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An Abnormal Resonance Light Scattering Arising from Ionic-Liquid/DNA/Ethidium Interactions

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Abstract: In the aqueous phase, ethidium bromide (EB) intercalates into the double helix structure of dsDNA (ds = double-stranded) with a notable enhancement in fluorescence and resonance light scattering (RLS). However, when dsDNA was extracted into an ionic liquid (IL), 1-butyl-3-methylimidazolium hexafluorophosphate ($BmimPF_6$), an abnormal RLS arising from the interactions of IL–DNA–EB was observed, with a substantial decrease of the recorded RLS. The cationic $Bmim^+$ groups of $BmimPF_6$ intercalate into the DNA helix structure, in which they interact with the P–O bonds of phosphate groups in DNA strands and result in a reduction of the base-pair interstice along with transformation of DNA conformations that consequently prohibits the intercalation of EB with DNA. Thus, in the IL phase, the interactions between ethidium and DNA were dominated by electrostatic interactions and hydrogen bonding, leading to a congregation of EB entities around the DNA strands that results in an increase of absorption by ethidium, and consequently the inner filter effect leads to a reduction of the RLS. The present observation has been applied to the direct quantification of DNA in an ionic-liquid phase after DNA from human whole blood was extracted into $BmimPF_6$.

Keywords: DNA · ethidium · ionic liquids · resonance light scattering

Introduction

Nucleic acids are among the most important biological macromolecules in life sciences. The quantification of trace level nucleic acids is therefore a critical step in a wide variety of biological and diagnostic applications. Among the various detection techniques, fluorescence-based approaches have attracted extensive attention attributed to its high sensitivity and superior tolerance to interferences, and a number of commercially available fluorescent probes have been employed, including ethidium bromide (EB),[1,2] Hoechst 33258,[3] YOYO-1,[4] M-hypocrellin A,[5] PicoGreen[6] and trivalent lanthanide cations.[7] Among those probes, the intercalation of EB into DNA double-helix structure provides an excellent performance for the quantification of dsDNA. In addition to its low cost, it is most widely employed probe used in the practice, although it is not the most sensitive fluorescent probe.

Since the development of resonance light scattering (RLS) technique during the last decade,[8] it has rapidly become very attractive for its applications in the assay of biological macromolecules including DNAs.[9–11] RLS is a unique scattering with an identical frequency for both the electromagnetic wave absorbed by electrons and that of the scattering, and determinations are usually performed based on the substantial enhancement of RLS intensity in the presence of nucleic acids. A number of dyes have been employed for RLS determination of nucleic acids;[12–15] however, ethidium bromide has not been applied for this purpose so far.

In our recent studies for conducting the exploitation of isolation/purification strategies for DNA in biological samples, we have demonstrated that dsDNA could readily be extracted into the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ($BmimPF_6$); this method provides an alternative approach for the purification of DNA.[16] The quantification of dsDNA transferred into the $BmimPF_6$ phase, however, proved to be unsuccessful by employing the various aqueous-only-applicable procedures available at hand. When EB-based procedures were considered, however, its intercalation into the double helix structure of DNA was excluded in $BmimPF_6$, and therefore its direct determination in the IL phase was prohibited. At this juncture, it is highly desirable to develop a practical quantification procedure for this specific purpose.

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With further investigations, an abnormal phenomenon for the resonance light scattering in the presence of EB and DNA was observed; that is, when DNA was extracted into ionic liquid $BmimPF_6$, the DNA–EB interaction causes a significant suppression on the recorded RLS. This is evidently an opposite effect to that frequently encountered in aqueous phase, in which both RLS and fluorescence were significantly enhanced when EB was intercalated into DNA structure. A novel procedure for the quantification of DNA in the phase of $BmimPF_6$ was thus developed based on the present observation by taking advantage of the linear relationship between DNA concentration and the decrease of the RLS intensity within a certain range. This procedure was successfully used for the determination of DNA in human whole blood. The mechanism of the present observation was also investigated.

