

Assay of Nucleic Acids at the Water/Tetrachloromethane Interface with Cetyltrimethylammonium Bromide by Total Internal Reflected Resonance Light Scattering

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In this contribution, nucleic acids, including calf thymus DNA (ctDNA), fish sperm DNA (fsDNA), and yeast RNA (yRNA), have been determined with good selectivity and sensitivity at the water/tetrachloromethane (H_2O/CCl_4) interface by a total internal reflected resonance light scattering (TIR-RLS) technique. Under optimal conditions, amphiphilic complexes produced by nucleic acids and cetyltrimethylammonium bromide (CTMAB) are absorbed at the H_2O/CCl_4 interface, resulting in a good separation of nucleic acids from the coexisting foreign substances in the aqueous phase and enrichment of nucleic acids at the interface. All of these induce significantly enhanced TIR-RLS signals with the maximum peak located at 370.0 nm at the interface. It was found that the enhanced TIR-RLS intensity is in good proportion to the concentration of nucleic acids in the range of 0.06–2.5, 0.015–3.5, and 0.046–3.5 $\mu g\,mL^{-1}$ for ctDND, fsDNA, and yRNA, respectively, and their limits of detection (3σ) are 6.0 $ng\,mL^{-1}$, 1.5 $ng\,mL^{-1}$ and 4.6 $ng\,mL^{-1}$ correspondingly. Artificial samples with complicated and highly interfering backgrounds were analyzed satisfactorily.

The adsorption of complexes at liquid/liquid interfaces has attracted the interest of researchers in various fields,[1,2] such as phase-transfer analysis,[3] colloidal chemistry, solvent extraction,[4] the ion or electron-transfer process, biomedical engineering, and pharmacology.[1,5,6] In particular, the immiscible liquid/liquid interface provides an excellent approximation to a cellular membrane where many important biological functions and processes occur. Therefore, to understand how material is transmitted at liquid/liquid interfaces is of utmost importance in application. Thus, it is compulsory to develop surface-sensitive techniques and molecular probes that are capable of obtaining the information about material adsorption at liquid/liquid interfaces.

Quantitative analysis of nucleic acids is critical in clinical assay and genetic diagnosis. Analytical methods of nucleic acids mainly involve microchip assay,[7,8] capillary electrophoresis (CE),[9,10] electrochemical techniques,[11,12] and spectroscopic techniques. Among these methods, the spectroscopic techniques, including spectrophotometry,[13] spectrofluorimetry,[14] and resonance light scattering (RLS),[15–17] are used more conveniently and commonly in the laboratory. However, these spectral measurements are all applied in aqueous bulk, and their selectivities and sensitivities are not satisfactory due to the complicated interferences. Since Hirschfeld introduced total internal reflection fluorometry (TIRF) in 1965,[18] TIRF technique has been developed into a conventional technique to study the adsorption at interfaces. When a light beam undergoes total internal reflection, it induces an electromagnetic field (evanescent field) in the optical less dense medium beyond the interface. The intensity of the evanescent field decays exponentially with perpendicular distance from the interface.[19,20] Therefore, the chemical species in the interfacial region can be highly selectively excited.[3] Since Pasternack et al. proposed the resonance light scattering (RLS) technique,[21,22] the RLS technique has been widely used for the sensitive analysis of biological molecules.[15,16] Similar to TIRF, by combining the resonance light scattering (RLS) technique with the total internal reflected light, we developed a TIR-RLS technique, which overcomes the disadvantages of the RLS technique in selectivity and sensitivity.[23]

Amphiphilic surfactants can form a monolayer at the liquid/liquid interface with the hydrocarbon chains of the surfactant oriented away from the aqueous phase. However, because of the electrostatic interactions of the polar head groups, the monolayer is in disorder.[24] Biomacromolecules, which are multiply charged, can be adsorbed by electrostatic interactions with the opposite charged surfactant at the liquid/liquid interface which is favorable for the formation of closely packed layers.[25] As we know, cetyltrimethylammonium bromide (CTMAB) is a cationic surfactant and nucleic acid is a highly negatively charged polymer due to its phosphate groups. Herein we proved that the coadsorption of CTMAB and nucleic acids led by electrical interaction, lead to the formation of an amphiphilic complex at the water/tetrachloromethane (H_2O/CCl_4) interface, resulting in strongly enhanced TIR-RLS signals. The TIR-RLS intensities of the complex are in good proportion to the concentration of nucleic acids.

