A novel fluorescence sensor based on covalent immobilization of 3-amino-9-ethylcarbazole by using silver nanoparticles as bridges and carriers

Shu-Zhen Tan\textsuperscript{a,}, Yan-Jun Hu\textsuperscript{b}, Fu-Chun Gong\textsuperscript{a}, Zhong Cao\textsuperscript{a}, Jiao-Yun Xia\textsuperscript{a}, Ling Zhang\textsuperscript{a}

\textsuperscript{a} College of Chemistry and Biological engineering, Changsha University of Science and Technology, Changsha, 410004, China
\textsuperscript{b} Institute of Quality Supervision and Inspection of Commodities Made in Human Province, Changsha, 410007, China

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\textbf{A B S T R A C T}

A novel technique of covalent immobilization of indicator dyes in the preparation of fluorescence sensors is developed. Silver nanoparticles are used as bridges and carriers for anchoring indicator dyes. 3-amino-9-ethylcarbazole (AEC) was employed as an example of indicator dyes with terminal amino groups and covalently immobilized onto the outmost surface of a quartz glass slide. First, the glass slide was functionalized by (3-mercaptopropyl)trimethoxysilane (MPS) to form a thiol-terminated self-assembled monolayer, where silver nanoparticles were strongly bound to the surface through covalent bonding. Then, 16-mercaptohexadecanoic acid (MHDA) was self-assembled to bring carboxylic groups onto the surface of silver nanoparticles. A further activation by using 1-\text{[3-dimethylaminopropyl]}\text{-}3-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) converted the carboxylic groups into succinimide esters. Finally, the active succinimide esters on the surface of silver nanoparticles were reacted with AEC. Thus, AEC was covalently bound to the glass slide and an AEC-immobilized sensor was obtained. The sensor exhibited very satisfactory reproducibility and reversibility, rapid response and no dye-leaching. Rutin can quench the fluorescence intensity of the sensor and be measured by using the sensor. The linear response of the sensor to rutin covers the range from 2.0 $\times$ 10\textsuperscript{-6} to 1.5 $\times$ 10\textsuperscript{-4} mol L\textsuperscript{-1} with a detection limit of 8.0 $\times$ 10\textsuperscript{-7} mol L\textsuperscript{-1}. The proposed technique may be feasible to the covalent immobilization of other dyes with primary amino groups.

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1. Introduction

Research on optical sensors has blossomed during the past two decades as a result of ever-increasing need for them in a wide range of applications. All of these owe to their advantages of easy operation, low cost, and high selectivity. Immobilization techniques of indicator dyes have significant effects on the performance of the optical sensors in terms of sensitivity and stability. The indicator dyes can be physically [1–10] and chemically [11–19] immobilized onto the support matrices. Physical techniques are very simple ways to immobilize indicator dyes and can give high yields at low cost, the durability of the sensing layers fabricated thereby is relatively poor since the indicator dyes can be easily leached out of the matrices. Covalent immobilization of indicator dyes has attracted research interest since it can prevent dyes from leaching out of the matrices, and results in an enhanced lifetime. The main technique of covalent immobilization reported was UV photopolymerization [16–19], the obtained sensors showed no dye-leaching, but it was difficult to control the thickness of the sensing films in the preparing process of sensors, and sometimes it was not easy to restore the fluorescence intensity of the sensor to the blank value by simply rinsing after the sensor was used. The reason is that the indicator dyes or dye-analyte complexes are distributed in the total sensing layer rather than on its outmost surface. To solve these problems, a novel technique of bonding indicator dyes was developed.

Perry and co-workers have reported a self-assembled layer of chromophores on a metal nanoparticle core, which allows for packing of ~2500 chromophores within a sphere of <10 nm diameter, they believe that the use of nanoparticles as carriers for large numbers of chromophores is promising and may have an impact on ultrasensitive detection [20]. In our previous work, gold nanoparticles were used as bridges and carriers for anchoring indicator dyes, the obtained sensors showed no dye-leaching, rapid response, good reproducibility and reversibility [21]. Compared with gold nanoparticles, silver nanoparticles are cheaper. In our present work, silver nanoparticles were employed as bridges and carriers to anchor indicator dyes while they were bound to the surface of a quartz glass slide via self-assembly. Self-assembly has become a popular surface...
the derivatization procedure, mostly owing to its simplicity, versatility, and the establishment of a high level of order on a molecular scale as a mean of preparing modified surfaces. The high organization and homogeneity, together with its molecular dimension, make it very attractive for tailoring surfaces with desired property. In our strategy for fabricating sensors, the technologies of covalent immobilization and self-assembly were combined together. 3-amino-9-ethylcarbazole (AEC) was chosen as an example of indicator dyes and covalently immobilized on the outermost surface of the quartz slide. An AEC-immobilized sensor was obtained, and had some advantages such as rapid response, no dye-leaching, good reproducibility and reversibility. The fluorescence intensity of the sensor can be quenched by rutin. Therefore, the sensor was employed as a detection device for rutin, showing a linear response to rutin in the range from $2.0 \times 10^{-6}$ to $1.5 \times 10^{-4}$ mol L$^{-1}$ with a detect limit of $8.0 \times 10^{-7}$ mol L$^{-1}$.