Results and Discussion

Opposite RLS behaviour of the DNA–ethidium system in $BmimPF_6$ and aqueous phases: Figure 1 illustrates the RLS spectra of the ethidium-containing systems obtained in both aqueous and $BmimPF_6$ phases in the presence of various amount of DNA. It is evident that in aqueous phase, the presence of dsDNA and the DNA–ethidium interaction gives rise to an increase of the RLS, as illustrated in Figure 1A recorded at an extraction acidity of pH 6.7; this increase could safely be attributed to the intercalation of ethidium into the double-helix structure of DNA.[17] Surprisingly, when dsDNA was transferred/extracted into the IL phase, its interaction with EB resulted in an entirely opposite observation for the recorded RLS as that in aqueous phase; that is, a suppression of the RLS was achieved in the presence of DNA bound with the IL entity (Figure 1B), and a further reduction of the RLS intensity was observed with the increase of DNA concentration transferred into IL. The interaction of DNA with $BmimPF_6$ has been investigated previously, in which a bond between the P–O bond of the phosphate groups in DNA strands and the cationic $Bmim^+$ group of $BmimPF_6$ was demonstrated,[16] and is thought to be closely related to the abnormal observation of RLS reported herein, as further discussed in the following sections.

In the ensuing investigations, consistent experimental conditions were employed as far as possible, in order to minimise the potential variable covariance arising from the inconsistency of the adopted experimental parameters.

DNA-ethidium interaction modes in aqueous and IL phases and the related RLS: The UV-Vis spectra for the DNA-ethidium system in both aqueous and $BmimPF_6$ phases are illustrated in Figure 2. In the aqueous phase, a decrease of the absorbance was recorded within the region around 510 nm, in accordance with the enhancement of RLS within a similar region (Figure 2A). In addition, an evident red shift of the absorption band with the increment of DNA concentration was also observed. These observations indicate the existence of two different kinds of interactions between the negatively charged DNA strands and the positive ethidium entities; that is, one is chemical bonding, while the other includes electrostatic and hydrogen bonding,[18] and both interactions were involved in an aqueous medium. The DNA–ethidium interaction modes in aqueous and $BmimPF_6$ phase are illustrated in Scheme 1. Figure 2B shows that in the contrast to the aqueous phase, an increase of the absorbance was recorded with the increase of DNA concentration when dsDNA is extracted into the IL phase, but no red shift of the absorption band was encountered. This observation clearly excluded the presence of chemical bonding between the EB moieties and the DNA strands, but indicates electrostatic and hydrogen-bonding interactions.

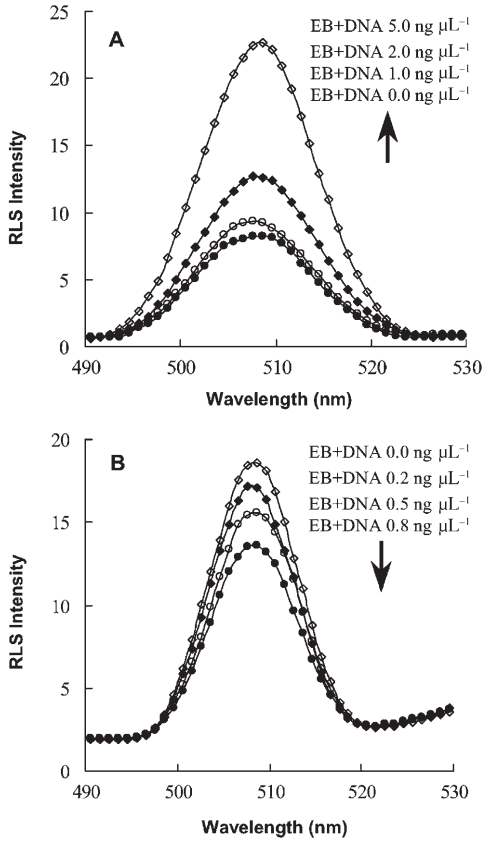


Figure 1. RLS spectra of DNA–ethidium systems. A) In aqueous phase with 0.0–5.0 ng μL^{-1} DNA and 10 ng μL^{-1} EB; B) DNA was extracted into $BmimPF_6$ (DNA solution: 200 μL; EB solution: 10 ng μL^{-1} in the final admixture; the volume of $BmimPF_6$: 100 μL; the volume of acetonitrile: 1000–2000 μL; the extraction time: 30 min).

In aqueous phase, EB intercalates into the DNA helix structure between the base pairs,[19] as generally the 3.4 Å base-pair separation in the DNA structure facilitates this.[17] The intercalation in turn results in the alteration of its local environment, and thus immerses itself into a hydrophobic region.[20] In such a circumstance, the intercalation of EB evidently leads to a reduction of the number of free ethidium entities, which corresponds to a decrease of its absorption.

