spectrofluorimetry, light scattering is a major source of interference, and should be minimized, but recently, a promising spectral technique, which was based on the measurement of enhanced Rayleigh light scattering (RLS),[12,13] has given rise to strong interest by analysts and biochemists.[14–20] The RLS signals have successfully been employed for DNA determination. Up to now, many kinds of compounds' RLS intensity can be increased after binding to DNA, such as: 1. Porphyrin and its derivatives: TAPP,[14,20] H_2TPPS [21] *etc.* 2. Alkaline dye: safranine T,[22] natural red,[23] methylene blue,[24] nile blue,[18,25] rhodamine B,[26] azur B[27] and TAAIPc[28] *etc.* 3. Metal cation complexes: Co(II)-5-Cl-PADAB[17] *etc.* 4. Cation surfactant: CTMAB[29] CPB, TPB, CDBAC *etc.*[30] All these compounds are characterized by a positive charge that is contrary to the DNA.

Molecular "light switches" are a kind of Ru(II) complex which are not photoluminescent in water but luminescent while intercalated into the base pairs of DNA. The fluorescence methods using molecular "light switches" for DNA determination in alkaline medium[8–11] have been developed by our group before. Here a novel RLS method for DNA assay is proposed in acidic condition. Little interference was observed from proteins, nucleosides, amino acids and many metal ions. This assay is characterized by high sensitivity, a wide linear range, rapid reaction, good stability and easy practices.

2. Experimental

2.1. Apparatus

All RLS measurements were performed using a PerkinElmer Model LS-55 spectrometer with a quartz cuvette (1 × 1 cm). A Shimadzu Model UV-1601 double-beam spectrophotometer was used for recording the absorption spectra. The pH was measured with a Model pHS-3C meter (Shanghai Leici Equipment Factory, China).

2.2. Reagents

All chemicals were analytical reagents of the best grade commercially available. All stock solutions were prepared using doubly distilled water.

The calf thymus DNA was purchased from HuaMei Biochemical Co. (China). The concentration of CT-DNA was calculated according to the absorption at 260 nm (50.0 μg ml^{-1} per OD). $Ru(phen)_2(dppz)(BF_4)_2{\cdot}2H_2O$, $Ru(phen)_2(dppx)(BF_4)_2{\cdot}3H_2O$, $Ru(bpy)_2(dppz)(BF_4)_2{\cdot}1.5H_2O$, $Ru(bpy)_2(dppx)(BF_4)_2{\cdot}2H_2O$ were synthesized according to refs. 31 and 32 and identified by ^{1}H NMR.[9] The stock solutions of $Ru(phen)_2(dppz)^{2+}$, $Ru(phen)_2(dppx)^{2+}$, $Ru(bpy)_2(dppz)^{2+}$, $Ru(bpy)_2(dppx)^{2+}$ (1.0×10^{-4} mol l^{-1}) were prepared by dissolving 19.1 mg $Ru(phen)_2(dppz)(BF_4)_2{\cdot}2H_2O$, 20.0 mg $Ru(phen)_2(dppx)(BF_4)_2{\cdot}3H_2O$, 17.9 mg $Ru(bpy)_2(dppz)(BF_4)_2{\cdot}1.5H_2O$ and 18.7 mg $Ru(bpy)_2(dppx)(BF_4)_2{\cdot}2H_2O$ in 200 ml water, respectively.

2.3. Preparation of samples

The bacteria used in this work were *Bacillus subtilis* DB104, which contained a recombinant plasmid PBE2. The procedure used for plasmid DNA extraction was as follows: An amount of