Experimental

Apparatus. TIR-RLS spectra and intensities were measured with a Hitachi F-2500 spectrofluorometer (Tokyo, Japan). The optical arrangement for the TIR-RLS spectra was illustrated in our previous report.[23,26] The bottom inside of an optical quartz cell (10 mm) was treated with dichlorodimethylsilane in toluene

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so as to make the lower inside wall hydrophobic and afford a flat H_2O/CCl_4 interface. Two right-angled prisms (10 mm × 10 mm × 10 mm, Huaguang Optics Co., Chongqing, China) were attached to the cell walls facing the excitation light source and the fluorescence detector, respectively. According to the Snell Law, the incident angle is 72.6°, which is sufficiently greater than the critical angle of 65.6° for total internal reflection of the H_2O/CCl_4 system. So the excitation light beam, passing through the quartz prism and organic phase, is irradiated on the H_2O/CCl_4 interface and undergoes total internal reflection.

An S-10A digital pH meter (Xiaoshan Scientific Instruments Company, Zhejiang, China) was used to measure the pH values of the aqueous solutions, and a MVS-1 vortex mixer (Beite Scientific Instrumental Ltd., Beijing, China) was used to blend the solutions.

Reagents. Stock solutions of nucleic acids were prepared by dissolving commercially purchased calf thymus DNA (ctDNA, Beitai Biochemical Co., Chinese Academy of Sciences, Beijing, China), fish sperm DNA, and yeast RNA (fsDNA, yRNA, Shanghai Institute of Biochemistry, Chinese Academy of Sciences, Shanghai, China) in doubly distilled water. The concentrations of nucleic acids were determined according to the absorbance at 260 nm after establishing that the absorbance ratio (A_{260}/A_{280}) was in the range of 1.80–1.90 for DNA and 1.90–2.00 for RNA.[27] The molarities of nucleic acids, when necessary, were calculated using $\varepsilon_{DNA} = 6600$ dm^3 mol^{-1} cm^{-1} and $\varepsilon_{RNA} =$ 7800 dm^3 mol^{-1} cm^{-1}, respectively. The working solutions of nucleic acids were 25.0 μg mL^{-1} (7.5×10^{-5} mol dm^{-3}).

A 1.0×10^{-4} mol dm^{-3} cetyltrimethylammonium bromide (CTMAB) solution was prepared by dissolving its crystal product (Shanghai Chemical Reagent Center, Shanghai, China) in doubly distilled water. A Britton–Robinson buffer solution was used to control the acidity of the aqueous medium, while 0.5 mol dm^{-3} and 1.0 mol dm^{-3} NaCl solutions were used to adjust the ionic strength of the aqueous medium. All other reagents were of analytical-reagent grade without further purification. Doubly distilled water was used throughout the experiment.

Standard Procedure. Into a 10.0 mL volumetric flask was added 1.50 mL of CTMAB solution, 1.0 mL of Britton–Robinson buffer solution, and appropriate nucleic acid solution successively. The mixture was agitated after the addition of all the interacting additives, and then it was diluted with doubly distilled water to 10.0 mL. The mixture was at last mixed thoroughly. 2.0 mL of this solution and 1.0 mL of CCl_4 were infused into the quartz cell of the optical arrangement, successively. Then the two immiscible phases were violently blended together and allowed to stand motionless for 15 min before the TIR-RLS measurement in order to eliminate all minute emulsions. All TIR-RLS spectra and intensities were measured against the blank treated in the same way without nucleic acid.

The TIR-RLS spectra were attained by scanning simultaneously the excitation and emission monochromators of the F-2500 spectrofluorometer from 220.0 to 700.0 nm with $\lambda_{ex} = \lambda_{em}$, with a slit width of 5.0 nm for both excitation and emission.

