A New “Turn-On” Fluorescence Probe for Al\(^{3+}\) Detection and Application Exploring in Living Cell and Real Samples

Zhi-Yan Gao \(^1\), Cui-Jiao Zhang \(^1\), Xian Zhang \(^1\)\(^*\), Shu Xing \(^2\), Jin-Shui Yao \(^1\), Cong-De Qiao \(^1\) and Wei-Liang Liu \(^1\)\(^\odot\)

\(^1\) School of Materials Science and Engineering, Qilu University of Technology, Shandong Academy of Sciences, Jinan 250353, China; zhiyangao98@126.com (Z.-Y.G.); 13256729106@163.com (C.-J.Z.); yaojsh@qlu.edu.cn (J.-S.Y.); cdqiao@qlu.edu.cn (C.-D.Q.); wlliu@sdu.edu.cn (W.-L.L.)

\(^2\) School of Chemistry and Pharmaceutical Engineering, Qilu University of Technology, Shandong Academy of Sciences, Jinan 250353, China; shuxingcareer@hotmail.com

* Correspondence: zhangx@qlu.edu.cn; Tel.: +86-531-89631-227

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Abstract: An excess of Al\(^{3+}\) will lead to biological disorders and even many diseases. Therefore, detecting the levels of Al\(^{3+}\) in the human body has drawn great attention for health monitoring. The fluorescence method has been broadly applied because of high sensitivity, real-time detection, and intracellular imaging. In this work, a new probe with “turn-on” fluorescence based on Schiff base derivative, 3,6-imine-triphenylamine-(9-ethyl) carbazole (ITEC), has been successfully synthesized and studied. The high selectivity and sensitivity of ITEC to Al\(^{3+}\) were verified by fluorescence spectra and the detection limit was 2.19 nmol/L. A 1:2 stoichiometry of ITEC-Al\(^{3+}\) was obtained by the \(^1\)H NMR spectra and Job’s plot. Furthermore, ITEC was successfully applied to the detection of Al\(^{3+}\) with different concentrations in living HeLa cells. The analog experiments about nature contamination of Al\(^{3+}\) in cells and real samples were finished.

Keywords: Al\(^{3+}\); Schiff base derivative; fluorescent probe; imaging; real samples

1. Introduction

Recently, the development and applications of multifarious fluorescence probes for metal ions detection in living organisms and the environment have drawn considerable attentions [1–5]. For all we know, aluminum is the richest metallic element on earth [6]. During cell growth and living body survival, aluminum is not a necessary element. However, it is used broadly in our daily life, such as food ingredients [7], medicines [8], water treatment [9], and the huge manufacturing industry [10], which lead to many aluminum ions discharged in our soil, water, and air [11,12]. Thus, the excess deposition of Al\(^{3+}\) in the human body from the food cycle may disturb the immune system and nervous system, and lead to numerous irreversible diseases including cerebral disease [13], Alzheimer’s disease [14], and Parkinson’s disease [15]. The World Health Organization (WHO) suggested that the daily intake of Al\(^{3+}\) should be approximate 3–10 mg per person [16,17]. In China, the National Food Safety Standard sets the aluminum content not to exceed 100 mg/kg in food and food additives (China GB 29924-2013). In view of the above, it is necessary to detect Al\(^{3+}\) in organisms and the environment [18]. Ion selective electrodes [19], voltammetry [20], and atomic absorption spectroscopy [21] are generally employed to detect metal ions. Nevertheless, these methods commonly have some disadvantages, such as being time-consuming, expensive, needing skilled operations, and having tedious synthetic processes [22]. However, the fluorescence method has attracted much interest in environmental chemistry, biology, and clinical science owing to its high sensitivity and easy operational procedure [16,23].
Currently, Schiff bases compounds are vastly recognized as fluorescence probes for metal ions with the high sensitivity, good selectivity, and high excitation coefficients, but a lot of them displayed fluorescence “on-off” behavior [24–26], which is a disadvantage for the visualization study. In recent years, a few “off-on” fluorescence probes have been developed, but the probes applied to detect Al\(^{3+}\) in living cells and real samples were scarce [27–30]. Hence, it is very meaningful to develop “off-on” probes for Al\(^{3+}\) monitoring in real application [31].