2. Experimental

2.1. Apparatus

Fluorescence measurements were performed with a Hitachi F-4500 Fluorescence Spectrometer. The reversibility and reproducibility of the sensor were carried out with a PerkinElmer LS-55 Luminescence Spectrometer. All pH measurements of buffer solutions were performed with a PHS-3C pH meter (Shanghai Analytical Instruments, Shanghai, China).

2.2. Reagents

(3-Mercaptopropyl) trimethoxysilane (MPS) and 3-amino-9-ethylcarbazole (AEC) were purchased from Acros Organics (Sweden). 16-mercaptohexadecanoic acid (MHDA) was obtained from Sigma. N-hydroxysuccinimide (NHS) was acquired from Aldrich and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was provided by Alfa Aesar. 3-mercaptopropionic acid was purchased from Fluka. K$_2$Cr$_2$O$_7$, AgNO$_3$, NaBH$_4$, KI, rutin and all other materials were purchased from Changsha Chemical Reagents (Changsha, China). B-R buffer solutions with different pH were prepared by mixing appropriate amounts of 0.2 mol L$^{-1}$ HOAc–H$_3$PO$_4$–H$_3$BO$_3$ with 0.2 mol L$^{-1}$ sodium hydroxide to the desired pH. All reagents were used as received without further purification. Doubly distilled water was used throughout. A stock solution of rutin ($1.0 \times 10^{-3}$ mol L$^{-1}$) was prepared by dissolving rutin in methanol. Working solutions of rutin with different concentrations were prepared by serial dilution of the stock solution with Britton-Robinson (B-R, pH 5.2) buffers.

2.3. Preparation of the silver colloid

Preparation of the silver colloid in solution was performed by using the procedure reported by Li et al. with minor modifications [22]. A total of 2.5 mL of 10$^{-2}$ mol L$^{-1}$ AgNO$_3$ was added to 75 mL...
of distilled water. A total of 5 mL of 10^{-2} \text{mol L}^{-1} mercaptoacetic acid was added as a stabilizer to the solution under stirring. After 10 min of mixing, 2.5 mL of 10^{-2} \text{mol L}^{-1} KI was dropped into the solution slowly, yielding a green–yellow AgI colloid. A total of 20 mg of NaBH₄ was added to the AgI colloidal solution, and the reaction mixture was continually stirred for about 20 min. The silver colloid was finally obtained. During the whole reaction, the color of the colloidal solution changed from green–yellow to nut-brown at the beginning, then to brown, and finally to orange.

2.4. Preparation of the AEC-immobilized sensor

The overall procedure is schematically illustrated in Fig. 1, and described in detail as follows: (1) Cleaning of a quartz glass slide. The slide was soaked in freshly prepared piranha solution (3:1 \text{H}_{2}\text{SO}_{4}:30\% \text{H}_{2}\text{O}_{2}) at 70 \degree C for 20 min to remove organic impurities, then rinsed thoroughly with water and dried in a stream of dry nitrogen gas. (2) Silanization of the slide. The cleaned slide was submerged in a 2% ethanolic solution of MPS for 2 h. Then, the slide was removed and rinsed successively with water and ethanol to remove unbound materials from the surface. This treatment causes a monolayer of MPS to be bonded to the surface of the slide. Finally, the slide was dried in a stream of dry nitrogen gas. A MPS-coated slide was acquired. (3) Attachment of silver nanoparticles to the slide. Silanization of MPS was adopted in order to introduce thiol groups onto the surface of the slide, then silver nanoparticles were linked to the slide via self-assembly. There are reports that fluorescence of fluorophores would be quenched by silver nanoparticles if they were in close proximity [23,24]. To solve this problem, we designed to put a long spacer between silver nanoparticle and indicator dye. Therefore, self-assembly of MHDA on the surface of silver nanoparticles. The silver-linked slide was soaked in 10 mM MHDA methanol solution for 3 h, then rinsed with methanol. Thus, a MHDA-capped slide was formed. (5) Activation of carboxylic groups. The MHDA-capped slide was submerged in a mixture of 50 mM NHS and 200 mM EDC (1:1, volume ratio) for 1 h. Unreacted NHS/EDC was removed from the slide by repetitively rinsing with methanol and water, then the slide was dried in a stream of dry nitrogen gas. (6) Covalent bonding of AEC. The slide obtained from the previous step was soaked in the AEC solution in acetonitrile (1 mg mL^{-1}) for 3–4 h, followed by repetitively rinsing with acetone and water to remove the unbound materials. By above-mentioned treatments, AEC was covalently immobilized onto the outmost surface of the slide, an AEC-immobilized sensor was obtained.

