Nephelometric determination of micro amounts of nucleic acids with protamine sulfate

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Nucleic acids can form large particle complexes with protamine sulfate by electrostatic forces, which results in strong light scattering. Based on this, a nephelometric method is described for sensitive and convenient determination of nucleic acids with protamine sulfate by using a common spectrofluorimeter. Maximum light scattering is produced in the range of pH 2.2–4.4 with the same excitation and emission wavelengths at 365 nm. Under optimal conditions, the calibration curves are linear in the range 0.05–60.0 \( \mu \text{g cm}^{-3} \) for nucleic acids. The corresponding detection limits are 12.5 ng cm\(^{-3}\) for calf thymus DNA, 9.0 ng cm\(^{-3}\) for fish sperm DNA, and 18.0 ng cm\(^{-3}\) for yeast RNA, respectively. Six synthetic samples are determined with satisfactory results. The relative standard deviation of five replicate measurements is 3.2% for 2.0 \( \mu \text{g cm}^{-3} \) calf thymus DNA.

The quantitative determination of nucleic acids is very important in biochemical studies and biological techniques. Direct use of the intrinsic fluorescence\(^1,2\) and ultraviolet absorption\(^3,4\) of nucleic acids for their determination has been severely limited by the low sensitivity and serious interference. Therefore, many techniques, based on the interaction between nucleic acids and extrinsic reagents, have been established for nucleic acid determination, such as spectrophotometric methods,\(^5,6\) a radioactive labeling method,\(^7\) and fluorimetric methods. Among them, the fluorescence dye-binding method with ethidium bromide\(^8\) has been the most widely used for many years. Many other fluorescence reagents, including the Hoechst dye 33258,\(^9\) ethidium homodimer (EthD),\(^10\) Thiazole Orange homodimer (YOYO),\(^11\) Oxazole Yellow homodimer (YOYO),\(^12\) and the lanthanide metal complexes,\(^13\) etc., have been studied to improve the sensitivity and selectivity for nucleic acid determination. The methods with TOTO and YOYO can detect as little as picogram amounts of nucleic acids. Nevertheless, the widespread use of these methods are restricted by the high cost of the fluorescence reagents.

Recently, the light scattering technique has become a new interesting method for determination of micro amounts of biomacromolecules. The light scattering analysis is attractive because the light scattering measurement can be performed by an ordinary spectrofluorimeter and for biomacromolecules the detection limit can be quite low. Huang \textit{et al.} firstly developed resonance light scattering for analytical purposes to determine micro amounts of nucleic acids with \( \alpha, \beta, \gamma, \delta \)-tetrakis[(4-trimethylammoniumyl)phenyl]prophine\(^14,15\) and the cobalt(II)/4-[(5-chloro-2-pyridyl) azo]-1,3-diaminobenzene complex.\(^16\) Then Ma \textit{et al.} established a series of high sensitivity determination systems for proteins by using an enhanced Rayleigh light scattering technique with Chrome Blue K,\(^17\) Bromopyrogallol Red,\(^18\) and Bromophenol Blue.\(^19\) Nephelometry, which is based on the large particle light scattering, has been used to determine various inorganic ions, organic compounds, and proteins.\(^20\) In recent years, the nephelometric determination has received much attention in the immunoassay.\(^21,22\) To our knowledge, however, the use of nephelometry for determination of nucleic acids has not been reported so far. In this paper, our focus is to develop nephelometry as a sensitive and convenient technique for the determination of micro amounts of nucleic acids.

It is well known that nucleic acids and protamine sulfate can form large particle complexes by electrostatic forces, which has generally been used for purification of nucleic acids\(^23\). We find the complex can cause a strong light scattering signal, by means of which nucleic acids can be sensitively detected. The light scattering responds linearly in the concentration range from 0.05 to 60.0 \( \mu \text{g cm}^{-3} \) with a relative standard deviation of less than 5%. The detection limits are 12.5 ng cm\(^{-3}\) for calf thymus DNA, 9.0 ng cm\(^{-3}\) for fish sperm DNA, and 18.0 ng cm\(^{-3}\) for yeast RNA, respectively. Particularly, almost no interference can be observed from proteins (HSA, BSA, and \( \gamma \)-G), nucleosides and most of the metal ions.