Results and Discussion

Features of the TIR-RLS Spectra at the H_2O/CCl_4 Interface. As Fig. 1 shows, without any addition of ctDNA, the TIR-RLS signals of the H_2O/CCl_4 interface are very faint in the whole scanning region, and it is hard to observe obvious changes in the TIR-RLS intensity with increasing CTMAB concentration. If a trace amount of ctDNA is added to the aqueous phase, a strongly enhanced TIR-RLS signal, characterized by a maximum peak signal at 370.0 nm, is observed in the 350–550 nm region, which is sufficient to indicate a new species has formed and been adsorbed at the interface. It has also been found that the degree of enhancement of the TIR-RLS spectra is directly proportional to the increase in concentration of nucleic acid, which reveals that the TIR-RLS signal is enhanced by nucleic acids and the assay of nucleic acids at the H_2O/CCl_4 interface by TIR-RLS is a reliable practice. Similar TIR-RLS spectra can be obtained when ctDNA is substituted by fsDNA or yRNA to interact with CTMAB.

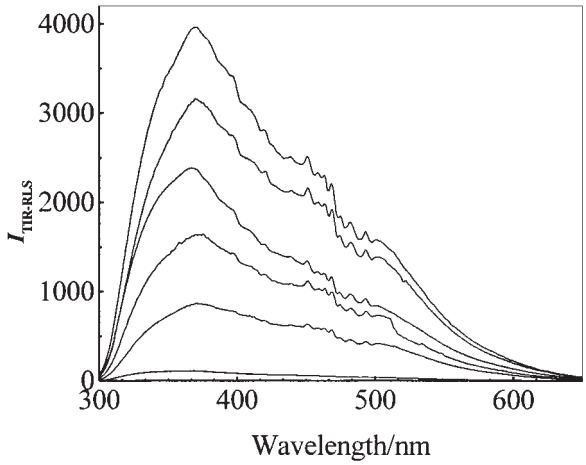

Fig. 1. TIR-RLS spectra of the complex of CTMAB and ctDNA in different concentration at the H_2O/CCl_4 interface. Concentrations: CTMAB, 1.5×10^{-5} mol dm^{-3}; ctDNA (from bottom to top, μg mL^{-1}) 0.0, 0.5, 1.0, 1.5, 2.0, and 2.5, respectively. pH of aqueous medium, 3.3; ionic strength of aqueous medium, 0.003 mol dm^{-3}.

Optimizations of the General Procedures. It is worth noting that the H_2O/CCl_4 interfacial properties are governed by various factors, such that a comprehensive study is required to obtain further detailed information about the characteristics of the H_2O/CCl_4 interface, which affect the state of the complex. It has been found that the TIR-RLS intensity displays different tendencies depending on the pH of the aqueous medium. As Fig. 2 shows, the enhanced TIR-RLS intensity is found to be stable and maximum in the range of pH 2.87–3.78. Otherwise, the TIR-RLS intensity decreases sharply regardless of the employment of ctDNA, fsDNA, or yRNA. The decrease in TIR-RLS intensity is possibly ascribed to the change of state of DNA at different pH values. In a medium of pH < 5.7, an indirect proton–phosphate interaction via water and the protonation of nitrogen atoms of bases could occur.[28] For example, protonation of cytosine N-3 ($pK_a = 4.24$) and adenine N-1 sites ($pK_a = 3.20$) occurs at pH 5.3. Decreasing the pH of the medium will lead to further protonation of nitrogen atom of other bases, for instance, guanine N-7 ($pK_a = 2.30$) will be protonated at pH < 3.22. The coincidence of protonation of the base with the optimal pH of the medium indicates that the interaction of nucleic acids with CTMAB involves the protonated nucleic acids. In this experiment, the pH value of the

