

Microdetermination of Nucleic Acids by Enhanced Resonance Light Scattering Spectroscopy of μ -oxotetraphenyl Porphyrinatoiron

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Abstract

A new method for the determination of nucleic acids at the nanogram level is proposed based on enhanced resonance light scattering (RLS) signal resulting from the interaction of μ -oxotetraphenyl porphyrinatoiron [(Fe(III)TPP)₂O] with nucleic acids. Under the optimum conditions, the reaction between (Fe(III)TPP)₂O and nucleic acids enhanced the weak light scattering signal of (Fe(III)TPP)₂O, and the enhanced light scattering intensity was proportional to the concentrations of nucleic acids in the range of 0.02-2.5 mg L⁻¹ for calf thymus DNA, 0.02-2.7 mg L⁻¹ for fish sperm DNA, and 0.03-3.1 mg L⁻¹ for yeast RNA. The detection limits (3 σ) are 1.0 ng mL⁻¹ for calf thymus DNA, 1.1 ng mL⁻¹ for fish sperm DNA, and 1.2 ng mL⁻¹ for yeast RNA, respectively. Synthetic samples were determined with satisfactory results.

Keywords: resonance light scattering, nucleic acid, μ -oxotetraphenyl porphyrinatoiron

Résumé

Nous proposons une nouvelle méthode pour la détermination des acides nucléiques au niveau de mesure du nanogramme en se basant sur le signal amplifié de la diffusion de lumière par résonance (RLS), qui résulte de l'interaction entre le μ -oxotétraphényl porphyrinatofer [(Fe(III)TPP)₂O] et les acides nucléiques. Sous les conditions optimales, la réaction entre (Fe(III)TPP)₂O et les acides nucléiques augmente le faible signal de diffusion de lumière de (Fe(III)TPP)₂O, dont l'intensité devient alors proportionnelle aux concentrations d'acides nucléiques dans la gamme 0.02-2.5 mg L⁻¹ pour

l'ADN de thymus de veau, 0.02-2.7 mg L⁻¹ pour celle de sperme de poisson, et 0.03-3.1 mg L⁻¹ pour l'ADN de levure. Les limites de détection (3 σ) sont de 1.0 ng mL⁻¹ pour l'ADN de thymus de veau, 1.1 ng mL⁻¹ celle de sperme de poisson, et 1.2 ng mL⁻¹ pour l'ADN de levure, respectivement. Des échantillons synthétiques ont été déterminés et ont donné des résultats satisfaisants.

Introduction

The quantitative analysis of nucleic acids is of great importance because it can offer valuable genetic and inheritance information, which is helpful for molecular biology and diagnosis applications. Some methods have been developed to determine the amount of nucleic acid, but they suffer from serious drawbacks. The classical ultraviolet absorption spectrometry is limited by serious interferences (1); the spectrophotometric method of using organic reagents has the disadvantages of low sensitivity and multiple procedural steps (2-4); the fluorometric method (5-7) is only restricted in such systems and some fluorescent dyes are toxic. Facing the serious challenge, it is vital for biochemists to develop an efficient method for the quantitation of nucleic acids.

Recently, resonance light scattering (RLS) technique (8-10) has stimulated strong interests amongst analytical chemists. On the basis of its simplicity, high sensitivity and good selectivity, it turns out to be an efficient tool for the determination of biomacromolecular and metal ions (11-16). Many probes, including various basic dyes (17-21) and poly(ethylenimine) (22), have been used to determine the amount of nucleic acids. However, the use of μ -oxotetraphenyl porphyrinatoiron (Fe(III)TPP)₂O (Figure 1) for the quantitation of nucleic acids by the RLS technique has not yet been reported.