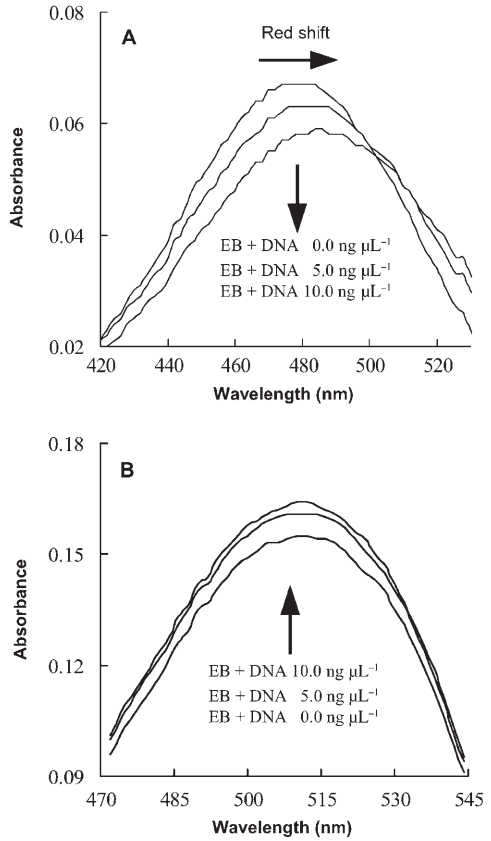
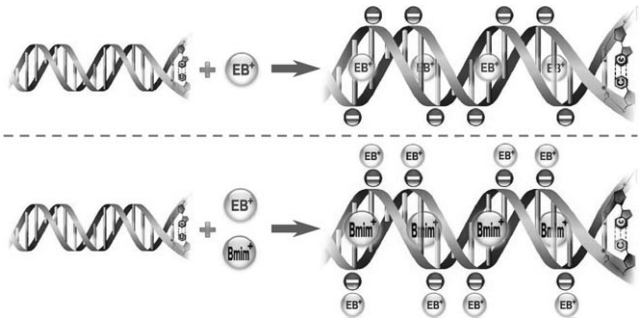


Figure 2. UV/Vis spectra of the EB–DNA systems. A) In aqueous phase with 0.0–10 ng μL^{-1} DNA and 10 ng μL^{-1} EB in the final admixture; B) DNA was extracted into $BmimPF_6$ phase (DNA solution: 0.0–10 ng μL^{-1}200 μL^{-1}; EB solution: 10 ng μL^{-1} in the final admixture; the volume of $BmimPF_6$: 100 μL; the volume of acetonitrile: 1000–2000 μL; the extraction time: 30 min).



Scheme 1. The DNA–EB interaction in aqueous phase and the IL–DNA–EB interaction in $BmimPF_6$ phase, illustrating the congregation of EB entities around DNA strands in the ionic liquid phase.

When DNA is extracted into the IL phase, the DNA strands are bound to the $BmimPF_6$ entity through strong interactions between the cationic $Bmim^+$ groups and the P–O bonds of phosphate groups in DNA strands as demonstrated in our previous work,[16] in which the cationic $Bmim^+$ groups intercalated into the helix structure of DNA strands. This interaction results in the transformation of DNA conformation along with a decrease of base-pair interstice; this decrease prohibits the intercalation of ethidium into the DNA structure. As a result, a congregation of EB moieties around the DNA strands takes place in the $BmimPF_6$ phase attributed to the DNA–EB interactions, which brings them into closer contact and increases their linear chromophore density as well as the strength of the electrostatic interaction.[21] Consequently, the relatively high concentration of pseudo-dissociative or pseudo-free EB moieties causes an increase in the absorption, and accordingly gives rise to a suppression of the RLS attributed to the inner filter effect as described in the following section.