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1.5 ml of bacteria cultured overnight was collected into a microtube, centrifuged at 12 000 rpm at room temperature for 45 s and the supernatant was removed as completely as possible by aspiration through a Pasteur pipette. The pellet was dissolved in 100 μl of solution A (50 mmol l^{-1} glucose, 25 mmol l^{-1} Tris–HCl, pH 8.0, 10 mmol l^{-1} EDTA). Then a 200 μl of solution B (0.2 mol l^{-1} NaOH, 1% SDS) was added to breakdown the cells and denature the nucleic acids. The tube was then put on ice for about 4 min, 150 μl of solution C (3 mol l^{-1} KAc, 11.5% HAc) was added to neutralise the solution. The tube was centrifuged at 12 000 rpm for about 8 min, the plasmid DNA remained in solution, whereas the chromosomal DNA and most of the cellular RNA and protein was precipitated. About 450 μl of the supernatant was transferred to a fresh tube and an equal volume of chloroform/isoamyl alcohol (1:1 mixture) was added and mixed completely. The suspension was then centrifuged at 12 000 rpm for 8 min, the upper phase (containing the nucleic acids) was carefully transferred to a fresh tube and 1 ml of 100% ethanol was added. The tube was allowed to stand at room temperature for 10 min, then the nucleic acids were precipitated by centrifugation at 12 000 rpm for 10 min. The supernatant was removed as completely as possible and the pellet (composed of plasmid DNA and some small molecular RNA) was dried at 65 °C for about 2 min. 250 μl of TE buffer (10 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA, pH 8.0) was then added to dissolve the sample.

2.4. Procedures

Samples containing appropriate concentrations of molecular "light switches", DNA, NaCl and BR buffer were made up to 10 ml. The solutions were kept in 1 cm quartz cuvettes and were measured 15 min after the solutions were thoroughly mixed. RLS spectra were gained by synchronously scanning with the same wavelength of excitation and emission through 250–600 nm. Based on the spectra, the RLS intensities were determined at 336 nm.

3. Results and discussion

3.1. Features of RLS spectra

The RLS spectra of four molecular "light switches" are shown in Fig. 1. All molecular "light switches" have low RLS intensity in acidic conditions (not shown in Fig. 1), but when DNA was added, the RLS intensity increased dramatically. Among the four complexes, the two "light switches" containing dppx ligand have a higher RLS intensity than those containing dppz ligand, which can be attributed to the large molecule and strong interaction with DNA.[9] As the four "light switches" have similar properties and $Ru(bpy)_2(dppx)^{2+}$ is the most sensitive RLS probe for the DNA assay, it was selected for the following studies. Among three RLS peaks: 336 nm, 396 nm and 483 nm, the RLS peak at 336 nm is the highest, so 336 nm was selected for DNA determination.

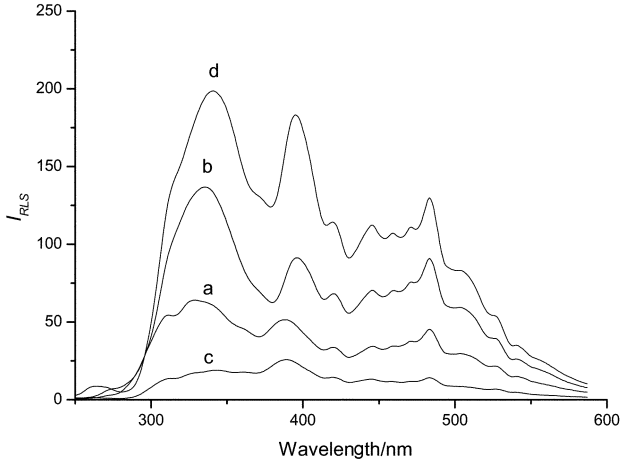

Fig. 1 RLS spectra of "light switches" (1.0×10^{-5} mol l^{-1}) in presence of ctDNA (1.60 μg ml^{-1}). a. $Ru(phen)_2dppz^{2+}$, b. $Ru(phen)_2dppx^{2+}$, c. $Ru(bpy)_2dppz^{2+}$ and d. $Ru(bpy)_2dppx^{2+}$.

3.2 Absorbance spectra study

The effect of DNA on the absorbance spectra of the four molecular "light switches" has been studied. The absorbance spectra of the four molecular "light switches" in the absence and presence of DNA show that there exists strong hypochromism and red shift after adding DNA. The results revealed that the "light switches" intercalate into the base pairs of DNA.[33–35]

The corresponding relationships between the RLS spectra and the absorption spectra are clearly shown. Taking $Ru(bpy)_2(dppx)^{2+}$ for example, there are three peaks at 336 nm, 396 nm and 483 nm and two valleys at 375 nm and 430 nm in the RLS spectrum (Fig. 1d). Correspondingly, there are two valleys about 340 nm and 405 nm and three peaks at 290 nm, 380 nm and 450 nm in the absorbance spectrum (Fig. 2). Furthermore, the RLS intensity increases in presence of DNA while the absorbance decreases. That is, high scattering intensity corresponds to low absorbance and the increase of RLS corresponds to the decrease of absorbance. These could be elucidated by the theory of resonance depolarized Rayleigh scattering.[12,13] The intensity of RLS of transparent isotropic media is in proportion to λ^{-4}, where λ is the wavelength of incident light in free space. If the intensity deviates from the dependence of λ, it is possible that the incident wavelength is near the absorption band of the analyte molecules.