A model compound of "cytochrome P-450", (Fe(III)TPP)₂O, catalyzes the reaction of alkyl hydrocar-

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bon hydroxylation (23) and acts as a neutral carrier for PVC membrane electrodes (24). Our experimental results showed that the weak RLS signal of $(\text{Fe(III)TPP})_2\text{O}$ could be sharply enhanced with the addition of nucleic acids under the optimum conditions. Moreover, the en-

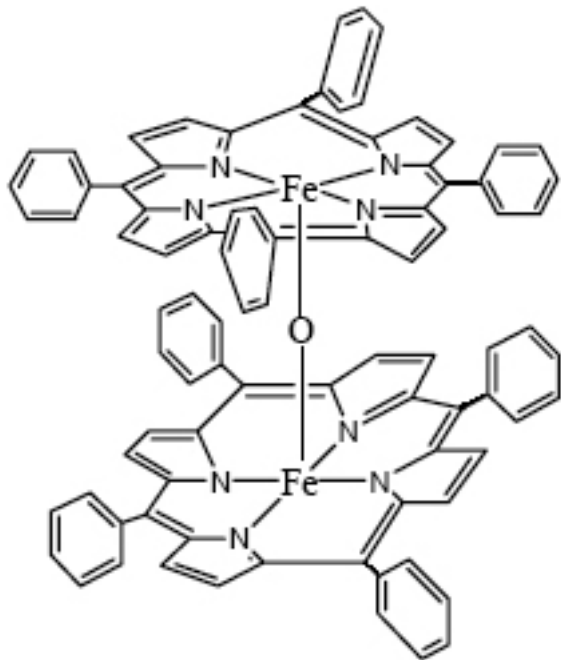


Figure 1. Structure of $(\text{Fe(III)TPP})_2\text{O}$.

hanced RLS signal was proportional to the concentration of nucleic acids. Based on this, a new method for the determination of nucleic acids is established. The method is simple, rapid, sensitive, and practical. Six synthesized samples were determined with satisfactory results. Our research also proved that the method involving $(\text{Fe(III)TPP})_2\text{O}$ was more sensitive than that of α , β , γ , δ -tetrakis[4-(trimethylammoniumyl)phenyl] porphyrin (TAPP) (11), which could be explained by their structural differences.

Experimental

Apparatus

The RLS spectra and intensities were measured with a Perkin-Elmer Model LS-55 fluorimeter spectrometer with a quartz cuvette (1×1 cm). All absorbance measurements were obtained using an Agilent 8453 ultraviolet spectrophotometer. An SA 720 laboratory instrument (Orion Research) was used to control the pH of the solution.

Chemicals

The stock solutions of nucleic acids were prepared by dissolving commercial products in double distilled

water. The concentration of the working solution was 5.0 mg L^{-1} . Calf thymus DNA (ctDNA) was purchased from Sino-American Biotechnology Company (China); fish sperm DNA (fsDNA) and yeast RNA (yRNA) were obtained from Sigma Chemical Company (USA). These stocks were stored at $0-4^\circ\text{C}$.

Tetraphenyl ferriporphyrin chorine [Fe(III)TPPCl] was synthesized according to the method proposed by Rothmund *et al.* (25). μ -Oxotetraphenyl porphyrinatoiron [$(\text{Fe(III)TPP})_2\text{O}$] was prepared by treating the chloroform solution of Fe(III)TPPCl with aqueous NaOH solution for 2h (26). Then, the organic layer was purified by chromatography using neutral Al_2O_3 column. The stock solution of $(\text{Fe(III)TPP})_2\text{O}$ was prepared by dissolving the crystallized product in the pure *N,N*-dimethylformamide (DMF) solution. The DMF solution used in the procedures was prepared with pure DMF and double distilled water (v/v 1:4). The working solution of $(\text{Fe(III)TPP})_2\text{O}$ was $1.0 \times 10^{-5} \text{ M}$.

The Britton-Robinson (BR) buffer solution was a mixture of 0.04 M phosphoric acid, 0.04 M acetic acid, 0.04 M boric acid and 0.2 M sodium hydroxide. A BR buffer solution of pH 6.80 was used to control the acidity. All chemicals used were of analytical grade or the best grade commercially available and double distilled water was used throughout.

Procedure

1.00 mL $(\text{Fe(III)TPP})_2\text{O}$ solution, 1.00 mL BR buffer solution and appropriate standard nucleic acids or sample solution were added to a 10 mL volumetric flask. They were mixed thoroughly and diluted to the mark with the prepared DMF solution.