In this manuscript, we reported a feasible method to synthesize a novel Schiff base, 3,6-imine-triphenylamine-(9-ethyl) carbazole (ITEC), as a “turn-on” fluorescence probe for selective detection of Al\(^{3+}\). The ITEC was further applied to detect Al\(^{3+}\) in living cells. In addition, the experiments of Al\(^{3+}\) detection in the real sample have been carried out.

2. Experimental

2.1. Materials and Instrumentation

Carbazole, triphenylamine, and Palladium/C catalysts were purchased from Aladdin. All other experimental materials were derived from the local commercial suppliers. All materials were not further purified. \(^1\)H NMR spectra were measured on a Bruker Avance 400 spectrometer. The electrospray ionization (ESI) mass spectra were recorded using an Agilent-6510-Q-TOF spectrometer. A UV-3600 spectrophotometer and a Thermo Scientific Nicolet IS10 Spectrometer were used to measure the UV-visible absorption spectra and Infrared spectra. Fluorescence spectra were obtained using a Hitachi F-7000 Fluorescence Spectrophotometer. The fluorescence images in living cells were obtained by an Olympus FV300 confocal microscope.

2.2. Synthesis of ITEC

Intermediates M1-M4 were synthesized according to the literature reports [32,33] and the detailed procedures were described in supporting information. The synthesis of 3,6-imine-triphenylamine-(9-ethyl) carbazole (ITEC) is shown in Scheme 1.

![Scheme 1. The synthesis route of 3,6-imine-triphenylamine-(9-ethyl) carbazole (ITEC).](image-url)
2.3. The Synthesis Procedure of ITEC

3-(Diphenyl amino) benzaldehyde (M1) (1.99 g, 11.5 mmol) and 3,6-diamino-9-ethyl-carbazole (1.1 g, 5 mmol) (M4) were dissolved in methanol (15 mL), and a drop of acetic acid was added to the above mixture. The mixture was reacted at 70 °C for 48 hours, then filtered and washed three times with ethanol. The brown solid was dried under vacuum. Yield: 65.6%. 1H NMR (400 MHz, DMSO) δ ppm: 8.68 (s, 2 H), 8.16 (s, 2 H), 7.8 (d, J = 8 Hz, 4 H), 7.6 (d, J = 8 Hz 2 H), 7.41 (m, 10 H), 7.13 (m, 12 H), 7(d, J = 8 Hz, 4 H), 4.45 (d, J = 8 Hz, 2 H), 1.32 (t, J = 6 Hz, 3 H). IR: (KBr, cm⁻¹): 2970 (N-H), 1687 (-CH=N), 1585 (C=C). Elemental analyses of C52H41N5 (%): calcd for C, 84.90; H, 5.58; N, 9.52, found C, 84.53; H, 5.46; N, 9.39. EI-MS (M⁺): calcd for 736.75, found 735.92.

2.4. Absorption and Fluorescence Studies

The stock solutions (2 × 10⁻² mol/L) of the nitrate salts of Cu²⁺, Co²⁺, Pb²⁺, Cd²⁺, Ag⁺, Zn²⁺ Al³⁺, Hg²⁺, Ca²⁺, Na⁺, Fe³⁺, and Ni²⁺ were prepared in ultrapure water. The stock solution of ITEC (1 × 10⁻² mol/L) was prepared in ethanol. Test sample solutions will be diluted to 4 × 10⁻⁵ mol/L for nitrate salts and 2 × 10⁻⁵ mol/L for ITEC before using. The fluorescence spectra of ITEC were excited at 365 nm.