3.3. Effect of pH

The effect of pH on the RLS was investigated. The scattering intensity of the assay system is greatly affected by the pH whereas the RLS of the reagent blank is not affected. As shown in Fig. 3, the highest RLS intensity occurs at pH 2.3 and decreases dramatically when the pH is higher than 2.7. Therefore, pH 2.3 was chosen for the assay. The dependence of the RLS intensity on the pH might be determined by the form of DNA. With the increasing acidity, the complex $Ru(bpy)_2(dppx)^{2+}$ is stable while part of the phosphate on the backbone is protonized, then DNA is easily aggregated, which

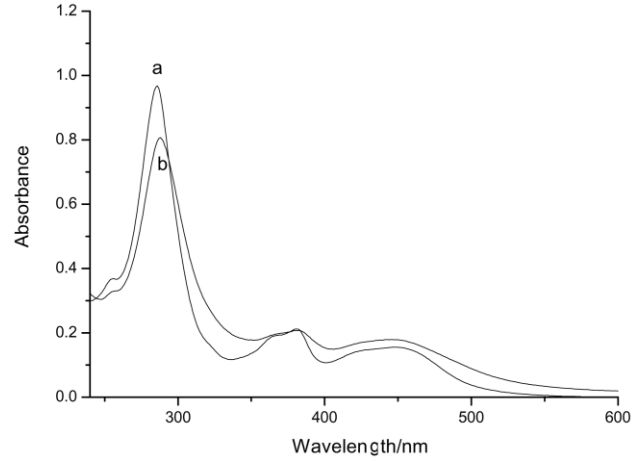

Fig. 2 Absorbance spectra of $Ru(bpy)_2dppx^{2+}$ (1.0×10^{-5} mol l^{-1}) in the absent (a) and the present (b) of ctDNA (3.20 μg ml^{-1}).

enlarges the particle size while binding with $Ru(bpy)_2(dppx)^{2+}$ and results in high RLS intensity. Furthermore, when the pH is lower than about 1.5, DNA itself can aggregate to form large particles whose dimensions are comparable to the wavelength of UV-Vis light and result in very strong light scattering.[36]

3.4 Effect of the Ru(bpy)2(dppx)$^{2+}$ concentration

The influence of $Ru(bpy)_2(dppx)^{2+}$ concentration on the RLS signal is demonstrated in Fig. 4. It was shown that the RLS intensity increased with the increasing concentration of $Ru(bpy)_2(dppx)^{2+}$. But when the concentration of $Ru(bpy)_2(dppx)^{2+}$ is higher than 1.0×10^{-5} mol l^{-1}, the RLS intensity slightly decreases. This may be due to the larger absorbance of a higher concentration of $Ru(bpy)_2(dppx)^{2+}$. Furthermore, the concentration of $Ru(bpy)_2(dppx)^{2+}$ will affect the linear range for the determination of DNA owning to the molar ratio of nucleic acids to $Ru(bpy)_2(dppx)^{2+}$.

3.5 Effect of the ionic strength

NaCl was used to control the ionic strength of the solution. Generally, with the increasing amount of NaCl, the reaction of the anion and the cation would be restrained. That is, the anion of phosphate on the backbone is shielded by the cation ion of the ionic strength controller. But the results show that a concentration of 0.0–0.2 M NaCl does not influence the RLS intensity of $Ru(bpy)_2(dppx)^{2+}$–DNA. This suggests that the interaction between $Ru(bpy)_2(dppx)^{2+}$ and DNA is not merely electrostatic interaction, but a strong interaction. As reported, the molecular "light switch" of $Ru(bpy)_2(dppx)^{2+}$ intercalates into the base pairs of DNA.[32,33]