RLS behaviour of the system with pure EB in the absence of DNA: Figures 3 and 4 illustrate the RLS and UV/Vis spectra for the systems containing pure EB in the absence of DNA in aqueous and IL phases. It is evident that in both phases, an almost linear decrease of the RLS at 510 nm occurs with the increase of ethidium concentration (Figure 3A and 3B). Accordingly, a significant increase of the absorption was recorded (Figure 4A and 4B); this observation is consistent with those frequently reported for conventional RLS systems.[22–24] Usually, light scattering is performed at a wavelength different from the absorption band, but for some species that aggregate, enhancements in light

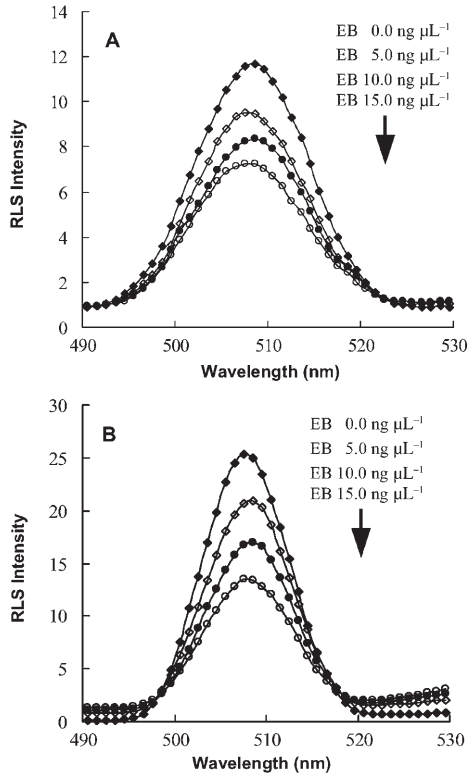


Figure 3. RLS spectra of the system with pure EB in the absence of DNA. A) In aqueous phase with 0.0–15 ng μL^{-1} EB. B) In the IL phase with 0.0–15 ng μL^{-1} EB in the final admixture; the volume of $BmimPF_6$: 100 μL; the volume of acetonitrile: 1000–2000 μL.

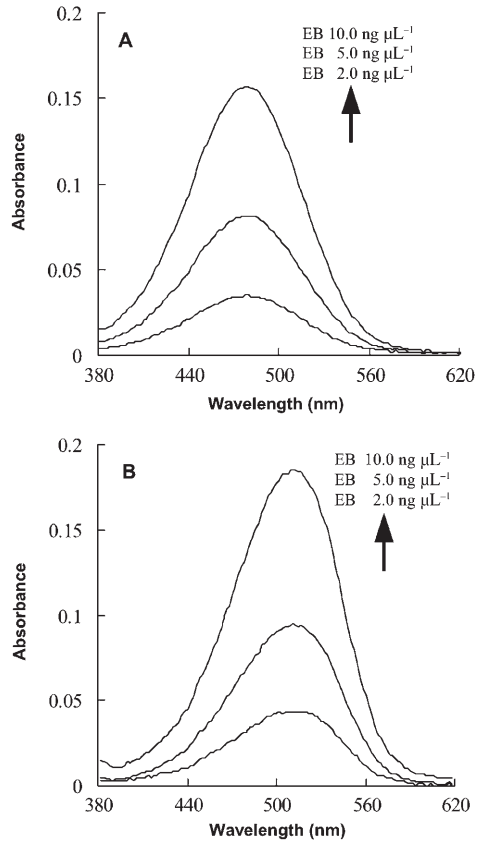


Figure 4. UV/Vis spectra of the systems containing pure EB. A) In aqueous phase with 2.0–10 ng μL^{-1} EB. B) In the $BmimPF_6$ phase with 2.0–10 ng μL^{-1} EB in the final admixture; the volume of $BmimPF_6$: 100 μL; the volume of acetonitrile: 1000–2000 μL.

scattering of several orders of magnitude can be observed at characteristic wavelengths of these species.[8]

Evident overlap of absorptions in the region of 450–540 nm with the emission and excitation band for RLS can be seen in Figure 4; this overlap facilitates the occurrence of the so-called inner filter effect (IFE). Generally speaking, IFE requires the presence of both an absorber and a fluorophore; the excitation or emission intensity of the latter is modulated by varying the absorption of the absorber.[25,26] In this particular case, ethidium acts as both the absorber and the fluorophore. The increase of ethidium concentration in both phases definitely increases the amount of free ethidium moieties, thus the IFE brings about an increase of the absorption and consequently a decrease of the RLS.