2.5. General Processes for Cell Culture and Fluorescence Imaging

HeLa cells were cultured in H-DMEM at 37 °C in humidified air and 5% CO₂. The cells were fully incubated with ITEC or ITEC-Al³⁺ solution for 20 min at 37 °C, and then washed three times with phosphate buffered saline (PBS) to remove extracellular ITEC and Al³⁺. Continuously cultured cells with 7.4 umol/L Al³⁺ daily for 3 days and followed by normal culture for 5 days to mimic bioaccumulation. Finally, confocal imaging was carried out (λ_ex = 405 nm, λ_em = 400–550 nm).

3. Results and Discussion

3.1. The Colorimetric Detection of ITEC to Al³⁺ by Naked Eyes

The observing method by the naked eyes is the convenient and inexpensive way of detecting metal ions. ITEC could successfully detect Al³⁺ by the naked eyes. In Figure 1a, the obviously blue fluorescence was found in the presence of Al³⁺ at the excitation wavelength of 365 nm when ITEC (20 μmol/L) solutions in ethanol were added various metal ions (40 μmol/L) including Co²⁺, Cu²⁺, Pb²⁺, Cd²⁺, Ag⁺, Zn²⁺ Al³⁺, Hg²⁺, Ca²⁺, Na⁺, Fe³⁺, and Ni²⁺. Moreover, the fluorescence intensities of ITEC solutions were gradually strengthened with the increasing concentrations of Al³⁺ in the range from 1 × 10⁻⁶ mol/L to 4 × 10⁻⁵ mol/L (Figure 1b). The limiting concentration (7.41 umol/L) of Al³⁺ is just in the range according to WHO regulations on drinking water, which indicates that it is possible to determine the level of Al³⁺ by naked eye observation [34].

![Figure 1](image-url)

**Figure 1.** (a) Color changes of 3,6-imine-triphenylamine-(9-ethyl) carbazole (ITEC) with various cations under 365 nm UV-light in ethyl alcohol. [ITEC] = 20 μM, [cations] = 40 μM. (b) Color changes of ITEC with different Al³⁺ concentrations (0 μM, 5 μM, 10 μM, 15 μM, 20 μM, 25 μM, 40 μM) under 365 nm UV-light, [ITEC] = 20 μM.
3.2. Selectivity of ITEC for Metal Ions

In view of the above easy way that ITEC response to Al$^{3+}$, a further optical investigation was carried out to evaluate potential application of ITEC for Al$^{3+}$ detection. The absorption and fluorescence spectra of ITEC was investigated after adding different metal ions (Al$^{3+}$, Ag$^{+}$, Ca$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Fe$^{3+}$, Hg$^{2+}$, Na$^{+}$, Ni$^{2+}$, Pb$^{2+}$, Zn$^{2+}$). In Figure 2a, a new absorption band at 475 nm was found after adding Cu$^{2+}$, Fe$^{3+}$, Hg$^{2+}$, and Al$^{3+}$. In Figure 2b, compared with other metal ions, the fluorescence of ITEC exhibited remarkable enhancements in the presence of Al$^{3+}$. Under the excitation wavelength of 365 nm, though the fluorescence of ITEC was enhanced in the presence of Fe$^{3+}$, the fluorescent intensity was far lower than that in the presence of Al$^{3+}$. Moreover, the two spectral shapes were absolutely difference. The possible reason for the enhanced fluorescence was the blocking of the photo-induced electron transfer process in the presence of Al$^{3+}$ [35,36].

The selective bars of ITEC to various metal ions were shown in Figure 2c. Here, F and F$_0$ are the fluorescent intensity of ITEC at 431 nm.

The fluorescence titration experiments were carried out by adding the various concentrations of Al$^{3+}$ (Figure 3a). With the increased concentrations of Al$^{3+}$ in the range from 2 μmol/L to 10 μmol/L, the linear fitting curve with fluorescence intensity of ITEC was plotted in Figure 3b. The concentration

![Figure 2](image-url).