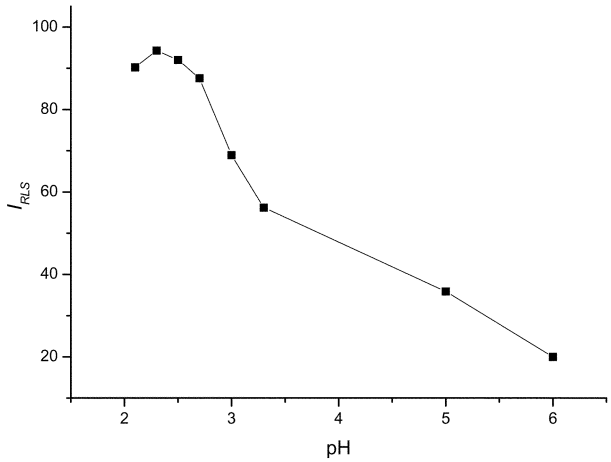

Fig. 3 Effect of pH on the RLS signals of $Ru(bpy)_2dppx^{2+}$(1.0×10^{-5} mol l^{-1}) in present of ctDNA (0.80 μg ml^{-1}).

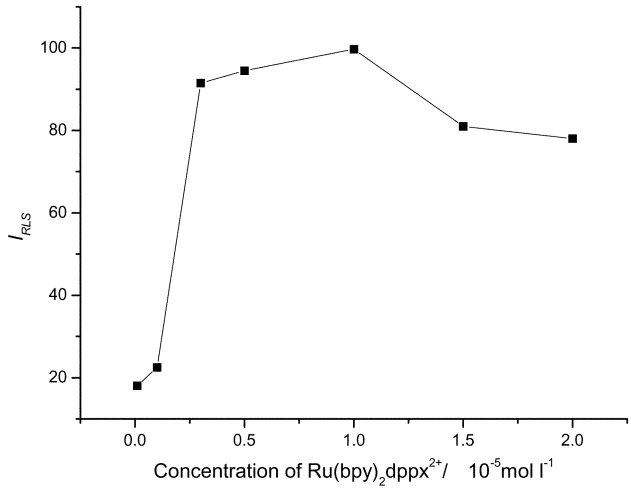

Fig. 4 Effect of $Ru(bpy)_2dppx^{2+}$ concentration on the RLS signals of $Ru(bpy)_2dppx^{2+}$–DNA. ctDNA: 0.80 μg ml^{-1}

3.6 Effect of the addition order of reagents

The addition of the reagents in different orders was investigated. The results showed that the order of addition of the reagents affects the RLS intensity of the system. The best procedure is mixing $Ru(bpy)_2(dppx)^{2+}$ and buffer solution first, and then adding the DNA.

3.7 Effect of reaction time

The effect of the reaction time was evaluated by detecting the RLS intensity every 2 min for 1 h. The result shows that the reaction had finished in 10 min and the RLS intensity did not change further in the 1 h (Fig. 5). Therefore, all the measurements could be carried out after mixing reaction solution for 15 min.

3.8 Influence of coexisting substances

The influence of foreign coexisting substances such as proteins, bases, and metal ions *etc.* were tested. The results are presented in Table 1. Nearly all the tested metal ions can be allowed up to relatively high concentrations of 50 μg ml^{-1} and the tolerance concentrations of BSA, bases and other biochemical reagents are 5 μg ml^{-1} or higher.

3.9 Calibration and assay of extracted samples

Under the optimum conditions of $Ru(bpy)_2(dppx)^{2+}$, four molecular "light switches" were use to determine ctDNA. The correlations between I_{RLS} and the concentration of ctDNA are shown in Table 2. Four complexes, especially two "light switches" containing dppx ligand, have wider linear ranges and lower detect limits for the DNA assay. Possibly the three other "light switches" would gain better results under their own optimum conditions, but the complex containing dppx was still the most sensitive RLS probe for DNA determination.[9]

The proposed method is applied to the determination of plasmid DNA extracted from *Bacillus subtilis* DB104 that

