Resonance Rayleigh Scattering as a Tool for Isoelectric Point Monitoring and Iron (III) Cation Determination

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Abstract

Resonance Rayleigh scattering (RRS) technique was utilized as a tool for isoelectric point monitoring and iron (III) cation determination. The spectral properties of some amphoteric molecules (proteins and a DNA sequence) were investigated using RRS technique. When the pH values were kept around their isoelectric points, especially high RRS signals could be obtained, which were much stronger than those at other pH values. By using C30 DNA sequence as a probe, iron (III) cation can be detected rapidly. After iron (III) was added to C30 solution, significantly decreased RRS signal was obtained. The sensing process can be finished within 10 minutes with a detection limit of 0.9 µM. Thus, a sensitive, selective, and label-free method was successfully developed for iron (III) detection.

Keywords: Resonance Rayleigh scattering; isoelectric point; proteins; DNA sequence; iron (III) detection
Introduction

The isoelectric point (pI) is the pH value where the net charge of molecule is zero, which is very significant for amphoteric molecules, such as peptides and proteins. Especially, the pI is important in protein purification as pI represents the pH at which the solubility of protein is minimal.\(^1\) Polyacrylamide gel and capillary isoelectric focusing are two common ways for determining pIs of proteins.\(^2\)-\(^4\) During isoelectric focusing, amphoteric molecules are separated based on their different pI, and the technique can therefore be used to detect the precise pI of an unknown component. However, the concentration of salt must be carefully controlled in the isoelectric focusing experiment. Thus, the development of simple and stable methods for pI detection is still in need.

Resonance Rayleigh scattering (RRS) method is a well-known analytical technique because of its high sensitivity, simplicity, and label-free analysis.\(^5\)-\(^7\) RRS light is greatly enhanced at wavelength near the absorption band due to the change in the complex refractive index. Characteristic and intensity of RRS spectrum can be greatly affected by the molecular conformation, size, and interfacial properties. Therefore, RRS spectra can be utilized to speculate molecular structure, charge distribution, and state of combination. Recently, RRS technique has been widely applied to sensing metal ions,\(^8\),\(^9\) biological molecules,\(^10\)-\(^12\) hazardous pollutants,\(^13\),\(^14\) drug molecules,\(^15\)-\(^17\) dyes,\(^18\)-\(^20\) and so on.\(^21\)-\(^23\)

Iron (III) cation is one of trace metal elements that play an important role in environmental and biological systems.\(^24\)-\(^26\) As a key index to evaluate water quality, iron (III) cation concentration needs to be monitored in environmental samples. In addition, because iron (III) cation accumulates within the spleen and liver, iron (III) cation level is of great importance for human health. In this work, RRS was utilized as a tool for pI monitoring and iron (III) cation determination (Fig. 1). For the proteins (albumin, haemoglobin, urease, and casein) and DNA sequences (C30 and A15), the RRS signals show the maxima around their pIs, providing a potential way for pI detection. With the addition of iron (III) target, reduced RRS intensity of C30 was found. The C30 sequence was employed as a probe for iron (III)
cation determination. Thus, a simple, rapid, and label-free RRS sensing method was developed for sensitive and selective detection of iron (III) cation.

**Experimental**

**Reagents and chemicals**

Albumin from chicken egg white and hemoglobin were purchased from Aladdin Reagent Ltd. (Shanghai, China). Urease was purchased from Sangon Inc. (Shanghai, China). The DNA sequences (C30, 5’-CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC-3’, and A15, AAA AAA AAA AAA AAA) was synthesized and HPLC-purified by Sangon Inc. (Shanghai, China). Sodium hydroxide (NaOH), LiNO₃, NaCl, KCl, MgCl₂·6H₂O, CaCl₂·2H₂O, Co(NO₃)₂·6H₂O, NiSO₄·6H₂O, Pb(NO₃)₂, Zn(NO₃)₂·6H₂O, Cu(NO₃)₂·3H₂O, Hg(NO₃)₂·H₂O, Al(NO₃)₃·9H₂O, and Fe(NO₃)₃·9H₂O were of analytical reagent grade and used as received. The 40 mM Britton-Robinson (BR) buffer was prepared according to the standard protocol. Ultrapure water with resistivity of 18.2 MΩ cm was utilized throughout this experiment.

**Apparatus**

An F-2700 spectrofluorophotometer (Hitachi, Japan) was utilized to measure the RRS spectra with equipment of a 150 W xenon lamp. The slits (Ex/Em) were kept 10.0/10.0 nm, and the photomultiplier voltage was set at 400 V. The RRS spectra were obtained by synchronous scanning wavelength from 220 to 650 nm at λ<sub>ex</sub> = λ<sub>em</sub>. A rapid mixing device (Ronghua Instrument Plant, Jiangsu, China) was employed for mixing solutions completely. A Five Easy Plus pH meter (METTLER TOLEDO, Shanghai, China) was used for adjusting different pH values.