**Experimental**

**Reagents**

All reagents were of analytical reagent grade without further purification. Doubly deionized water was used throughout. Stock solutions of nucleic acids (100 \( \mu \text{g cm}^{-3} \)) were prepared at 0–4 °C by dissolving calf thymus DNA and fish sperm DNA (Baitai Biochemical Co., Chinese Academy of Sciences, Beijing, China) in water, and dissolving yeast RNA (Shanghai Institute of Biochemistry, Chinese Academy of Sciences, Shanghai, China) in 0.5% NaCl aqueous solution. Working standard solutions were obtained by appropriate dilution of the stock solutions. A 1.0 mg cm\(^{-3}\) protamine sulfate solution was prepared by dissolving 0.1 g of protamine sulfate (Sigma, St Louis, MO, USA) in 100 cm\(^3\) water.

**Apparatus**

The light scattering spectra and the intensity of light scattering were measured and recorded by a Shimadzu Model RF-540 spectrofluorimeter (Kyoto, Japan) with a quartz cuvette (1 × 1 cm). The hydrodynamics radius (\( R_h \)) of nucleic acids was measured by a laser light-scattering spectrometer (laboratory-built by Peking University, Beijing, China) equipped with an argon ion laser (Spectra-Physics Lasers San Jose, CA, USA; Stabilite 2017, output power = ~400 mW at \( \lambda = 514.5 \text{ nm} \)) as the light source. The pH values were measured with a Model 821 digital pH meter (Zhong Shan University, China).
General procedure

In a 10 cm$^3$ standard flask, a known volume of nucleic acids or sample solution, 2.0 cm$^3$ Britton–Robinson buffer solution (pH 3.3) is added and approximately diluted to 8.5 cm$^3$ with water. Then 1.0 cm$^3$ protamine sulfate (1.0 mg cm$^{-3}$) is transferred in. The solution is mixed and diluted to volume. The light scattering intensity is measured at 365 nm with slit width at 10.0 nm for the excitation and emission. The light scattering spectrum is obtained by scanning simultaneously with the same excitation and emission wavelengths by the RF-540 spectrofluorimeter. The light scattering signals of the complex of the nucleic acids with protamine sulfate reported in the paper are net intensities of light scattering in arbitrary units of the instrument, where the background light scattering signal of the protamine sulfate in the absence of the nucleic acid has been subtracted for each value except for the light scattering spectra.

Results and discussion

Spectral characteristics

Fig. 1 shows the light scattering spectra of calf thymus DNA, protamine sulfate, and their mixture. As can be seen from this figure, the light scattering intensity of both the DNA and protamine sulfate is very small when they exist separately. However, when the DNA is mixed with protamine sulfate, the intensity of light scattering is strongly enhanced in the wavelength range 242–700 nm and reaches a maximum at 365 nm ($\lambda_{\text{max}}$). Moreover, in the wavelength range 337.5–387.5 nm, where $\lambda_{\text{max}}$ is central, the light scattering intensity is almost constant. Therefore, $\lambda_{\text{max}}$ is chosen as the optimum wavelength for light scattering measurements of nucleic acids.

The strong enhancement of light scattering for fish sperm DNA and yeast RNA by protamine sulfate is also observed, and the pattern of the light scattering spectra is same as that of calf thymus DNA. However, the light scattering intensity differs for different nucleic acids at the same concentration. The experiments show the intensity order of light scattering as follows: fish sperm DNA > calf thymus DNA > yeast RNA, which accords with the order of the molecular mass of the nucleic acids.

In addition, the light scattering spectra not only depend on the nature of the complex of nucleic acids with protamine sulfate, but also reflect the characteristics of the instrument (such as the convolution of the lamp spectrum, the transmission of the monochromators, and the spectral response of the photomultiplier tube). Therefore, we suggest that the optimum wavelength should be determined again when a different model of spectrofluorimeter or light scattering spectrometer is used for light scattering measurements of nucleic acids.