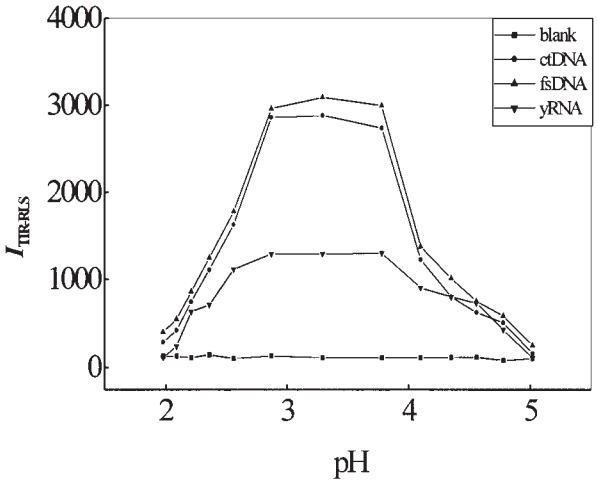

Fig. 2. Effects of pH values of aqueous medium on TIR-RLS intensity. Concentration: CTMAB, in ctDNA system, 1.5×10^{-5} mol dm^{-3}; in fsDNA and yRNA systems 1.75×10^{-5} mol dm^{-3}; nucleic acids, 2.0 μg mL^{-1}. Ionic strength in aqueous medium, 0.003 mol dm^{-3}.

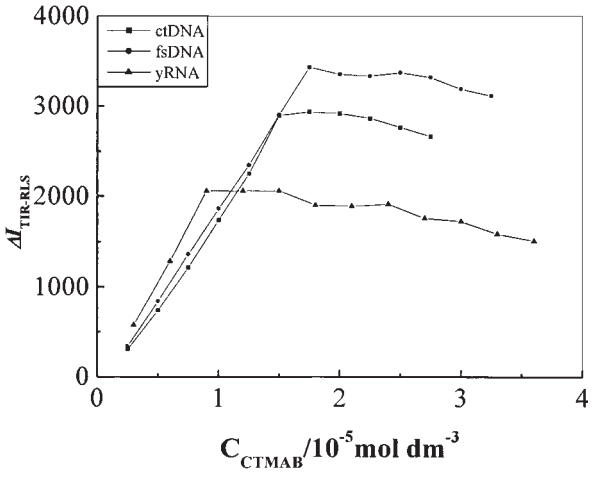

Fig. 3. Dependence of the TIR-RLS intensity at H_2O/CCl_4 interface on the CTMAB concentration. The concentration of nucleic acids, 2.0 μg mL^{-1}. pH of aqueous medium, 3.3; ionic strength in aqueous medium, 0.003 mol dm^{-3}.

aqueous medium was controlled to pH 3.3 by 1.0 mL Britton–Robinson buffer.

The ionic strength of the aqueous medium seems to have a significant effect on the TIR-RLS signal at the H_2O/CCl_4 interface. The TIR-RLS signals of the complex are sensitive to increasing NaCl concentration in the aqueous medium, and the TIR-RLS intensity declines sharply with increasing ionic strength from 0.003 to 0.053 mol dm^{-3}. When the ionic strength continues to increase, the TIR-RLS intensity becomes very small. This can possibly be ascribed to the increased shielding effect of charges on both the CTMAB anion and the phosphate groups of nucleic acids, which obstructs the interaction between CTMAB and nucleic acid. As a result, the ionic strength was kept at 0.003 mol dm^{-3} in the aqueous medium during the experiment without extra addition of NaCl in order to obtain strong TIR-RLS signals.

Molar Ratio of CTMAB to Nucleic Acid at H_2O/CCl_4 Interface. Figure 3 displays the role that CTMAB played in this experiment. When the concentrations of nucleic acids are all kept at 2.0 μg mL^{-1} (6.0×10^{-6} mol dm^{-3}), it can be seen that the enhanced TIR-RLS signals strongly depend on the CTMAB concentration. If the CTMAB concentration is too small in this experiment, the TIR-RLS intensities will be very faint, which can be explained by the solubility of nucleic acids in aqueous medium and surface-inactive, and can not be adsorbed at the interface. The TIR-RLS signals get correspondingly stronger with gradually increasing CTMAB concentration, indicating that nucleic acid gathers at the interface region because of the formation of the amphiphilic complex. When the CTMAB concentration is 2.5 times (1.5×10^{-5} mol dm^{-3}) the concentration of ctDNA, the enhanced TIR-RLS intensity reaches its maximum, indicating all CTMAB and DNA have reacted completely at that point. Then the TIR-RLS intensity begins to decrease gradually with a further increase in CTMAB concentration. As for fsDNA and yRNA, when the concentration of CTMAB is 3.0 (1.75×10^{-5} mol dm^{-3}) and 1.5 (0.9×10^{-5} mol dm^{-3}) times the concentration of nucleic acids, respectively, the TIR-RLS signals reach the maximum. The molar ratio (R) of CTMAB to DNA is in agreement with the result of the other report where the experiment occurred in aqueous bulk.[17] The concentrations of CTMAB and DNA in the report were 1.5×10^{-5} mol dm^{-3} and 0.6×10^{-5} mol dm^{-3}, respectively. The molar ratio R can also be identified by keeping the total concentration of CTMAB and nucleic acids (ctDNA, fsDNA, and yRNA) as 2.1×10^{-5} mol dm^{-3}, 2.35×10^{-5} mol dm^{-3}, and 1.5×10^{-5} mol dm^{-3}, respectively, which are equal to the total concentration of CTMAB and nucleic acids at the running points in Fig. 3, while the concentrations of the two components of the complex are changed simultaneously. As Fig. 4 shows, when the fractions of ctDNA, fsDNA and yRNA in the complexes are 0.26, 0.25 and 0.40, respectively, the enhanced TIR-RLS of the complexes all reach their maxima, revealing the molar ratios R of CTMAB to ctDNA, fsDNA, and yRNA