**Experiment procedures**

For recording RRS spectra of the albumin solution, 10 μL of albumin solution (20 mg/mL) was added to 990 μL of BR buffer. The RRS spectra were recorded after 5 minutes. By changing the pH values of BR buffer solutions, the RRS intensities of albumin at different pH values were obtained. Similarly, the RRS signals of haemoglobin (0.02 mg/mL)
urease (0.01 mg/mL), and casein (0.06 mg/mL) solution at different pH values were recorded, respectively.

For exploring the properties of C30 DNA, 8 µL of C30 solution (100 µM) was mixed with 992 µL of BR buffer (pH 2.0 – 4.0). Five minutes later, the corresponding RRS spectra were measured. Similarly, the RRS signals of A15 (1.1 µM) solution at different pH values were recorded. For iron (III) cation detection, 10 µL of iron (III) solution with various concentrations was added to 990 µL of C30 solution. After 5 min, the RRS spectra were collected. For water sample analysis, tap water from the laboratory and river water from the Jialing River (Chongqing, China) were collected. To remove the suspended impurities, the water samples were purified with a 0.22 µm membrane. Then, different concentrations of iron (III) were added to the water samples. The following procedures were the same as above.

Results and discussion

RRS properties of the proteins

As is known, proteins are amphiprotic biomolecules, consisting of one or more long chains of amino acid residues. To explore the RRS property of proteins at different pH values, four kinds of proteins (albumin, haemoglobin, urease, and casein) were chosen and investigated. As shown in Fig. 2A, the RRS intensity of albumin at 310 nm is gradually enhanced with increasing pH values, and the RRS intensity reaches the maximum at pH 5.0. However, the RRS intensity becomes decreased with further increase of pH values. The corresponding RRS spectra of albumin are exhibited in Fig. 2B, and the maximum RRS peak is located near 310 nm.

The RRS properties of haemoglobin, urease, and casein were also discussed. As shown in Figs. 2C, 2E, and 2G, the variation of their RRS intensity at 310 nm shows the similar tendency. The maximum RRS peaks of haemoglobin, urease, and casein are also located around 310 nm (Figs. 2D, 2F, and 2H). The RRS intensity is increased firstly and then deceased with increasing pH values. The RRS intensities of haemoglobin and urease get the
maximum at pH 7.0, 5.0, and 5.0, respectively. It should be noticeable that the pH values obtained from the maximum RRS intensity are in accordance with the pIs of these proteins, which may be the universal property of different proteins. It is inferred that proteins interact with each other well, and aggregate at the pI because of electrostatic interaction, resulting in enhanced RRS signal.

**RRS properties of DNA sequence**

To investigate whether the rule can be adapted to other kind of ampholytes, the DNA sequences C30 and A15 were chosen to detect their RRS signals. As can be seen in Fig. 3A, the RRS intensity of C30 at 302.5 nm is first increased and reaches the maximum at pH 3.0. With further increase of pH value, the RRS signal is gradually decreased. The A15 shows the similar RRS property as C30 with pI of 2.4 (Fig. 3C). These DNA sequences are composed of bases and backbone phosphate group. When the pH value of solution is above 2.0, phosphodiester linkage is kept negatively charged. According to the report, the pKₐ values of cytosine and adenine are 4.2 and 3.5, respectively. When the pH value of solution is below their pKₐ values, cytosine and adenine are protonated and thus positively charged. The maximum RRS peak of C30 and A15 are around 302.5 and 290 nm, which are blue-shifted compared with those of proteins (Fig. 3B, 3D). The RRS peak locations of different molecules are related to their construction. As a result, RRS can be an alternative tool to measure the pIs of different amphoteric biomolecules.

**The interaction between DNA and cations**

To further demonstrate the mechanism of obtained high RRS signal, the property of C30 sequence was explored with addition of different metal ions. As shown in Fig. 4A, C30 solution exhibits high RRS signal at pH 3.0 (curve a). However, when the iron (III) cation solution is added, the RRS intensity of C30 is significantly decreased (curve b). The sole iron (III) solution shows very low RRS intensity (curve c). In order to understand whether the selectivity for iron (III) derives from the unique property of C30 DNA, the A15 DNA has been examined. As shown in Fig. 4B, the RRS intensity of A15 solution is gradually decreased with addition of increasing iron (III). As a result, the A15 DNA exhibits similar
response as C30 DNA when iron (III) is added. Thus, it is inferred that backbone phosphate group of DNA plays an important role in the response of DNA for iron (III). The decrease of RRS intensity can be attributed to the strong interaction between iron (III) and backbone phosphate group of DNA. Iron (III) can interact with backbone phosphate group, and destroy the electrostatic interaction between DNA sequences, resulting in decreased DNA RRS signal.