The RLS spectra were obtained by scanning simultaneously the excitation and emission monochromators of the LS-55 spectrofluorometer from 250 nm to 700 nm with $\Delta\lambda = 0.0$ nm. Both the excitation and emission slit widths were kept at 5.0 nm. The RLS intensity of $(\text{Fe(III)TPP})_2\text{O}$ was measured at 444 nm. The enhanced RLS intensity of $(\text{Fe(III)TPP})_2\text{O}$ -ctDNA is represented as $\Delta I = I - I_0$, where I and I_0 are the intensities of the system with and without nucleic acids, respectively.

Results and Discussion

Features of RLS spectra

The RLS spectra of $(\text{Fe(III)TPP})_2\text{O}$ and $(\text{Fe(III)TPP})_2\text{O}$ -ctDNA are shown in Figure 2. It was found that the RLS signal of $(\text{Fe(III)TPP})_2\text{O}$ was faint in the whole scanning region, but increased sharply after the addition of ctDNA,

which proved that the reaction between $(\text{Fe(III)TPP})_2\text{O}$ and ctDNA had taken place. The RLS enhancement was attributed to the aggregation of $(\text{Fe(III)TPP})_2\text{O}$ on the surfaces of nucleic acids (8). Moreover, the enhanced RLS intensity was linear with respect to the concentration of nucleic acid. It suggested that a novel assay of nucleic acids should be developed. The RLS intensity of $(\text{Fe(III)TPP})_2\text{O}$ reached the maximum at 444 nm, which was selected as the excitation and emission wavelength.

Absorption spectra

The effect of nucleic acid on the absorption spectrum of $(\text{Fe(III)TPP})_2\text{O}$ is presented in Figure 3. In the region of the Soret band, the absorption peak of $(\text{Fe(III)TPP})_2\text{O}$ was located at 422 nm. However, it shifted to 423 nm after nucleic acids were added. The slight red shift in the absorption spectra showed that a new complex was produced. Besides this, the absorbance decreased with the addition of nucleic acid. When the metalloporphyrin compound intercalated into nucleic acids, the density of the electron cloud of the porphyrin ring was changed. The electron cloud of the bases of nucleic acids influenced the density of the electron cloud of the porphyrin ring, therefore, the hypochromic effect took place in the absorption spectra (27-28).

The corresponding relationships between the RLS spectra and the absorption spectra were clearly described. On one hand, there was a peak at 444 nm and one valley at 420 nm in the RLS spectra. Likewise, there was one wide valley near 450 nm and one peak at 423 nm in the absorption spectra. On the other hand, the RLS intensity increased in the presence of nucleic acid while the absorbance decreased. All of the above data were in

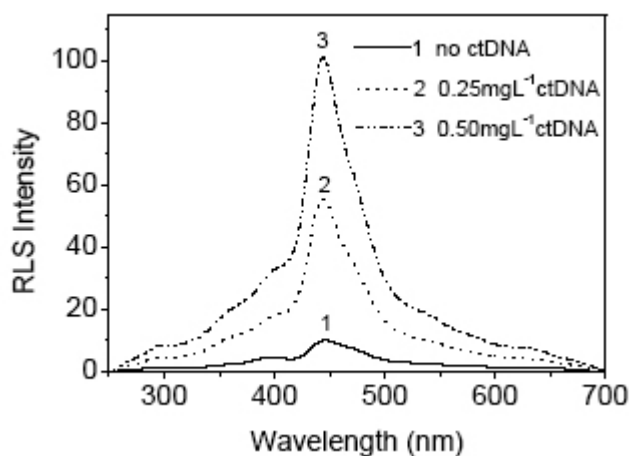


Figure 2. Resonance light scattering spectra. Conditions: $[(\text{Fe(III)TPP})_2\text{O}] = 1.0 \times 10^{-6} \text{ M}$; $\text{pH} = 6.80$.

agreement with the RLS theory (10).