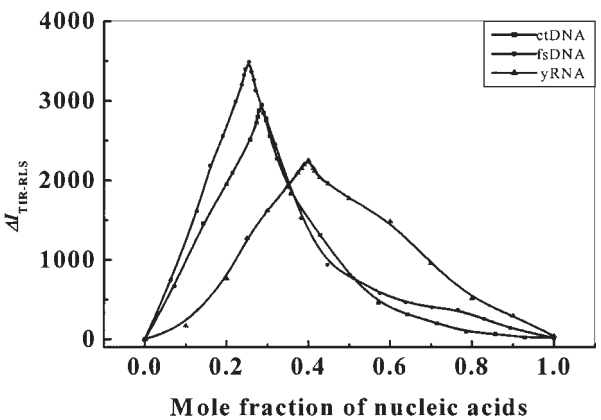

Fig. 4. Binding molar ratios of CTMAB to nucleic acids in the complexes. The total concentrations of CTMAB and ctDNA, fsDNA, and yRNA were kept as 2.1×10^{-5} mol dm^{-3}, 2.35×10^{-5} mol dm^{-3}, and 1.5×10^{-5} mol dm^{-3}, respectively. pH of aqueous medium, 3.3; ionic strength in aqueous medium, 0.003 mol dm^{-3}.

are 2.5:1, 3.0:1, and 1.5:1, respectively. The mole ratios are identical to the results from Fig. 3.

Tolerance of Coexisting Foreign Substances. The tolerance of this assay with a tolerance level between +5% and −5% is investigated by the use of 2.0 $\mu g\,mL^{-1}$ (1.2×10^{-5} $mol\,dm^{-3}$) nucleic acid in the general procedure by premixing it with the interference substances. The influence of various metal ions, proteins, sugar, surfactants, bases, and nucleotides was tested under optimal conditions according to the standard procedure. Thanks to the coadsorption of amphiphilic complexes and the enrichment at the interface, the interference coming from aqueous medium decreases greatly.[23] The comparisons of the tolerance level of foreign materials both with the TIR-RLS and with the RLS technique[17] are listed in Table 1.

For instance, the tolerance level of common metal ions at interfaces is about 100 times larger than in aqueous solution. Some metal ions, such as Mg^{2+}, Ca^{2+}, Cd^{2+}, Pb^{2+}, Ba^{2+}, and Co^{2+}, can be tolerated at high concentration levels (larger than 10^{-3} $mol\,dm^{-3}$). Some common substances including K^+, Na^+, NH_4^+, and urea do not have any effect on the TIR-RLS intensity, even if they are at high concentrations, about 1.0×10^{-2} $mol\,dm^{-3}$. Proteins can also be tolerated at a high concentration level. Sugar and β-CD also have no interference on the system. Thus, it is obvious that this method has high tolerance levels, further supporting previous research that had verified the superiority of this technique.[23,29] As a result, it is evident that this analytical method has good selectivity and can be directly utilized for the assay of nucleic acids in practical samples in complicated backgrounds without separation of coexisting foreign substances. The RLS technique established in bulk does not enjoy this advantage.