The effect of pH for the original DNA aqueous solution on the RLS behaviour of the $BmimPF_6$–DNA–EB system: The ionic liquid used in the present investigation has been prewashed so that is has a neutral pH value (pH 6.5);[16] hence the acidity of the DNA aqueous solution adopted for extraction not only decides the pH value of the extraction system, but plays an important rule in the RLS of the $BmimPF_6$–DNA–EB system after extraction. The recorded RLS spectra of the $BmimPF_6$-DNA-EB system in the IL phase at various pH values of the original DNA aqueous solution are illustrated in Figure 5. The investigated pH range was confined within 4–8 in order to ensure that dsDNA was not destroyed during the investigations. It is evident that at lower pH values, that is, pH < 6, higher RLS intensities were recorded, and meanwhile a significant decrease of the RLS was observed with the increase of pH. Thereafter, the RLS intensity remained virtually unchanged with the variation of pH value in the range of 7–8. These results are consistent with the DNA extraction mechanism by using $BmimPF_6$ detailed in our previous studies,[16] in which we noted that at lower pH values, although a large part of the DNA was extracted into $BmimPF_6$, there was some residual DNA remained in aqueous phase. In this case, the net reduction of RLS was assigned not only to the decrease of RLS caused by the $BmimPF_6$–DNA–EB interaction, that is, the congregation of EB moieties around the DNA strands, but also to the increase of RLS arising from the residual free DNA fragments in aqueous phase.

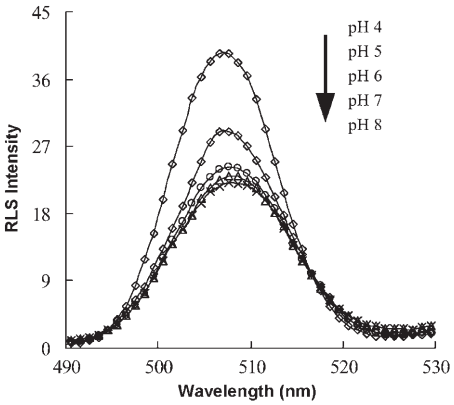


Figure 5. The effect of pH on the RLS of the IL–DNA–EB system. DNA solution: 2.0 ng μL^{-1} 200 μL^{-1}; EB solution: 10 ng μL^{-1} in the final admixture; the volume of $BmimPF_6$: 400 μL; the volume of acetonitrile: 1000–2000 μL; the extraction time: 30 min.

A higher pH value facilitates the extraction of dsDNA into the IL phase and helps to improve the extraction efficiency, which leads to a decrease of the residual DNA concentration in the aqueous phase. At a pH value > 7, a trace level of dsDNA was quantitatively extracted into the IL phase and virtually no residual DNA left in the aqueous phase. In this case, the reduction of RLS was attributed solely to the interaction of $BmimPF_6$–DNA–EB system in $BmimPF_6$. This observation evidently offers a figure of merit when using this system for DNA quantification in IL phase.

The effect of various amount of IL on the RLS: The effect of various amount of $BmimPF_6$ on the RLS behaviour of the system with and without DNA extraction into the ionic-liquid phase are shown in Figure 6. It is clear that in the absence of DNA (Figure 6A), a significant increment of the RLS was recorded purely with the increase in the amount of

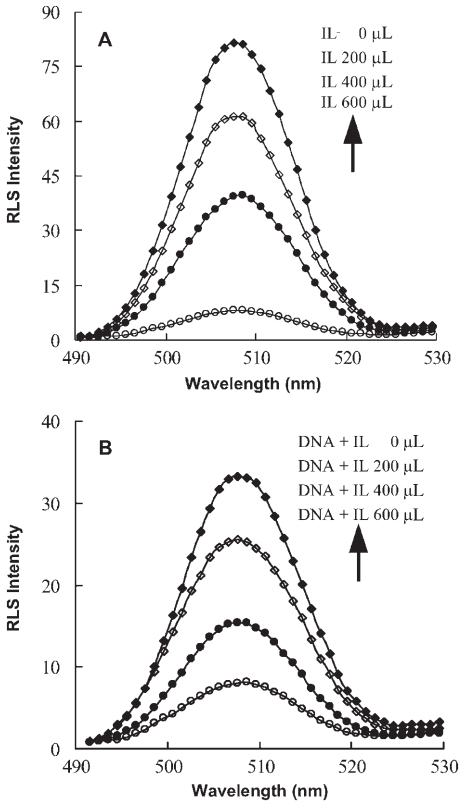


Figure 6. RLS spectra recorded in the presence of various amounts of $BmimPF_6$. A) In the absence of DNA (EB solution: 10 ng μL^{-1} in the final admixture; the volume of acetonitrile: 1000–2000 μL). B) In the presence of DNA (DNA solution: 2.0 ng μL^{-1} 200 μL^{-1}; EB solution: 10 ng μL^{-1} in the final admixture; the volume of acetonitrile: 1000–2000 μL; the extraction time: 30 min).