2.5. Fluorescence measurements

Fluorescence intensities of the sensor were measured at 386 nm. The slits were set at 10 and 5 nm for excitation and emission, respectively, and a maximum excitation wavelength of 245 nm was used. The sensor was placed in a quartz cuvette filled with rutin solutions.

Rutin solutions with various concentrations were kept in pH 5.2 B-R buffer solution. In order to recover the fluorescence intensity of the sensor, after each measurement, a blank pH 5.2 B-R buffer solution was used to wash the sensor till the blank fluorescence reading is recovered.

3. Results and discussion

3.1. Preparation of the fluorescence sensor

In the present investigation, silver nanoparticles were chosen as bridges and carriers for anchoring indicator dyes onto the quartz glass slide. It was necessary that silver nanoparticles were attached to the slide firstly. Silanization of MPS was adopted in order to introduce thiol groups onto the surface of the slide, then silver nanoparticles were linked to the slide via self-assembly. There are reports that fluorescence of fluorophores would be quenched by silver nanoparticles if they were in close proximity [23,24]. To solve this problem, we designed to put a long spacer between silver nanoparticle and indicator dye. Therefore, self-assembly of MHDA was employed to bring long spacer –(CH₂)₁₅– and carboxylic groups onto the surface of the silver nanoparticles. After this, the carboxylic terminal amino group to be anchored covalently. We selected AEC as an example of indicator dyes, the AEC-immobilized sensor was fabricated.
3.2. Effect of pH on fluorescence intensity of the AEC-immobilized sensor

Effect of pH was investigated by measuring fluorescence intensity of the sensor when the sensor was contacted with different pH buffers containing 1.0 × 10^{-5} mol L^{-1} rutin. Results showed that fluorescence intensity of the sensor was nearly independent of pH in the range from 3 to 7. In subsequent experiments, a B-R buffer with pH 5.2 was used.

3.3. Dye-leaching test of the AEC-immobilized sensor

Firstly, the AEC-immobilized sensor was placed in a B-R buffer with pH 5.2, its fluorescence intensity was measured immediately. Then, the sensor was soaked for 72 h at room temperature. After this, the sensor was taken out, rinsed with water and dried, followed by measuring its fluorescence intensity in a fresh B-R buffer (pH 5.2) again. Experimental results in Fig. 2 indicate that there are no measurable dye to be leaked into the solution. This is anticipated because the covalent immobilization of the indicator dye is very firm, efficiently preventing the leakage of the dye from the support matrix and resulting in a significantly enhanced lifetime.

3.4. Reproducibility, reversibility and response time of the AEC-immobilized sensor

The reproducibility and reversibility were evaluated by alternately exposing the sensor into 1.0 × 10^{-5} mol L^{-1} rutin solution and blank B-R buffer solution (pH 5.2). Fig. 3 exhibited that the reproducibility and reversibility of the sensor were very satisfactory. The mean fluorescence intensity values with the standard deviation were found to be 822.83 ± 82.82 (n = 5, in the blank B-R buffer solution) and 629.09 ± 2.99 (n = 5, in 1.0 × 10^{-5} mol L^{-1} rutin solution). When the AEC-immobilized sensor was contacted with rutin solutions with different concentrations, its fluorescence intensities reached stable values less than 10 s. The response time is really short. This is anticipated because the indicator dyes are covalently immobilized onto the outmost layer of the support matrix rather than in the whole of the support matrix, and the diffusion limitation of the dyes into the support matrix is overcome. Also, it was found that the proposed sensor could be restored easily by simply rinsing with water or blank buffer after it was used in rutin solutions with different concentrations.

3.5. Response spectra, linear response range, detection limit of the AEC-immobilized sensor to rutin

The fluorescence excitation and emission spectra of the sensor in serial pH 5.2 B-R suffer solutions containing rutin were recorded in Fig. 4. The excitation spectra were obtained by fixing the maximum emission wavelength at 386 nm. The emission spectra were obtained by fixing the maximum excitation wavelength at 245 nm. The fluorescence intensity of the proposed sensor was quenched by rutin, which made it possible to assay rutin by using this sensor. Measuring principle of the sensor is similar to that reported elsewhere [25]. The relative fluorescence intensity (α) is defined as

\[
\alpha = \frac{[\text{AEC}] - [\text{AEC}]_t}{[\text{AEC}]_t} = \frac{F_0}{F}
\]