Particle size

In Britton–Robinson buffer solution (pH 3.3), the hydrodynamics radius ($R_g$) of the complex of calf thymus DNA with protamine sulfate at different concentrations is measured by a dynamic laser scattering method according to the literature. Fig. 2 shows that the $R_g$ linearly increases with increasing DNA concentration. This phenomenon proves that the complexes aggregate to form large particles, whose dimensions are comparable to the wavelength of UV/VIS light. In addition, when the concentration of nucleic acids (including calf thymus DNA, fish sperm DNA, and yeast RNA) is greater than 20 mg cm$^{-3}$, the particles in suspension can be obviously observed in the solutions. Therefore, the proposed technique using scattered light for the determination of nucleic acids can be judged as nephelometry.

Influence of pH

Fig. 3 shows the influence of pH on the light scattering intensity of calf thymus DNA and the complex of calf thymus DNA with protamine sulfate, respectively. The Britton–Robinson buffer solution was used to adjust the pH. As shown in Fig. 3, the light scattering intensity of calf thymus DNA is almost same in the pH range of 2.2–9.6, but it shows a stronger value at pH 1.8, possibly as a result of the denaturation of the DNA in acidic solution. The complex shows a higher light scattering at the pH range between 2.2–4.4. The pH 3.3 was selected as the optimum pH because at this value the complex shows a maximum intensity of light scattering. Different buffer solutions (Britton–Robinson, acetic acid–sodium acetate, citric acid–sodium citrate) were tested for the adjustment and the Britton–Robinson buffer gave the best results.
Optimization of protamine sulfate concentration

The optimization of protamine sulfate concentration was investigated for calf thymus DNA (1.0–60.0 µg cm\(^{-2}\)) using different protamine sulfate concentrations (10.0–200 µg cm\(^{-2}\)). As shown in Fig. 4, when the protamine sulfate is 10.0, 20.0 and 50.0 µg cm\(^{-2}\), the calibration curves bend down at the DNA concentration of 20.0, 40.0 and 40.0 µg cm\(^{-2}\), respectively. When the protamine sulfate concentration is 100 µg cm\(^{-2}\), there is a good linear relationship between the light scattering intensity and the DNA concentrations at the range of 1.0–60.0 µg cm\(^{-2}\). When the protamine sulfate concentration is 200 µg cm\(^{-2}\), the calibration curve bends upward at the DNA concentration of 40.0 µg cm\(^{-2}\). However, in the linear range, the calibration curves have the same slope at different protamine sulfate concentrations. Therefore, 100 µg cm\(^{-2}\) of protamine sulfate is chosen as optimum concentration for subsequent work.

Effect of ionic strength

The effect of ionic strength on the light scattering intensity of the complex of calf thymus DNA with protamine sulfate was studied by the addition of NaCl; the results indicate that the light scattering intensity decreases 9.4% at a NaCl concentration of 1.0%. It is obvious that the ionic strength has little effect on the light scattering intensity of the complex, which indicates that the electrostatic forces of protamine sulfate binding on the DNA is very strong.

Stability of light scattering signal

The stability of the light scattering signal is very important for the nephelometric determination. The influence of incubation time on light scattering intensity was investigated in a 120 min period immediately after mixing the calf thymus DNA and protamine sulfate in Britton–Robinson buffer solution (pH 3.3). The experiment shows that the maximum intensity of light scattering is reached immediately when the solutions are mixed, and remains constant for 50 min. However, when the incubation time is longer than 50 min, the light scattering intensity begins to decrease. The results express that the stability of the light scattering signal is practical for the nephelometric determination of nucleic acids.

Interference study

In order to study the potential interference of various substances with the nephelometric determination of nucleic acids, the standard solution containing 1.0 µg cm\(^{-2}\) calf thymus DNA was premixed with foreign substances, then the intensity of light scattering is detected according to the general procedure and compared with that of the standard solution itself. The tolerance concentrations of proteins, nucleotides, and several metal ions for the nephelometric determination are summarized in Table 1. From Table 1, it can be seen that proteins and nucleotides do not interfere with the nephelometric determination of the DNA. Although the metal ions have a positive effect on the light scattering intensity of the determination system, the metal ions can be tolerated at very high concentration except Fe\(^{3+}\). However, the tolerated concentration of Fe\(^{3+}\) (1 × 10\(^{-6}\) mol dm\(^{-3}\)) is generally greater than that in biological samples.