3.3. Sensitivity of ITEC to Al$^{3+}$

The fluorescence titration experiments were carried out by adding the various concentrations of Al$^{3+}$ (Figure 3a). With the increased concentrations of Al$^{3+}$ in the range from 2 μmol/L to 10 μmol/L, the linear fitting curve with fluorescence intensity of ITEC was plotted in Figure 3b. The concentration
of Al\(^{3+}\) (7.41 \text{ umol/L}) is within this linear range according to WHO regulations on drinking water [34]. According to the basis of 3 \(\sigma/k\), the detection limit of ITEC was calculated to be \(2.19 \times 10^{-9}\) \text{ mol/L} (\(k = 8.861 \times 10^8\), \(\sigma = 0.647\)) \([38,39]\), which was lower than those of many reported fluorescence probes for Al\(^{3+}\) (Table 1).

**Figure 3.** (a) Change in fluorescence spectra of ITEC (10 \(\mu\text{M}\)) after adding Al\(^{3+}\) (0 \(\mu\text{M}\), 0.6 \(\mu\text{M}\), 0.8 \(\mu\text{M}\), 2.0 \(\mu\text{M}\), 2.4 \(\mu\text{M}\), 3.0 \(\mu\text{M}\), 4.4 \(\mu\text{M}\), 5.0 \(\mu\text{M}\), 6.4 \(\mu\text{M}\), 7.0 \(\mu\text{M}\), 9.0 \(\mu\text{M}\), 10.0 \(\mu\text{M}\), and 14.0 \(\mu\text{M}\), respectively) with an excitation at 365 nm. (b) The curve was plotted with the fluorescence intensity of ITEC in 460 nm versus Al\(^{3+}\) concentrations (2.4 \(\mu\text{M}\) –10 \(\mu\text{M}\)).

**Table 1.** Comparison of probes structure for Al\(^{3+}\) detection by fluorescence method.

<table>
<thead>
<tr>
<th>Structure of Probes</th>
<th>Detection Limit (mol/L)</th>
<th>Modes (probe:Al(^{3+}))</th>
<th>Application</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Structure 1" /></td>
<td>1.0 (\times 10^{-7})</td>
<td>1:1</td>
<td>-</td>
<td>[40]</td>
</tr>
<tr>
<td><img src="image2" alt="Structure 2" /></td>
<td>4.79 (\times 10^{-8})</td>
<td>1:1</td>
<td>-</td>
<td>[41]</td>
</tr>
<tr>
<td><img src="image3" alt="Structure 3" /></td>
<td>1.0 (\times 10^{-7})</td>
<td>1:1</td>
<td>-</td>
<td>[42]</td>
</tr>
<tr>
<td><img src="image4" alt="Structure 4" /></td>
<td>7.4 (\times 10^{-9})</td>
<td>1:1</td>
<td>Real samples</td>
<td>[43]</td>
</tr>
<tr>
<td>Tyrosine-stabilized fluorescent gold nanoclusters</td>
<td>3.0 (\times 10^{-7})</td>
<td>-</td>
<td>Real samples</td>
<td>[44]</td>
</tr>
<tr>
<td>Salicylaldehyde modified MOF (UiO-66-NH2-SA)</td>
<td>6.98 (\times 10^{-6})</td>
<td>-</td>
<td>-</td>
<td>[45]</td>
</tr>
<tr>
<td>Nitrogen-doped graphene quantum dot</td>
<td>1.3 (\times 10^{-6})</td>
<td>1:1</td>
<td>Cell imaging</td>
<td>[46]</td>
</tr>
<tr>
<td><img src="image5" alt="Structure 5" /></td>
<td>7.4 (\times 10^{-9})</td>
<td>1:2</td>
<td>Cell imaging</td>
<td>This work</td>
</tr>
</tbody>
</table>
3.4. The Interaction Mechanism of ITEC-Al$^{3+}$

