GOLD NANOCLUSTER BASED FLUORESCENCE SENSOR FOR NOREPINEPHRINE

8.1 Introduction
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8.3 Results and discussion
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This chapter describes the application of protein stabilized gold nanoclusters (AuNCs) as the fluorescent probe for the determination of norepinephrine (NE). The aqueous solution of the bovine serum albumin protected AuNCs was deep brown in color under visible light and exhibited bright-red fluorescence under UV irradiation. Upon addition of NE, the fluorescence emission intensity of AuNCs increased remarkably, which can be attributed to the restricted intramolecular rotation resulting from the aggregation of AuNCs. Under optimum conditions, the relative fluorescence intensity, \( I/I_0 \) showed a linear relationship with the concentration of NE in the range \( 4.76 \times 10^{-7} \text{ M} \) to \( 4.98 \times 10^{-8} \text{ M} \), and the limit of detection was found to be \( 8.55 \times 10^{-9} \text{ M} \). The proposed method was applied for the determination of NE in urine sample and the results were compared with HPLC method.
8.1 Introduction

Norepinephrine (NE) otherwise known as noradrenaline (Figure 8.1), is one of the most important catecholamine neurotransmitter in the mammalian central nervous system, whose structure is closely related to dopamine. It is the major chemical messenger of the sympathetic nervous system. Many automatic functions of the body, such as heart rate and blood pressure are controlled by the sympathetic nervous system. NE is an endogenous hormone secreted by the adrenal medulla, and relinquished as a neurotransmitter from nerve endings in the sympathetic nervous system and some areas of the cerebral cortex.\textsuperscript{351}

![Figure 8.1: Structure of NE](image)

NE has crucial role in muscle and tissue control. It decreases peripheral circulation, stimulates arteriole contraction and activates lipolysis in adipose tissue.\textsuperscript{352} The low level of NE is related with mental depression, DNA breakage in cardiac myoblast cells, heart failure, and diabetes. Recent studies revealed that abnormal level of NE enhances adhesion of human immune deficiency virus-1 (HIV-1)-infected leukocytes to cardiac microvascular endothelial cells and also accelerates HIV replication via protein kinase.\textsuperscript{353,354} NE is one of the stimulants that have been prohibited in 2005 by World Anti-Doping Agency.\textsuperscript{355} Hence the development of a sensitive and convenient method for quantitative determination of NE in biological fluids is of great significance.
in clinical medicine, since it provides useful information on physiological functions and the diagnosis of some diseases.

Various analytical methods such as spectrophotometry, capillary electrophoresis, gas chromatography, ion chromatography, high-performance liquid chromatography (HPLC) and voltammetric methods have been employed for the determination of NE. Nevertheless, most of these techniques have several inconveniences. Since NE being an electroactive compound, the commonly used method for its determination is voltammetric techniques. But the main problem related to voltammetric method is the interference from the associated compounds such as uric acid and ascorbic acid, which usually results in overlapping of signals due to their very similar oxidation peak potentials. The requirement of sample treatment with ion pair reagents makes GC-MS method tedious and time consuming. HPLC based methods have several draw backs since they normally require a previous extraction and purification of the sample. Fluorescence has proved to be a more powerful optical technique for the detection of low concentration of analytes, owing to its simplicity and excellent selectivity.

Recent advances in nanotechnology have given rise to a new class of fluorescent materials namely metal nanoclusters (NCs), which consist of several to hundred atoms and exhibit molecule like properties. As the density of states is insufficient to merge the valence and conduction bands, NCs show molecule like transitions. These materials emerged as a hot area of research since they provide the missing link between atomic and nanoparticle behaviour in metals. They exhibit remarkable properties including the discrete electronic states and size-dependent fluorescence.
Among the various metal NCs, AuNCs have attracted significant attention in sensing and imaging applications due to their biocompatibility and strong fluorescence.\textsuperscript{370} Fluorescent AuNCs possess special features such as strong photoluminescence, good photostability, high emission rates, large stokes shift and size-dependent tunable fluorescence.\textsuperscript{371,372} Compared to other fluorophores such as semiconductor nanoparticles, carbon nanodots and dye doped nanoparticles, AuNCs have many advantages like simplicity in preparation, good water solubility, biocompatibility and low toxicity.\textsuperscript{373}

The surface modification of AuNCs endows a number of new applications for them like targeting, imaging and therapeutic effects. The stabilizing or capping ligand has a profound role on the photoluminescence of AuNCs.\textsuperscript{374,375} In recent years, many proteins such as bovine serum albumin (BSA), human serum albumin (HSA), lysozyme, trypsin, pepsin and insulin have been used as protecting agents for the preparation of fluorescent AuNCs, since they contain active sites for metal ion accumulation and reduction.\textsuperscript{145,146,376-378}