However, some nucleotides can be allowed in a low concentration, while surfactants such as CTMAC, Zeph, SDBS, SDS, SLS, and Triton-100 can also be tolerated at a lower concentration. The reason for the former may be that nucleotides react with CTMAB and remain at the interface, while that for the latter is the possible encumbrance of the complex into the interfacial region as a result of the increased disorder assay of excessive surfactants at the interface.[24]

Calibration Curves and Sample Determinations. According to the general procedures, a linear relationship between the TIR-RLS intensity at the interface and the concentration of nucleic acids was constructed. All the analytical parameters are presented in Table 2 with a comparison of the RLS method established in aqueous bulk. These values show the TIR-RLS method is much more sensitive than RLS. In terms of the slope of the regression equations, the sensitivities of the TIR-RLS method for different nucleic acids have the following sequence: fsDNA ≈ ctDNA > yRNA.

Table 1. Comparison of the Tolerance Level of Foreign Materials Both with TIF-RLS and RLS Technique

No.	Interference substances	Concentration/10^{-6} $mol\,dm^{-3}$		Change of ΔI/%	
		TIR-RLS	RLS	TIR-RLS	RLS
1	Urea	20000	—	−3.1	—
2	$NH_4^+Cl^-$	14000	670	+2.8	+7.7
3	$K(I)Cl^-$	10000	500	−5.5	−5.6
4	$Na(I)Cl^-$	8000	500	−1.4	−6.2
5	$Mg(II)Cl^-$	5000	500	−3.8	−2.1
6	$Ca(II)Cl^-$	5000	500	−2.8	−12
7	$Pb(II)Cl^-$	2400	0.02	+5.7	−2.7
8	$Ba(II)Cl^-$	1500	—	+0.8	—
9	$Cd(II)Cl^-$	1000	1.0	−8.9	−3.2
10	$Co(II)Cl^-$	1000	0.01	−5.9	−12
11	Protein, HAS	2.0[a)]	0.25[a)]	+2.2	+12
12	Protein, BAS	2.0[a)]	0.25[a)]	+1.5	+13
13	β-Cyclodextrin	100	3.0	+3.4	−2.5
14	Glucose	50	1.0	−4.1	−2.1
15	Lactose	50	50	−2.7	−2.9
16	Maltose	50	50	−3.6	−2.5
17	Sucrose	50	5.0	−4.0	+5.0
18	Triton X-100	0.2[b)]	0.4[b)]	−3.9	−7.1
19	SLS	9	—	−4.6	—
20	SDBS	3	—	−3.5	—
21	CTMAC	0.4	—	+6.2	—
22	SDS	0.1	2.5	−4.3	+13
23	Zeph	0.1	—	+2.7	—
24	5′-AMP	1.0	1.0	−1.6	−9.5
25	5′-CMP	1.0	1.0	+2.3	−6.2
26	5′-GMP	1.0	1.0	−3.6	−11

RLS data were obtained in aqueous bulk according to Ref. 17, while TIR-RLS data were obtained at H_2O/CCl_4 interface in this article. a) $\mu g\,mL^{-1}$. b) %. Concentrations: CTMAB, 1.5×10^{-5} $mol\,dm^{-3}$; ctDNA, 2.0 $\mu g\,mL^{-1}$. pH of aqueous medium, 3.3; ionic strength in aqueous medium, 0.003 $mol\,dm^{-3}$.

Table 2. Comparison of Analytical Parameters of the Determination Both with RLS and TIR-RLS Technique

Nucleic acid	Detection method	Linear range /μg mL^{-1}	Linear regression equation (c/μg mL^{-1})	LOD (3σ/ng mL^{-1})	Correlation coefficient
ctDNA	TIR-RLS	0.06–2.5	$\Delta I = -13.9 + 1539.9c$	6.0	0.9999
	RLS	0.14–3.5	$\Delta I = 1.7 + 31.7c$	14.2	0.9992
fsDNA	TIR-RLS	0.015–3.5	$\Delta I = 18.7 + 1628.5c$	1.5	0.9995
	RLS	0.046–3.5	$\Delta I = -1.3 + 30.7c$	4.6	0.9994
yRNA	TIR-RLS	0.048–3.5	$\Delta I = 4.5 + 712.8c$	4.8	0.9999

RLS data were obtained in aqueous bulk according to Ref. 17 which the results of yRNA were not reported, while TIR-RLS data were obtained at H_2O/CCl_4 interface in this article. The concentrations of CTMAB are 1.5×10^{-5} mol dm^{-3} for ctDNA, 1.75×10^{-5} mol dm^{-3} for fsDNA and yRNA, respectively. pH in aqueous medium, 3.3; ionic strength in aqueous medium, 0.003 mol dm^{-3}. All data were obtained with $\lambda_{ex} = \lambda_{em}$ kept at 370.0 nm.