A Job's plot was drawn according to our previous method to obtain the complex ratio of ITEC-Al$^{3+}$ [47]. From Figure 4a, the fluorescent spectrum vertex reached the mole fraction of 0.7, which indicated a 1:2 stoichiometry between ITEC and Al$^{3+}$ [48,49]. To further investigate interaction mechanism, the $^1$H NMR spectra of ITEC and ITEC-Al$^{3+}$ were measured and showed in Figure 4b. The integral intensities of peaks from a to c at 7.71 ppm improve continually with the increase of Al$^{3+}$ concentrations, and the same phenomena is also found at 6.87, which indicates that the proton signal of HC=N will change with the addition of Al$^{3+}$. The coordination of Al$^{3+}$ with the N on amide bond can availably reduce their electron-donating ability, so the photoinduced electron transfer (PET) process from the N to the triphenylamine is suppressed, which leads to a large increase of fluorescence intensity. Based on the above studying, a possible sensing mechanism of ITEC toward Al$^{3+}$ has been proposed as shown in Figure 4c. At the same time, there was obvious blue fluorescence after adding Al$^{3+}$. In addition, a 2:1 stoichiometry between ITEC and Fe$^{3+}$ was found. The binding mode of ITEC and Fe$^{3+}$ was explored and detailed in Figure S6–S8 are supporting information.

![Figure 4](image)

**Figure 4.** (a) The Job’s plot for ITEC-Al$^{3+}$ complex, [ITEC] + [Al$^{3+}$] = 10 μM; (b) $^1$H NMR (400 MHz) spectra of ITEC, ITEC with Al$^{3+}$ (1.0 equiv.), and ITEC with Al$^{3+}$ (2.0 equiv.) in DMSO; (c) The proposed binding mechanism of ITEC-Al$^{3+}$ and the color change of ITEC solution (20 μM) after adding Al$^{3+}$ (40 μM).

3.5. Fluorescence Imaging in Cells

The application of ITEC for the detection of Al$^{3+}$ in HeLa cells was studied. The cells were incubated with 5 μmol/L of ITEC and 0 μmol/L, 2 μmol/L, and 5 μmol/L Al$^{3+}$ solution, respectively, in PBS (2% DMSO, pH = 7.4) for 20 min at 37 °C, then washed three times with PBS to remove the excess ITEC and Al$^{3+}$. As depicted in Figure 5, no obvious fluorescence was observed in the cells incubated only with ITEC. However, the fluorescence was founded in Figure 5 (2a–3a) after adding Al$^{3+}$. Moreover, it displayed enhanced fluorescence with the increased concentrations of Al$^{3+}$ (2 μmol/L, 5 μmol/L) in cells. Therefore, the results in cells indicated that ITEC could be used as an effective Al$^{3+}$ imaging agent.
As we all know, Al\(^{3+}\) is a non-essential element of the human body. It will accumulate in the body from water or food containing Al\(^{3+}\). Considering the complicated process, we mimic the phenomenon of bioaccumulation and performed cell imaging (Figure 6). The cells were cultured with 7.4 \(\mu\)mol/L Al\(^{3+}\) daily for 3 days and followed by normal culture for 5 days to achieve natural concentration, then incubated with ITEC solution (5 \(\mu\)mol/L) for 20 min. The cell has been successfully imaged, which demonstrates the applicability of the technique.

![Fluorescence microscope images of ITEC (5 \(\mu\)M) in living HeLa cells with different concentrations of Al\(^{3+}\) (0 \(\mu\)M, 2 \(\mu\)M, and 5 \(\mu\)M, respectively).](image1)

**Figure 5.** Fluorescence microscope images of ITEC (5 \(\mu\)M) in living HeLa cells with different concentrations of Al\(^{3+}\) (0 \(\mu\)M, 2 \(\mu\)M, and 5 \(\mu\)M, respectively).

![Image of cell cultured with 7.4 \(\mu\)M Al\(^{3+}\) daily for 3 days followed by normal culture for 5 days, then incubated with 5 \(\mu\)M ITEC for 20 min.](image2)

**Figure 6.** The cell cultured with 7.4 \(\mu\)M Al\(^{3+}\) daily for 3 days followed by normal culture for 5 days, then incubated with 5 \(\mu\)M ITEC for 20 min.