Effect of pH

Figure 4 shows the effect of pH on I/I_0 , where I and I_0 represent the RLS intensities of the system with and without nucleic acid, respectively. The RLS intensity was greatly affected by the pH of the solution. In strong acidic solution, I/I_0 was small because the dissociation of the phosphate groups of the nucleic acid was inhibited. Thus, the combination between $(\text{Fe(III)TPP})_2\text{O}$ was weak. In the near neutral solution, the phosphate groups dissociated and were charged negatively. The negatively charged phosphate groups combined with the positive iron ion of the $(\text{Fe(III)TPP})_2\text{O}$ by electrostatic force to produce a new supermolecule complex $(\text{Fe(III)TPP})_2\text{O}$ -ctDNA. The RLS intensity of the system was the most strongest at that point. In alkaline solution, I/I_0 decreased steeply because of the denaturation of the double helix structure of DNA. In the alkaline medium, the hydrogen bonds between the nucleic acid strands in the duplex were destroyed, which resulted in two single-stranded chains. The pH dependence of the RLS intensity proved that electrostatic attraction played a great role in the interaction between $(\text{Fe(III)TPP})_2\text{O}$ and nucleic acids.

The ratio I/I_0 was almost the same and reached the maximum in the pH range of 6.37-7.24, which meant that the difference of RLS intensity between $(\text{Fe(III)TPP})_2\text{O}$ and $(\text{Fe(III)TPP})_2\text{O}$ -ctDNA reached the maximum. This explained why pH 6.80 was chosen as the optimum.

Effect of $(\text{Fe(III)TPP})_2\text{O}$ concentration

The effect of $(\text{Fe(III)TPP})_2\text{O}$ concentration on I/I_0 is

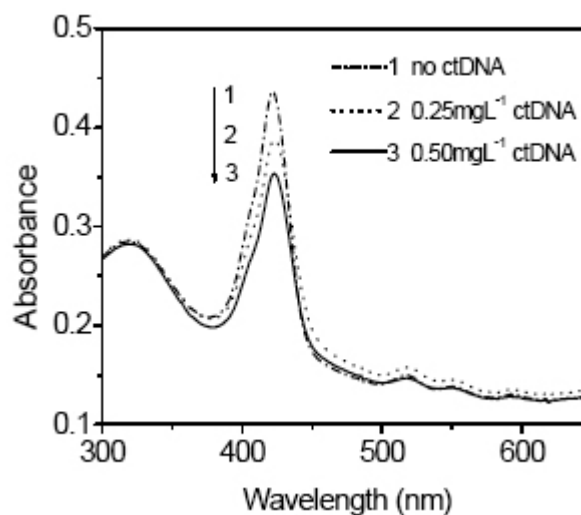


Figure 3. Absorption spectra. Conditions: $[(\text{Fe(III)TPP})_2\text{O}] = 1.0 \times 10^{-6} \text{ M}$; $\text{pH} = 6.80$.

shown in Figure 5. Insufficient $(\text{Fe(III)TPP})_2\text{O}$ hardly bound to DNA to form $(\text{Fe(III)TPP})_2\text{O}$ -ctDNA such that I/I_0 is small. On the contrary, high concentrations of $(\text{Fe(III)TPP})_2\text{O}$ tended to decrease the RLS signal due to the strong absorbance of the molecule, which was disadvantageous to the RLS enhancement. It was found that a final $(\text{Fe(III)TPP})_2\text{O}$ concentration from 8.0×10^{-7} M to 1.2×10^{-6} M gave the maximum I/I_0 under the given conditions. 1.0×10^{-6} M was selected as the optimum concentration.

Effect of ionic strength

The effect of ionic strength on the RLS intensity is shown in Figure 6. With increasing NaCl concentration, the RLS intensity of $(\text{Fe(III)TPP})_2\text{O}$ changed slightly. However, that of $(\text{Fe(III)TPP})_2\text{O}$ -ctDNA decreased greatly. This could be due to the sodium ions, as counter ions, which hindered the binding of $(\text{Fe(III)TPP})_2\text{O}$ with phosphate groups on the DNA backbone. Besides this, it also proved that $(\text{Fe(III)TPP})_2\text{O}$ bound with nucleic acid using electrostatic force. As counter ions for $(\text{Fe(III)TPP})_2\text{O}$, chlorine ions decreased slightly during RLS intensity enhancement because the concentrations of chlorine ions were low. If the concentrations of chlorine ions were high, they would also weaken the association of $(\text{Fe(III)TPP})_2\text{O}$ and DNA.