$BmimPF_6$ employed in the interaction system. A similar trend was also observed when certain amount of dsDNA was extracted/transferred into $BmimPF_6$ (Figure 6B), but the recorded RLS intensity at the same level of IL concentration was evidently lower than that achieved in the absence of DNA due to the signal reduction caused by congregation of EB moieties. In addition to the enhancement of RLS attributed to the increase of the amount of $BmimPF_6$, the suppression effect of $BmimPF_6$–DNA–EB interactions on the RLS as described previously should definitely be included in this case. It is therefore necessary to keep the amount of the $BmimPF_6$ employed constant, when directly quantifying DNA contents in the IL phase.

Applications of the system for direct quantification of DNA in $BmimPF_6$: When applying $BmimPF_6$ as a solvent for DNA extraction and purification from real world biological samples, it is highly desirable that the DNA concentration transferred into $BmimPF_6$ can be directly quantified in the ionic-liquid phase. We found that at the experimental conditions cited herein, there exists a linear relationship between the DNA concentration within a narrow range that is transferred into $BmimPF_6$ and the net decrease of the RLS intensity of the $BmimPF_6$–DNA–ethidium system. A novel procedure for direct determination of DNA content in the IL phase is therefore proposed as detailed in the Experimental Section, for which a series of standards were prepared similarly by mixing $BmimPF_6$ (100 μL) directly with the sample (200 μL) and EB solution (10 μL), followed by the addition of acetonitrile (1000–2000 μL) in order to make a homogenous solution. The limit of detection of the procedure was derived to be approximately 0.01 ng μL^{-1} (Calf thymus DNA).

The practical applicability of this procedure was first demonstrated by measuring DNA concentrations in both aqueous and $BmimPF_6$ phases after certain amounts of DNA were extracted into the $BmimPF_6$ phase. The experimental results indicated that a low DNA concentration level transferred into $BmimPF_6$, quantified by direct measurement in the ionic-liquid phase, agreed well with those derived from the DNA residual concentrations in aqueous phase, indicating the suitability of this procedure for direct quantification of DNA in ionic liquid after extraction.

DNA extraction and quantification in human whole blood: The practical applicability of this procedure was further demonstrated by measuring the DNA content in human whole blood after extraction into the ionic-liquid phase, by adopting the following sample pretreatment (cell-lysis) procedure.

A portion of human whole blood (1000 μL) was centrifuged for 10 min at 10000 rpm. The supernatant plasma was discarded following the addition of a cell-lysis solution (500 μL: 0.01 mol L^{-1} Tris-HCl, 0.1 mol L^{-1} EDTA, 0.5% SDS, pH 8.0) and a Proteinase K solution (10 μL: 20 μg μL^{-1}). The mixture was incubated in a water bath under agitation at 37°C for 2 h, followed by 50°C for 3 h. A 5 μL aliquot of the final solution was transferred into a centrifugal tube and diluted to 200 μL, which was afterward extracted with $BmimPF_6$ (400 μL) following the same procedure as detailed in the Experimental Section. After phase separation, 100 μL of the IL phase was taken to mix with appropriate amount of EB solution, and the mixture was shaken for 20 s to facilitate the EB–DNA interaction. Finally, acetonitrile (1000–2000 μL) was introduced to obtain a homogenous solution, and the RLS of the system was measured at 510 nm by transferring the admixture into a 10 mm quartz cell.

Spiking recoveries were performed by introducing a certain amount of calf thymus DNA into the human whole blood sample before the cell-lysis procedure. The results achieved were summarised in Table 1.

Table 1. The analytical results for DNA contents in human whole blood obtained by employing the present procedure.