### 3.6. The Test of the ITEC in Real Sample

The lake water and tap water samples were gained from the Changqing Lake (Jinan, China) and the Qilu University of Technology, respectively. The bottled water samples were purchased from the local supermarket. The water samples were centrifuged and filtrated, and then added to Al\(^{3+}\) solutions with the concentrations of 3 \(\mu\)mol/L, 5 \(\mu\)mol/L, and 10 \(\mu\)mol/L, respectively. The proposed method was used to detect Al\(^{3+}\) in real samples. The results in Table 2 showed the recoveries were in the range...
from 97.6% to 109.3% with lower relative standard deviation (RSD < 1%), which clearly indicates the reliability and accuracy of the proposed method.

Table 2. Determinations of Al\(^{3+}\) in real samples \((n = 3)\).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added (µmol/L)</th>
<th>Measured (µmol/L)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>River water 1</td>
<td>3</td>
<td>2.99</td>
<td>99.7</td>
<td>0.40</td>
</tr>
<tr>
<td>River water 2</td>
<td>5</td>
<td>5.27</td>
<td>105.4</td>
<td>0.48</td>
</tr>
<tr>
<td>River water 3</td>
<td>10</td>
<td>10.3</td>
<td>103.3</td>
<td>0.60</td>
</tr>
<tr>
<td>Tap water 1</td>
<td>3</td>
<td>3.17</td>
<td>105.5</td>
<td>0.50</td>
</tr>
<tr>
<td>Tap water 2</td>
<td>5</td>
<td>5.25</td>
<td>104.9</td>
<td>0.12</td>
</tr>
<tr>
<td>Tap water 3</td>
<td>10</td>
<td>9.76</td>
<td>97.6</td>
<td>0.36</td>
</tr>
<tr>
<td>Bottle water 1</td>
<td>3</td>
<td>2.99</td>
<td>99.8</td>
<td>0.88</td>
</tr>
<tr>
<td>Bottle water 2</td>
<td>5</td>
<td>5.46</td>
<td>109.3</td>
<td>0.23</td>
</tr>
<tr>
<td>Bottle water 3</td>
<td>10</td>
<td>9.88</td>
<td>98.8</td>
<td>0.53</td>
</tr>
</tbody>
</table>

RSD: relative standard deviation.

4. Conclusions

In a word, we developed a new Schiff base derivative, which has good selectivity and sensitivity towards Al\(^{3+}\). The detection limit was 2.19 nmol/L. The fluorescence titration tests and the Job’s plot elucidated that a stoichiometric ratio of 1:2 for ITEC-Al\(^{3+}\) was calculated. Furthermore, ITEC can be successfully regarded as a fluorescence “turn-on” probe for the detection of Al\(^{3+}\) in cells imaging, which supplied the potential application of ITEC to study the effect of Al\(^{3+}\) in biological systems. In addition, ITEC was highly efficient for the monitoring of Al\(^{3+}\) in real samples.

Supplementary Materials: The following are available online at [http://www.mdpi.com/2076-3417/9/3/577/s1](http://www.mdpi.com/2076-3417/9/3/577/s1), Figure S1: The \(^1\)H NMR spectrum of intermediate M1 in DMSO; Figure S2: The \(^1\)H NMR spectrum of intermediate M4 in DMSO; Figure S3: The \(^1\)H NMR spectrum of ITEC in DMSO; Figure S4: Mass spectrum of ITEC; Figure S5: The FTIR spectrum of ITEC; Figure S6: The Job’s plot for determining the stoichiometry between ITEC and Fe\(^{3+}\); Figure S7: \(^1\)H NMR (400 MHz) spectra of ITEC and ITEC with Fe\(^{3+}\) (1.0 equiv.); Figure S8: The proposed binding mechanism of ITEC with Fe\(^{3+}\).

Author Contributions: Conceptualization, X.Z. and J.-S.Y.; Data Curation, C.-D.Z. and S.X.; Formal Analysis, X.Z. and J.-S.Y.; Writing—Original Draft Preparation, Z.-Y.G.; Visualization, W.-L.L.; Funding Acquisition, X.Z.

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Conflicts of Interest: The authors declare no conflict of interest.

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