Effect of thermal denaturation of nucleic acid

The effect of thermal denaturation on the RLS intensity is also studied in Figure 7. The ctDNA solution was

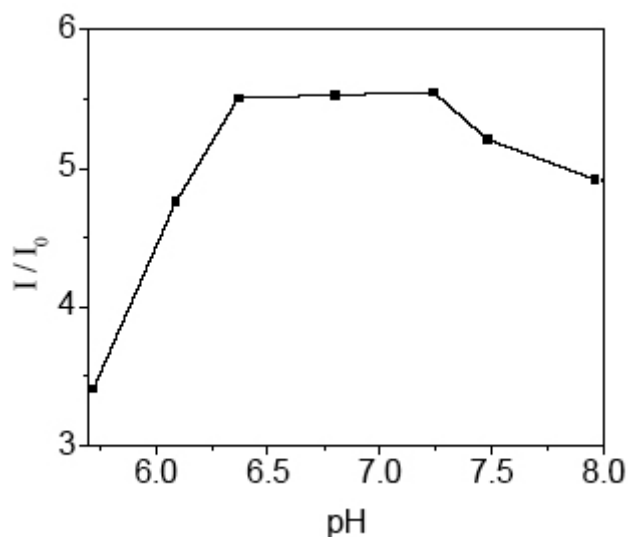


Figure 4. Effect of pH on I/I_0 . Conditions: $[(\text{Fe(III)TPP})_2\text{O}] = 1.0 \times 10^{-6}$ M; $[\text{ctDNA}] = 0.25$ mg L^{-1} .

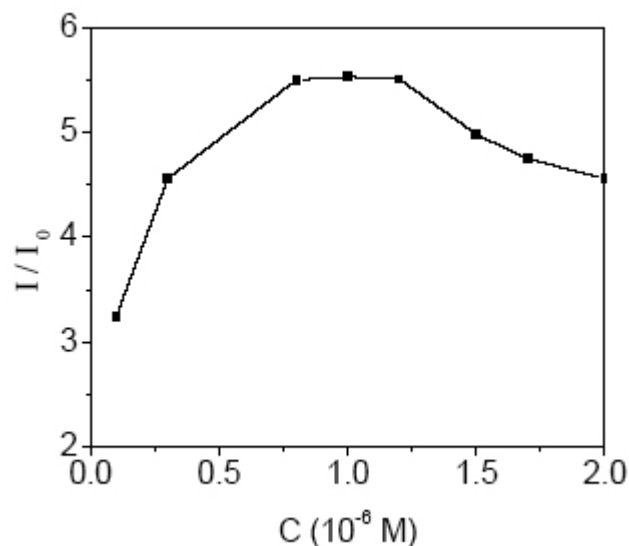


Figure 5. Effect of $(\text{Fe(III)TPP})_2\text{O}$ concentrations on I/I_0 . Conditions: $\text{pH} = 6.80$; $[\text{ctDNA}] = 0.25$ mg L^{-1} .

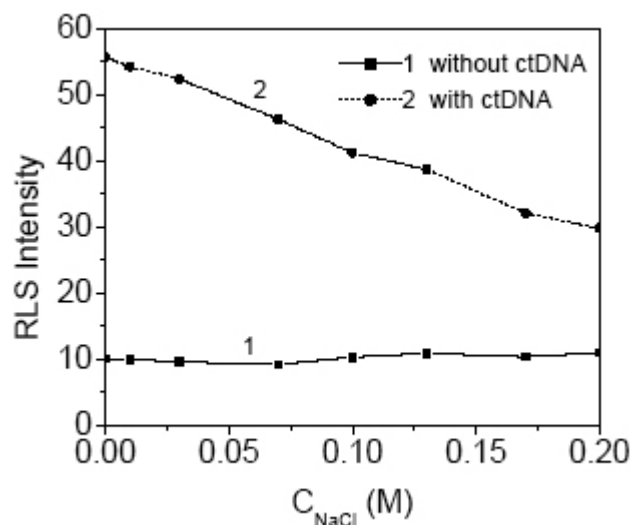


Figure 6. Effect of ionic strength on the RLS intensity. Conditions: $[(\text{Fe(III)TPP})_2\text{O}] = 1.0 \times 10^{-6}$ M; $[\text{ctDNA}] = 0.25$ mg L^{-1} ; $\text{pH} = 6.80$.

heated in a boiled water-bath for about 30 min., and then it was immediately placed in an ice water-bath for about 10 min. to prevent it from renaturation. The cooled denatured ctDNA was finally utilized for the experiment.