Calibration curves

According to above general procedure, the calibration curves for the nephelometric determination of nucleic acids were constructed under the optimal conditions. All the analytical parameters are presented in Table 2. As Table 2 shows, there are good linear relationships between the light scattering intensity and the concentration of nucleic acids over a wide range.

Determination of nucleic acids in synthetic samples

With the calibration curves, six synthetic samples constructed on the basis of the interference of foreign substances were simultaneously determined under the same conditions. The determination results are listed in Table 3. As Table 3 shows, the results are satisfactory.

Conclusions

The light scattering intensity of protamine sulfate can be enhanced greatly by the addition of nucleic acids due to the formation of large particle complexes by strongly electrostatic interactions. Based on this, a nephelometric method has been developed for the determination of nucleic acids. The proposed method has high sensitivity, which can be comparable to that of classical fluorescence methods with ethidium bromide\(^8\) and Hoechst dye.\(^9\) Though it is less sensitive than the fluorescence methods using TOTO and YOYO,\(^12\) it can be characterized with
obvious advantages in respect of (1) widely linear range (0.05–60.0 \( \mu \text{g cm}^{-2} \)), (2) short incubation time ( < 1 min at room temperature), (3) simple operation by using a common spectrophotometer without any expensive reagents and (4) tolerance of most interfering substances. Because proteins and nucleotides do not interfere in the nephelometric determination of DNA, we assume that this method can offer a convenient means of quantitative analysis for the polymerase chain reaction and other biotechnologies.

Acknowledgements

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References

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Table 1  Tolerance concentrations of interfering substances

<table>
<thead>
<tr>
<th>Substance</th>
<th>Change in ( I ) (%)</th>
<th>Substance</th>
<th>Change in ( I ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ( \mu \text{g cm}^{-2} ) BSA</td>
<td>-3.2</td>
<td>1 ( \times 10^{-5} ) mol dm(^{-3} ) Cd(^{2+} ) chloride</td>
<td>3.1</td>
</tr>
<tr>
<td>10 ( \mu \text{g cm}^{-2} ) HSA</td>
<td>-0.50</td>
<td>1 ( \times 10^{-5} ) mol dm(^{-3} ) Mn(^{2+} ) sulfate</td>
<td>0.90</td>
</tr>
<tr>
<td>10 ( \mu \text{g cm}^{-2} ) ( \gamma )-G</td>
<td>1.7</td>
<td>1 ( \times 10^{-5} ) mol dm(^{-3} ) Cu(^{2+} ) sulfate</td>
<td>2.0</td>
</tr>
<tr>
<td>10 ( \mu \text{g cm}^{-2} ) AMP</td>
<td>-0.35</td>
<td>1 ( \times 10^{-5} ) mol dm(^{-3} ) Co(^{2+} ) chloride</td>
<td>4.3</td>
</tr>
<tr>
<td>10 ( \mu \text{g cm}^{-2} ) GMP</td>
<td>-0.53</td>
<td>1 ( \times 10^{-5} ) mol dm(^{-3} ) Ni(^{2+} ) nitrate</td>
<td>1.7</td>
</tr>
<tr>
<td>10 ( \mu \text{g cm}^{-2} ) CMP</td>
<td>0.90</td>
<td>1 ( \times 10^{-5} ) mol dm(^{-3} ) Pb(^{2+} ) nitrate</td>
<td>3.4</td>
</tr>
<tr>
<td>10 ( \mu \text{g cm}^{-2} ) TMP</td>
<td>-0.62</td>
<td>1 ( \times 10^{-5} ) mol dm(^{-3} ) Fe(^{3+} ) nitrate</td>
<td>2.2</td>
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<tr>
<td>10 ( \mu \text{g cm}^{-2} ) UMP</td>
<td>0.40</td>
<td>1 ( \times 10^{-5} ) mol dm(^{-3} ) Fe(^{3+} ) sulfate</td>
<td>1.6</td>
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<tr>
<td>10 ( \mu \text{g cm}^{-2} ) dAMP</td>
<td>1.8</td>
<td>1 ( \times 10^{-5} ) mol dm(^{-3} ) Fe(^{3+} ) chloride</td>
<td>3.6</td>
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<tr>
<td>10 ( \mu \text{g cm}^{-2} ) dCMP</td>
<td>-1.1</td>
<td>1 ( \times 10^{-5} ) mol dm(^{-3} ) Fe(^{3+} ) chloride</td>
<td>3.6</td>
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<tr>
<td>10 ( \mu \text{g cm}^{-2} ) dGMP</td>
<td>-0.70</td>
<td>1 ( \times 10^{-5} ) mol dm(^{-3} ) Al(^{3+} ) sulfate</td>
<td>1.3</td>
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<tr>
<td>Yeast RNA</td>
<td>1.0</td>
<td>1 ( \times 10^{-5} ) mol dm(^{-3} ) Mg(^{2+} ) chloride</td>
<td>5.4</td>
</tr>
<tr>
<td>Fish sperm DNA</td>
<td>3.07</td>
<td>1 ( \times 10^{-5} ) mol dm(^{-3} ) Mg(^{2+} ) chloride</td>
<td>4.6</td>
</tr>
</tbody>
</table>