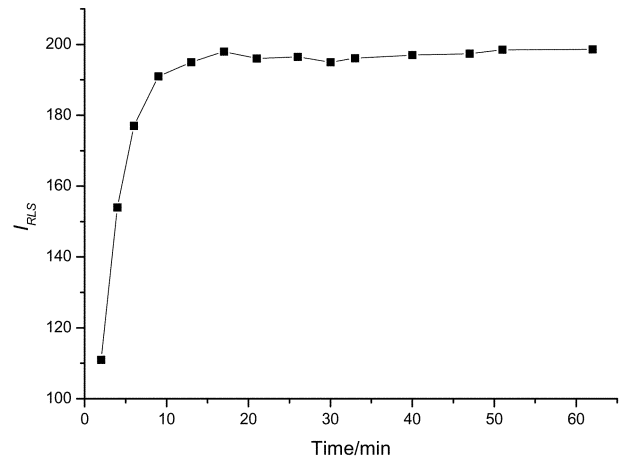

Fig. 5 Effect of reaction time on the RLS signals of $Ru(bpy)_2dppx^{2+}$–ctDNA. ($Ru(bpy)_2dppx^{2+}$: 1.0×10^{-5} mol l^{-1}, ctDNA: 1.60 μg ml^{-1})

Table 1 Tolerance of foreign substances. (pH = 2.3, $Ru(bpy)_2dppx^{2+}$: 1×10^{-5} mol l^{-1}, ctDNA: 1.2 μg ml^{-1})

Foreign substance	Concentration/ μg ml^{-1}	Change of ΔI_{RLS} (%)	Foreign substance	Concentration/ μg ml^{-1}	Change of ΔI_{RLS} (%)
$Mn^{2+}(MnSO_4 \cdot H_2O)$	50	−1.47	Glycine	10	−3.48
$Co^{2+}(CoCl_2 \cdot 6H_2O)$	50	3.4	Glucose	50	−3.62
$Mg^{2+}(MgSO_4)$	50	−3.79	BSA	5	3.15
$Ca^{2+}(CaCl_2)$	50	−1.86	Guanine	5	−4.29
EDTA	50	5.39	Thymine	5	−3.06
Lactose	50	−2.01	Uracil	5	−5.39
Citric acid	50	−0.49	Histidine	50	−1.59

Table 2 Analytical parameters for determination of ctDNA by use of molecular "light switch" complexes (1.0×10^{-5} mol l^{-1})

"Light switch" complexes	Linear regression equation (c: μg ml^{-1})	Correlation coefficient (r^b)	Limit of detection (3σ)/ng ml^{-1}	Line range/ ng ml^{-1}
$Ru(phen)_2dppz^{2+}$	$I_{RLS} = 1.4 + 31.37c$	0.9966	13.0	40–4000
$Ru(phen)_2dppx^{2+}$	$I_{RLS} = 7.4 + 97.25c$	0.9997	4.2	40–9000
$Ru(bpy)_2dppz^{2+}$	$I_{RLS} = 2.4 + 7.93c$	0.9885	51.5	100–5000
$Ru(bpy)_2dppx^{2+}$	$I_{RLS} = 1.0 + 136.4c$	0.9993	3.0	40–6000

Table 3 Recovery of ctDNA in real samples

Sample	Plasmid DNA/ μg ml^{-1}	ctDNA		
		Added/ μg ml^{-1}	Found/ μg ml^{-1}	Recovery (%)
1	0.1	1.2	1.25	104.8
		2.4	2.27	94.6
2	0.3	1.2	1.29	107.5
		2.4	2.40	100.1
3	0.5	1.2	1.32	110.7
		2.4	2.50	104.3

contained a recombinant plasmid PBE2. To detect the applicability of the method, the addition and recovery of ctDNA in the extracted samples were also studied. As shown in Table 3, the values found for the three samples are identical with the expected ones, the recoveries are within 94.6–110.7%. Therefore, the proposed method is applicable.

4. Conclusion

A sensitive method is proposed based on the enhancement of Rayleigh light scattering signals of molecular "light switches" by DNA. However, sensitivity is always contrary to precision for any analytical method to a certain degree. Compared with the fluorescence method, RLS method has relatively higher sensitivity and lower precision. But RLS technique is still a significant method for nucleic acids analysis. As future work, it would be interesting to see the effect of DNA thermal denaturation on the RLS of molecular "light switches" to study structural effects. Also, it would be interesting to see if RNA shows the same RLS effect with these molecular "light switches".

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