The results showed that denatured ctDNA- $(\text{Fe(III)TPP})_2\text{O}$ had lower RLS intensity than that of natural DNA- $(\text{Fe(III)TPP})_2\text{O}$ under the same conditions, which indicated that the double helical structure of DNA was of great importance in the formation of the complex $(\text{Fe(III)TPP})_2\text{O}$ -ctDNA. The double helical structure of DNA turned gradually into two single-stranded chains

under high temperature such that $(\text{Fe(III)TPP})_2\text{O}$ could not bind to DNA.

Stability

The stability of $(\text{Fe(III)TPP})_2\text{O}$ -ctDNA is shown in Figure 8. It was found that the reaction between $(\text{Fe(III)TPP})_2\text{O}$ and DNA occurred rapidly at room temperature and the RLS intensity of $(\text{Fe(III)TPP})_2\text{O}$ -ctDNA reached the maximum after 4 min. Moreover, the RLS intensity remained stable for 2h. Thus, this assay did not require crucial timing.

Tolerance of foreign substances

The effect of foreign substances on the quantitation of DNA was tested by pre-mixing DNA with foreign substances. According to Table 1, few substances interfered with the determination of DNA. Common hard ions, for instance, K(I) , Ca(II) , Mg(II) , could be tolerated at high concentrations because they combined with DNA by electrostatic forces to stabilize the DNA double helix. However, some soft ions, for example Hg(II) , Pb(II) and Ag(I) , could only be tolerated at very low concentration, which was attributed to the fact that they coordinated with nitrogen atoms on DNA bases to form stable metal complexes. Dilution with water could minimize all these interferences in the samples. Border-line ions, such as Cu(II) , Cr(III) , Mn(II) , were allowed at intermediate concentration levels because they bound to either phosphate groups or bases of nucleic acids. Proteins could also be tolerated at high levels, which suggested that this method should be practical.

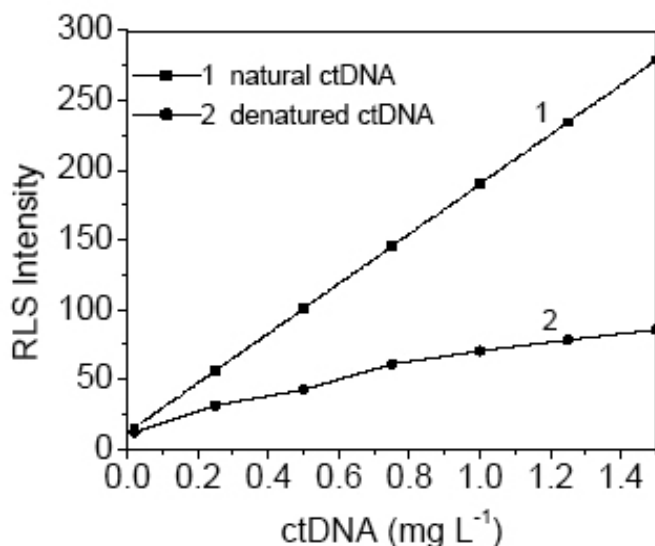


Figure 7. Effect of natural ctDNA and denatured ctDNA on the RLS intensity of the system. Conditions: $[(\text{Fe(III)TPP})_2\text{O}] = 1.0 \times 10^{-6} \text{ M}$; $\text{pH} = 6.80$.

Calibration and detection limit

The analytical parameters of the assay were presented in Table 2. The calibration curves of ctDNA, fsDNA and yRNA were constructed with increasing the concentrations of nucleic acids according to the standard procedures. The limits of detection (LOD) were given by $3S_0/S$, where 3 is the factor at the 99% confidence level, S_0 is the standard deviation of the blank measurements ($n = 10$), and S is the calibration. Judged by the slope of the linear regression equation, the sensitivities of the method were as follows: $\text{ctDNA} > \text{fsDNA} > \text{yRNA}$. It occurred to us that the interaction of $(\text{Fe(III)TPP})_2\text{O}$ and nucleic acids depended on the double helices of nucleic acid.