Table 3. Determination of Trace Amounts of Nucleic Acids in Synthetic Samples

Nucleic acid in sample/μg mL^{-1}	Main additives[a)] in samples	Found /μg mL^{-1}	Recovery /%, $n = 5$	RSD /%, $n = 5$
ctDNA (25.0)	K(I), Mg(II), Ca(II), Co(II)	24.92	97.9–103.2	2.4
ctDNA (15.0)	β-CD, SDS, BSA	15.26	99.2–103.4	1.5
fsDNA (20.0)	Ba(II), Cd(II), Triton X-100, Urea	20.08	98.1–104.8	2.8
fsDNA (10.0)	5′-AMP, 5′-CMP, 5′-GMP	9.92	94.4–103.9	6.7
yRNA (25.0)	Glucose, Lactose, Maltose, Sucrose	25.45	97.1–104.6	3.0
yRNA (15.0)	Mg(II), Ca(II), K(I), β-CD	14.85	93.9–104.1	3.9

a) Concentrations of additives: BSA, 0.05 μg mL^{-1}; Nucleotide, 1.5×10^{-7} mol dm^{-3}; Sugar, 5.0×10^{-6} mol dm^{-3}. Mg(II), 2.5×10^{-3} mol dm^{-3}; Ca(II), 2.5×10^{-3} mol dm^{-3}; Co(II), 5.0×10^{-5} mol dm^{-3}; K(I), 5.0×10^{-4} mol dm^{-3}; Cd(II), 5.0×10^{-5} mol dm^{-3}; Ba(II), 7.3×10^{-5} mol dm^{-3}; SDS, 5.0×10^{-8} mol dm^{-3}; β-CD, 1.0×10^{-5} mol dm^{-3}; Triton X-100, 1.0×10^{-4}%; Urea, 5.0×10^{-4} mol dm^{-3}. CTMAB concentrations are 1.5×10^{-5} mol dm^{-3} for ctDNA, 1.75×10^{-5} mol dm^{-3} for fsDNA and yRNA, respectively. pH of aqueous medium, 3.3; ionic strength in aqueous medium, 0.003 mol dm^{-3}. All the data were obtained with $\lambda_{ex} = \lambda_{em}$ kept at 370.0 nm.

From the different slopes of the regression equations and R of the three nucleic acids, we can get information about the interaction mechanism of CTMAB and the nucleic acids. Natural ctDNA and fsDNA are double-stranded, while yRNA is single-stranded. That is to say, the negative charges of DNA are twice those of yRNA. In this experiment, the slopes of the regression equations and mole ratio R for DNA are double those concerning RNA. Hence we can conclude that the interaction of CTMAB with nucleic acids is strand-dependent with regards to the nucleic acids molecules.

To test the practical feasibility of this assay, six artificial samples in which a series of foreign substances displayed in Table 3 were added were analyzed. As Table 3 shows, the results for these artificial samples were reproducible and reliable. All of these data indicate that this assay method of nucleic acid with CTMAB is sensitive, feasible, and reproducible in practical samples.

Conclusion

In this paper, we developed a total internal reflected resonance light scattering (TIR-RLS) technique to quantitatively investigate of nucleic acids at the H_2O/CCl_4 interface. It was shown that this technique is a powerful tool to study the liquid/liquid interface and has two typical advantages of the separation and enrichment of analytes. Due to the analytes being separated from the bulk and their enrichment at the interface, the analytes can be detected with high selectivity and sensitivity. However, the other spectroscopic techniques established for aqueous bulk don't possess these benefits. Therefore, the TIR-RLS technique is promising for application in the determination of samples in which the contents of coexisting substances are very high and, especially, biological samples in which the interference of coexisting substances is serious.

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