Here, [AEC] and [AEC]_t are the concentration of free AEC and the total concentrations of AEC in the phase of sensing film, respectively. F and F_0 are fluorescence intensity of the AEC-immobilized sensor in presence and absence of rutin (R), respectively. Theoretical curves of α versus log[R] were plotted in Fig. 5. These curves were predicated by adjusting a complexing ratio (m:n) and equilibrium constant (K). It can be seen that the curve corresponding to a 1:1 complexing ratio and K = 5 × 10^4 gives the best fit to the experimental data (Fig. 5, curve c). Therefore, a 1:1 complex was formed between AEC and rutin. Fig. 6 is the plot of experimental values of F_0/F versus [R], showing that a linear relationship between F_0/F and [R] exists in the range of 2.0 × 10^{-6} to 1.5 × 10^{-4} mol L^{-1} with following regression equation:

\[
F_0 = 4.017 + 4.485 \times 10^4 [R] \quad (r = 0.9955)
\]

Eq. (2) provides the quantitative basis for the determination of rutin. The detection limit, defined as the concentration of rutin when the signal change is three-times the standard deviation (n = 12) of the blank buffer signal, was found to be 8.0 × 10^{-7} mol L^{-1}.

3.6. Recovery experiments

The validity of the proposed sensor was examined by recovery experiments. The results in Table 1 showed that the recoveries were in the range of 97.8–105.0%. It seems that the AEC-immobilized sensor is feasible for the determination of rutin.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added (mol L⁻¹)</th>
<th>Found (mol L⁻¹)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.0 × 10⁻⁶</td>
<td>(4.899 ± 0.999) × 10⁻⁶</td>
<td>97.8</td>
</tr>
<tr>
<td>2</td>
<td>1.0 × 10⁻⁵</td>
<td>(1.059 ± 0.739) × 10⁻⁵</td>
<td>105.0</td>
</tr>
<tr>
<td>3</td>
<td>2.0 × 10⁻⁵</td>
<td>(2.079 ± 0.609) × 10⁻⁵</td>
<td>103.5</td>
</tr>
</tbody>
</table>

a. Mean values from three determinations.

b. Standard deviation.

### Table 2

Interference of different materials to the determination of rutin.

<table>
<thead>
<tr>
<th>Interferent</th>
<th>Relative fluorescence intensity change a (F₂−F₁) × 100 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinchonine</td>
<td>2.2</td>
</tr>
<tr>
<td>Vitamin B₁</td>
<td>−4.6</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>−2.6</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>−1.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>−0.8</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.6</td>
</tr>
<tr>
<td>Glycine</td>
<td>−1.0</td>
</tr>
<tr>
<td>KCl</td>
<td>0.6</td>
</tr>
<tr>
<td>KI</td>
<td>−0.8</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>−4.1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>−0.6</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>−1.0</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.1</td>
</tr>
</tbody>
</table>

a. F₂ and F₁ are fluorescence intensity of the AEC-immobilized sensor exposed to 1.0 × 10⁻⁵ mol L⁻¹ rutin in a pH 5.2 B-R buffer with and without an interferent, respectively.
3.7. Selectivity of the AEC-immobilized sensor

Some ions present in biological samples and several medicines were chosen as interferents to study the selectivity of the AEC-immobilized sensor. To pH 5.2 B-R buffer containing rutin \(1.0 \times 10^{-5} \text{ mol L}^{-1}\), an interferent \(1.0 \times 10^{-3} \text{ mol L}^{-1}\) was added, fluorescence intensity of the AEC-immobilized sensor was recorded before and after the interferent was added. The results in Table 2 indicated that the changes in fluorescence intensity arising from the interferents were less than 5.0%, which could be recognized to be tolerable.

4. Conclusion

A novel method for fabricating fluorescence sensors based on covalent immobilization of indicator dyes was investigated. Silver nanoparticles were employed as bridges and carriers for anchoring indicator dyes. The main procedure includes silanization of the quartz glass slide, attachment of silver nanoparticles, self-assembly of MHDA, activation of the carboxylic groups by using EDC and NHS, and bonding of indicator dyes. The prepared sensor has some advantages such as very satisfactory reproducibility and reversibility, rapid response, no dye-leaching. These were attributed to the fact that indicator dyes were covalently immobilized on the outermost layer of the support matrix, and the diffusion limitation of the dyes into the support matrix was overcome. AEC was chosen as an example of indicator dyes, and the AEC-immobilized sensor was acquired. This sensor was employed to assay rutin, showing a linear response to rutin in the range from \(2.0 \times 10^{-6}\) to \(1.5 \times 10^{-4} \text{ mol L}^{-1}\) with a detect limit of \(8.0 \times 10^{-7} \text{ mol L}^{-1}\).

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