\( ^a \) Average value from three measurements. \( ^* \) I is the light scattering intensity.

Table 2  Analytical parameters for nucleic acid determination by nephelometry

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Linear range/ ( \mu \text{g cm}^{-2} )</th>
<th>Linear regression equation (( C(\mu \text{g cm}^{-2}) ))</th>
<th>Detection limit (3( \sigma )ng cm(^{-2} ))</th>
<th>( r )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf thymus DNA</td>
<td>0.05–60.0</td>
<td>( I = 1.03 + 27.4 C )</td>
<td>12.5</td>
<td>0.995</td>
</tr>
<tr>
<td>Fish sperm DNA</td>
<td>0.05–60.0</td>
<td>( I = 1.10 + 23.7 C )</td>
<td>18.0</td>
<td>0.9989</td>
</tr>
</tbody>
</table>

Table 3  The results of determinations in synthetic samples

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Conc./ ( \mu \text{g cm}^{-2} )</th>
<th>Main additives(^a)</th>
<th>Found/ ( \mu \text{g cm}^{-2} )</th>
<th>Recovery (%, ( n = 5 ))</th>
<th>RSD (%, ( n = 5 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf thymus DNA</td>
<td>3.00</td>
<td>BSA, Fe(^{3+} ), Ca(^{2+} ), Mg(^{2+} )</td>
<td>3.07</td>
<td>97.8–103.8</td>
<td>3.1</td>
</tr>
<tr>
<td>Fish sperm DNA</td>
<td>2.00</td>
<td>AMP, GMP, CMP, TMP, UMP</td>
<td>1.94</td>
<td>93.3–102.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Yeast RNA</td>
<td>3.00</td>
<td>HSA, Zn(^{2+} ), Cd(^{2+} ), Hg(^{2+} )</td>
<td>3.01</td>
<td>98.9–102.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Yeast RNA</td>
<td>3.00</td>
<td>( \gamma )-G, Cu(^{2+} ), Co(^{2+} ), Ni(^{2+} )</td>
<td>2.98</td>
<td>96.4–104.9</td>
<td>4.3</td>
</tr>
</tbody>
</table>

\( ^a \) The concentration of additives is as follows: BSA, HAS, \( \gamma \)-G, 10 \( \mu \text{g cm}^{-1} \); AMP, GMP, CMP, TMP, UMP, 2.0 \( \mu \text{g cm}^{-1} \); Fe\(^{3+} \), 1 \( \times 10^{-5} \) mol dm\(^{-3} \); Ca\(^{2+} \), Mg\(^{2+} \), Zn\(^{2+} \), Cd\(^{2+} \), Hg\(^{2+} \), Co\(^{2+} \), Ni\(^{2+} \), 1 \( \times 10^{-5} \) mol dm\(^{-3} \).