Samples	Found [ng μL^{-1}, $n=4$]	Spiked [ng μL^{-1}]	Recovery [%]
human whole blood 1	36.0 ± 3.9	40.0	95.4
human whole blood 2	30.7 ± 4.0	40.0	102.6

Conclusion

When dsDNA is extracted into ionic-liquid $BmimPF_6$, the cationic $Bmim^+$ group in $BmimPF_6$ intercalates into the DNA helix structure. The strong interactions between the P–O bond of phosphate groups in DNA strands and the $Bmim^+$ lead to the transformation of DNA conformation, which excludes the intercalation of ethidium with DNA. The electrostatic interaction between the ethidium entities and the DNA strands bound with IL results in the congregation of EB around the DNA strands. This gathering effect leads to closer contact of EB entities and increases their linear chromophore density as well as the strength of the electrostatic interaction, which consequently results in an increase of the absorption because of inner filter effect and accordingly a suppression of the resonance light scattering (RLS). Based on this abnormal observation of RLS, a novel procedure for the direct quantification of DNA in ionic-liquid phase was developed. The use of green solvent $BmimPF_6$ for DNA extraction offers clear advantages over the conventional approaches by employing organic solvents.[16]

Experimental Section

Reagents: Deoxyribonucleic acid activated from calf thymus (Calf thymus DNA, Sigma, D4522) was used as purchased from Sigma. Hexafluorophosphoric acid (Kunshan Fine Chemicals, China), 1-chlorobutane (Beijing Chemicals, China), 1-methylimidazole (Kaile Chemicals, China), ethidium bromide (EB) (Life technologies, Gaithersburg, USA), Proteinase K (Takara Biotechnology, China), tris(hydroxymethyl)aminomethane (Tris, Sinopharm Chemical Reagent Co. China-SCRC), sodium dodecyl sulfate (SDS, SCRC) and EDTA (SCRC) were used as received.

The ionic-liquid 1-butyl-3-methylimidazolium hexafluorophosphate was prepared by adopting a slightly modified documented pathway.[27–29] The chemical shifts obtained from the ^{1}H NMR spectra for the obtained $BmimPF_6$ were consistent with those of the reported values.[28,29]

A stock solution of EB in deionised water (2.0 $\mu g\,\mu L^{-1}$) was prepared, working solutions of different concentrations were obtained by step-wise dilution of the stock solution.

A stock solution of DNA in deionised water (1.0 $\mu g\,\mu L^{-1}$) was prepared and the DNA content was determined roughly by measuring its absorbance at 260 nm.

Other chemicals employed were at least of analytical reagent grade and were used without further purification. 18 MΩ cm deionised water was used throughout.

Apparatus: The RLS spectra of the system were obtained by scanning synchronously the excitation and emission monochromators ($\lambda_{ex}=\lambda_{em}$) from 250.0 to 700.0 nm. The RLS intensities were recorded at 510 nm by using an F-4500 fluorimeter (Hitachi, Japan), with the slit widths for excitation and emission set at 10 nm. The UV/Vis spectra were obtained by using a UV/Vis spectrophotometer (Purkinje General Instruments, Beijing, China). The ^{1}H NMR spectrum of the prepared ionic liquid $BmimPF_6$ was recorded in $(CD_3)_2CO$ at 293 K on a Bruker AVANCE 500 spectrometer (Bruker, Switzerland), with chemical shifts referenced to tetramethylsilane (TMS).

The RLS and UV/Vis spectra of DNA-EB system in aqueous and IL phases: An aliquot (2.0 mL) of the aqueous DNA solution (with concentrations within 0.0–5.0 ng μL^{-1}) was added into a 5 mL centrifugal tube followed by the EB solution (10 μL; 2 $\mu g\,\mu L^{-1}$) to give a reaction mixture that contained 10 ng μL^{-1} EB with a pH 6.7. The mixture was then shaken for 20 s in order to facilitate the intercalation of EB into the DNA structure, and afterwards the RLS spectra for the aqueous system were recorded, as illustrated in Figure 1A.

An aqueous solution of DNA (200 μL; with concentrations within 0.0–0.8 ng μL^{-1}) or sample solution was taken into a 5 mL centrifugal tube, following the introduction of $BmimPF_6$ (400 μL). After the mixture was agitated vigorously in an oscillator for 30 min in order to facilitate the extraction of dsDNA into IL, the two phases were separated by centrifugation. An aliquot (100 μL) of the IL phase was taken into a 5 mL cuvette and was mixed with the EB solution (10 μL; 2 $\mu g\,\mu L^{-1}$), giving a final EB concentration of about 10 ng μL^{-1}. The mixture was shaken for 20 s to facilitate the EB–DNA interaction. A portion of acetonitrile (1000–2000 μL) was then introduced to make a homogenous solution. The RLS of the system was measured at 510 nm by transferring the admixture into a 10 mm quartz cell.

The UV/Vis spectra of the above DNA–ethidium systems were also recorded within the range of 200–900 nm (Figure 2).

Acknowledgements

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