Compared with that of TAPP (11), the proposed method had higher sensitivity, as shown in Table 3. This can be explained from the structure of $(\text{Fe(III)TPP})_2\text{O}$. Besides coordinating with nitrogen atoms on the porphyrin ring, the iron ion combined with the oxygen atom, thus the radii of the iron ion reduced so it could coordinate with the porphyrin ring in the same plane. The rigidity and coplanarity of the molecule were improved greatly, which was profitable to the RLS enhancement. Because of better rigidity and coplanarity, the method of using $(\text{Fe(III)TPP})_2\text{O}$ was more sensitive than that of TAPP.

Analysis of synthesized samples

The analysis of synthetic samples was presented in Table 4. Synthetic samples, being made up of nucleic acids and foreign co-existing ions, were determined by the standard addition method. The results showed that this assay was reproducible and reliable.

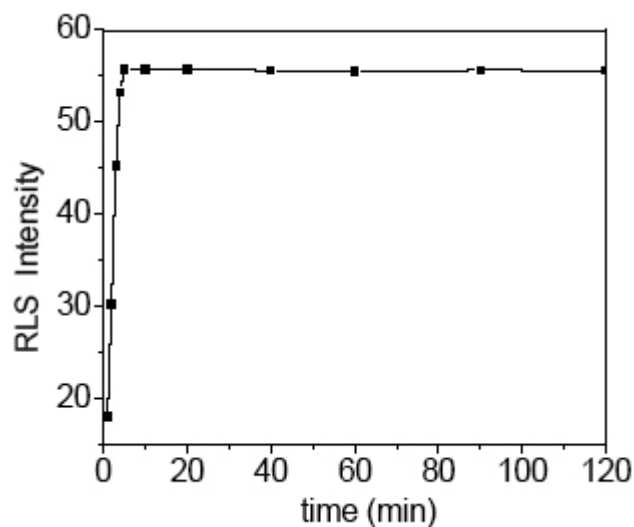


Figure 8. Effect of reaction time on the RLS intensity of the system. Conditions: $[(\text{Fe(III)TPP})_2\text{O}] = 1.0 \times 10^{-6} \text{ M}$; $[\text{ctDNA}] = 0.25 \text{ mg L}^{-1}$; $\text{pH} = 6.80$.

Table 1. Tolerance of foreign substances.*

Substances	Concentration	Change of I_{RLS} (ΔI_{RLS}) (%)
K(I), Cl ⁻	150.0 μM	-2.9
Na(I), Cl ⁻	150.0 μM	-2.0
Ca (II) , Cl ⁻	150.0 μM	-1.7
Mg(II) , Cl ⁻	70.0 μM	-2.5
Al(III), SO ₄ ²⁻	70.0 μM	-3.1
Zn(II) , Cl ⁻	40.0 μM	-2.3
Hg(II) , Cl ⁻	0.06 μM	-10.9
Ag(I), NO ₃ ⁻	0.02 μM	-12.6
Pb(II) , NO ₃ ⁻	0.03 μM	-11.5
Cd(II) , Cl ⁻	0.07 μM	-12.3
Cr(III) , Cl ⁻	4.0 μM	-4.8
Co(II) , Cl ⁻	3.0 μM	-5.6
Cu(II), NO ₃ ⁻	5.0 μM	-3.5
Mn(II) , Cl ⁻	2.0 μM	-6.7
Fe(II), SO ₄ ²⁻	2.5 μM	-5.9
Fe(III) , Cl ⁻	2.5 μM	-5.4
NH ₄ ⁺ , Cl ⁻	60.0 μM	-7.1
H ₂ PO ₄ ⁻	30.0 μM	1.9
Maltose	5.0 mg L ⁻¹	-4.0
Maltose	5.0 mg L ⁻¹	-4.0
Glucose	5.0 mg L ⁻¹	-3.8
Sucrose	5.0 mg L ⁻¹	-2.5
Gelation	0.5 mg L ⁻¹	4.3
BSA. **	0.4 mg L ⁻¹	6.4
HSA. ***	0.6 mg L ⁻¹	5.6
Adenine (A)	0.5 mg L ⁻¹	7.8
Guanine (G)	0.5 mg L ⁻¹	8.5
Cytosine (C)	0.4 mg L ⁻¹	5.2
Thymine (T)	0.4 mg L ⁻¹	7.1
Uracil (U)	0.6 mg L ⁻¹	3.9