The RRS property of C30 was investigated with some other cations, such as Li⁺, Na⁺, K⁺, Mg²⁺, Ca²⁺, Co²⁺, Pb²⁺, Zn²⁺, Cu²⁺, Hg²⁺, Al³⁺, and Fe²⁺. As shown in Fig. 4C, the RRS intensity difference (ΔRRS) at 302.5 nm induced by iron (III) is much higher than other cations (ΔRRS = I₀ − I. I₀ and I represent the RRS intensities of C30 before and after the addition of different cations, respectively.). Compared with iron (III), other cations cannot interact with backbone phosphate group of C30 well, and thus no obvious changes of RRS spectra were observed. Considering the good selectivity of C30 probe for iron cation (III), the C30 probe exhibits great potential for iron (III) detection.

The iron (III) cation detection with C30 probe

As the RRS intensity of C30 probe can be affected by iron (III), the RRS spectra of C30 solutions with different concentrations of iron (III) were tested. As can be seen in Fig. 5A, in absence of iron (III), the RRS signal of C30 solution is very strong. Upon addition of iron (III), the RRS intensity of C30 solution is decreased. With increasing iron (III) concentration, the RRS intensity is continually decreased. As shown in Fig. 5B, the RRS intensity difference (ΔRRS) at 302.5 nm is first increased sharply and then tends to balance (ΔRRS = I₀ − I. I and I₀ represent the RRS intensities of C30 with and without the addition of iron (III), respectively.). From the inset in Fig. 5B, the RRS intensity difference exhibits a good linear relationship with 5 – 50 μM iron (III) (R² = 0.99). Based on 3Sb/slope (Sb represents the standard deviation of the background signals), the limit of detection (LOD) of iron (III) was calculated to be 0.9 μM. The obtained LOD is much lower than the safety limit 0.036 mM according to the “Guidelines for Drinking-Water Quality of World Health Organization (WHO)”. The sensitivity of the proposed method is compared with some
reported methods and the data are listed in Table 1. The performance of C30 probe is comparable with these methods. In addition, six repeated trials of 50 μM iron (III) were operated, and the relative standard deviation (RSD) was obtained as 2.59%, suggesting good dependability of the method. The C30 probe used for iron (III) detection is label-free, and the method is very sensitive and selective. The detection process for iron (III) is simple, rapid, and can be completed in 15 min.

**Application in real water samples**

As the RRS signal can be affected by the potential interferents, it remains a challenge for iron (III) detection in complicated real samples. To make sure the method can be applied to real samples, tap water and Jialing River water samples were investigated. As shown in Table 2, the amount of iron (III) is detected in blank water samples. After spiked with iron (III) standard solution, the water samples show decreased RRS signal and the results are coincident with the spiked values. The recoveries of iron (III) in the water samples are from 91.5 to 106.0%. The relative standard deviations (RSD) of five repetitive assays are calculated as 3.1 – 4.9%, suggesting acceptable repeatability of the method. The results indicate great potential of the RRS sensor for iron (III) detection in real samples.

**Conclusions**

In this work, RRS properties of different amphoteric molecules were investigated, including four proteins and two DNA sequences. The strong RRS intensity was observed near their pI values. With a rapid response rate and easy operation procedure, the RRS technique can be a potential tool for pI monitoring. By utilizing C30 as a label-free probe, iron (III) cation determination was realized. The iron (III) sensing method is highly sensitive and selective, exhibiting great potential applications in real environmental samples.

**Acknowledgements**

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References

Table 1: Comparison of different methods for iron (III) detection.