* Conditions: $[(\text{Fe(III)TPP})_2\text{O}] = 1.0 \times 10^{-6} \text{ M}$; $[\text{ctDNA}] = 0.25 \text{ mg L}^{-1}$; $\text{pH} = 6.80$.

** Bovine serum albumin; *** Human serum albumin.

Table 2. Analytical parameters of this method.*

Nucleic acid	Linear range (mg L ⁻¹)	Regression equation	Detection limit (ng mL ⁻¹)	Correlation coefficient (r)
ctDNA	0.02-2.5	$\Delta I_{\text{RLS}} = 1.75 + 178.02c$	1.0	0.9991
fsDNA	0.02-2.7	$\Delta I_{\text{RLS}} = 2.45 + 162.86c$	1.1	0.9966
yRNA	0.03-3.1	$\Delta I_{\text{RLS}} = 2.90 + 153.95c$	1.2	0.9973

* Conditions: $[(\text{Fe(III)TPP})_2\text{O}] = 1.0 \times 10^{-6} \text{ M}$; pH = 6.80.

Table 3. Comparisons with other assays of nucleic acids.

Method	Reagents	Detection Limit (ng mL ⁻¹)	References
Abs	Diphenylamine	3000	[2]
Flu	Ethidium Bromide	86	[5]
Flu	Hoechst 33258	20	[6]
Flu	Tb ³⁺ -phenanthroline	100	[7]
RLS	TAPP	41	[11]
RLS	Co(II)/5-Cl-PADAB	1.4	[12]
RLS	Nile Blue Sulphate	1.4	[17]
RLS	Janus Green B	7.5	[18]
RLS	Acridne Red and CTMAB	1.28	[19]
RLS	Alcian Blue 8GX	6.9	[20]
RLS	Thionine	3.5	[21]
RLS	Poly(ethylenimine)	5.3	[22]
RLS	(Fe(III)TPP) ₂ O	1.0	This work

Table 4. Recoveries of nucleic acids in synthetic samples.*

Nucleic acids in samples (mg L ⁻¹)	Main additives**	Found value (mg L ⁻¹ , n = 5)	Recovery range % (n = 5)
ctDNA 1.25	BSA, Ca(II), Mg(II)	1.23	98.4
ctDNA 1.25	H ₂ PO ₄ ⁻ , A, T, C, G	1.22	97.6
fsDNA 2.20	BSA, Zn(II), Cu (II)	2.28	103.6
fsDNA 2.20	H ₂ PO ₄ ⁻ , A, T, C, G	2.23	101.4
yRNA 2.00	BSA, Co(II), Fe(II)	1.91	95.5
yRNA 2.00	H ₂ PO ₄ ⁻ , A, U, C, G	1.88	94.0

* Conditions: $[(\text{Fe(II)TPP})_2\text{O}] = 1.0 \times 10^{-6} \text{ M}$; pH = 6.80.

** Concentration of additives: BSA, HSA, 0.3 mg L⁻¹; Ca(II), 100 μM ; Mg(II), Zn(II), 30 μM ; Cu(II), 3.0 μM ; Mn(II), Fe(II), 1.0 μM ; A(adenine), T(thymine), C(cytosine), G(guanine), U(uracil), 0.3 mg L⁻¹; H₂PO₄⁻, 20 mg L⁻¹.

Conclusion

A new method for the determination of nucleic acids was established based on the enhancement of resonance light scattering effect on (Fe(III)TPP)₂O with nucleic acids. The assay had high sensitivity, good selectivity and simplicity. It was also free of interferences from most metal ions or other coexistent substances. As future work, it would be interesting to develop new medicine for antiviral and anti-cancer by the study of the interaction

between (Fe(III)TPP)₂O metalloporphyrin and nucleic acids (29).

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