<table>
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<tr>
<th>Method</th>
<th>System</th>
<th>Linear range/μM</th>
<th>LOD/μM</th>
<th>Interference</th>
<th>Refs.</th>
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<tr>
<td>Electrochemistry</td>
<td>MOF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1 – 1.2</td>
<td>0.1</td>
<td>Cr&lt;sup&gt;3+&lt;/sup&gt;</td>
<td>30</td>
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<tr>
<td>Electrochemistry</td>
<td>Fe (III)–RD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15 – 350</td>
<td>3.3</td>
<td>/</td>
<td>31</td>
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<tr>
<td>Fluorescence</td>
<td>Gold Nanoclusters</td>
<td>5.0 – 1280</td>
<td>3.5</td>
<td>/</td>
<td>32</td>
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<tr>
<td>Fluorescence</td>
<td>Polyphenyl derivative</td>
<td>4 – 20</td>
<td>4.0</td>
<td>/</td>
<td>33</td>
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<tr>
<td>Fluorescence</td>
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<td>Hg&lt;sup&gt;2+&lt;/sup&gt;</td>
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<tr>
<td>Colorimetry</td>
<td>PProDOTCB-salt&lt;sup&gt;c&lt;/sup&gt;</td>
<td>47 – 84</td>
<td>23.0</td>
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<td>29</td>
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<td>Colorimetry</td>
<td>AgNPs&lt;sup&gt;d&lt;/sup&gt;</td>
<td>/</td>
<td>125.0</td>
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<td>RRS</td>
<td>Label-free C30</td>
<td>5 – 50</td>
<td>0.9</td>
<td>/</td>
<td>This work</td>
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<sup>a</sup> Metal-organic anion framework. <sup>b</sup> RD: Rhodamine dimer.
<sup>c</sup> Poly(3,4-propylenedioxythiophene) derivative. <sup>d</sup> Silver nanoparticles.

Table 2: Iron (III) detection in several water samples using the developed sensor (n = 5).

<table>
<thead>
<tr>
<th>Sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Concentration/μM</th>
<th>Recovery, %</th>
<th>RSD, %</th>
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<td>Added</td>
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<td>Tap water</td>
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<td></td>
<td>50.0</td>
<td>106.0</td>
<td>3.1</td>
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<tr>
<td></td>
<td>0</td>
<td>12.5</td>
<td>/</td>
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<tr>
<td>Jialing River water</td>
<td>20.0</td>
<td>91.5</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>93.8</td>
<td>4.6</td>
</tr>
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<sup>a</sup> The tap water was collected from our lab. <sup>b</sup> ND, not detected.
Figure captions

Fig. 1 The schematic diagram of RRS method for pI and iron (III) determinations.

Fig. 2 RRS intensities at 310 nm (A) and spectra (B) of albumin at different pH values (curves a-m: pH 3.0, 3.5, 4.0, 4.2, 4.5, 4.8, 5.0, 5.3, 5.5, 5.8, 6.0, 7.0, and 8.0). RRS intensities at 310 nm (C) and spectra (D) of haemoglobin at different pH values (curves a-i: pH 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, and 10). RRS intensities at 310 nm (E) and spectra (F) of urease at different pH values (curves a-m: pH 2.5, 3.0, 3.5, 4.0, 4.5, 4.8, 5.0, 5.2, 5.5, 6.0, 6.5, 7.0, and 8.0). RRS intensities at 310 nm (G) and spectra (H) of casein at different pH values (curves a-i: pH 4.0, 4.5, 4.8, 5.0, 5.2, 5.5, and 6.0). Conditions: albumin (0.2 mg/mL), haemoglobin (0.02 mg/mL), urease (0.01 mg/mL), casein (0.06 mg/mL), BR buffer (pH 3.0 – 10).

Fig. 3 RRS intensities at 302.5 nm (A) and spectra (B) of C30 at different pH values (curves a-g: pH 2.0, 2.5, 2.7, 3.0, 3.2, 3.5, and 4.0). RRS intensities at 290 nm (C) and spectra (D) of A15 at different pH values (curves a-i: pH 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.8, and 3.0). Conditions: C30 (0.8 μM), A15 (1.1 μM), BR buffer (pH 2.0 – 4.0).

Fig. 4 (A) The RRS spectra of C30 (curve a), C30 and Fe$^{3+}$ (curve b), and Fe$^{3+}$ (curve c) solutions. (B) The RRS spectra of A15 (curve a), A15 and 25 μM Fe$^{3+}$ (curve b), and A15 and 50 μM Fe$^{3+}$ (curve c) solutions. Conditions: A15 (1.1 μM), BR buffer (pH 2.5). (C) The RRS intensity difference of C30 at 302.5 nm with addition of different cations. Conditions: C30 (0.8 μM), BR buffer (pH 3.0). The concentrations of both iron (III) and other cations are 50 μM.

Fig. 5 (A) RRS spectra of C30 with different concentrations of iron (III) cation (from top to bottom: 0 – 300 μM) added. (B) The relationship between RRS intensity difference at 302.5 nm of C30 and iron (III) concentration from 5 to 300 μM. The inset is the linear range for iron (III) in the 5 – 50 μM range. Conditions: C30 (0.8 μM), BR buffer (pH 3.0).
Fig. 1 The schematic diagram of RRS method for pH and iron (III) determinations.
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