AuNCs based fluorescence sensor has been designed for monitoring of NE in body fluids, since it can be used as a powerful indicator for an early warning sign of certain diseases. Fluorescent AuNCs were synthesized using BSA as reducing and capping agent\textsuperscript{110} and employed as a novel platform for simple and sensitive determination of NE. The fluorescence intensity of AuNCs increased with increasing concentration of NE and the limit of detection was found to be $8.55 \times 10^{-9}$ M. The mechanism of fluorescence enhancement of AuNCs by the addition of NE is discussed. Application study of the developed sensor has been carried out in urine sample and results are validated with HPLC method.
8.2 Experimental

8.2.1 Synthesis of BSA stabilized AuNCs

Highly fluorescent BSA protected AuNCs were synthesized by the method explained in section 6.2.1 in chapter 6.

8.2.2 Sample preparation

A stock solution of NE (1.00 \times 10^{-2} \text{ M}) was prepared by dissolving 0.169 g NE in 10 mL water and desired concentrations were prepared by the serial dilution of stock solution using water.

8.2.3 Analytical procedure

AuNCs@BSA solution was mixed with phosphate buffer solution of pH 7 in the volume ratio 1:4. Appropriate amount of NE solution was added to 2 mL of the above solution, and the fluorescence emission spectrum was recorded. The luminescence intensity of AuNCs@BSA before and after the addition of NE was assigned as \( I_0 \) and \( I \) respectively.

8.3 Results and discussion

8.3.1 Characterization of AuNCs

The synthesized BSA protected AuNCs were characterized by UV-Vis spectroscopy, fluorescence spectroscopy, FTIR spectroscopy and TEM as explained in chapter 6.

8.3.2 Fluorescence enhancement of AuNCs by NE

Upon excitation at 400 nm, the photoluminescence spectrum of AuNCs@BSA showed an emission maximum at 653 nm. The fluorescence
intensity of AuNCs were strongly enhanced upon the addition of NE (Figure 8.2), and the enhancement of fluorescence intensity at 653 nm was proportional to the concentration of NE. Emission spectra of AuNCs@BSA displayed a slightly red shifted emission peak at 666 nm instead of 653 nm in the presence of NE. The observed red shift in the fluorescence emission wavelength of AuNCs is about 13 nm, which indicate the changes in the surface states of AuNCs@BSA arising from the interaction of NE molecules. Based on the fluorescence enhancement of AuNCs by NE, a novel turn on sensor was developed for the quantification of this neurotransmitter.

![Fluorescence spectra of AuNCs before and after the addition of NE](image)

**Figure 8.2: Fluorescence spectra of AuNCs before and after the addition of NE**

### 8.3.3 Effect of buffer solutions

NE induced fluorescence enhancement of AuNCs was studied in various types of buffer solutions, such as phosphate buffer, acetate buffer and citrate buffer solutions. Maximum relative fluorescence intensity, \(I/I_0\) for NE-AuNCs system was observed in phosphate buffer solution, indicating that phosphate buffer solution was preferable for the interaction between NE.
and BSA stabilized AuNCs. Hence, phosphate buffer solution (PBS) was chosen for the subsequent studies.

In addition, the influence of the volume of buffer solution on the fluorescence intensity of NE-AuNC system was also examined. Five different volume ratios were studied and the maximum \( I/I_0 \) value was obtained when the ratio of AuNCs and PBS was 1:4 (Figure 8.3).

![Figure 8.3: Effect of volume of PBS on the fluorescence enhancement of AuNCs by NE](image)

**8.3.4 Effect of pH**

pH value of the system played a crucial role in the sensing of NE by AuNCs. In order to study the effect of pH on the relative fluorescence intensity of NE-AuNCs system, PBS with different pH values (2 to 9) were employed (Figure 8.4a). Maximum \( I/I_0 \) value was obtained at pH 7 (Figure 8.4b) and it was selected as optimal pH for further studies.
8.3.5 Effect of Time

To investigate the kinetic stability of the developed assay, fluorescence intensity of AuNCs before and after the addition of different concentrations
of NE was recorded at different time intervals (Figure 8.5). It was observed that the interaction between NE and AuNCs was rapid and fluorescence intensity reached maximum immediately after mixing. The emission intensity remains almost constant at least for about 35 min indicating the high stability of the assay.

![Figure 8.5: Effect of time on the fluorescence intensity of (a) AuNCs (b) AuNCs + 1.48 × 10^{-7} M NE (c) AuNCs + 4.31 × 10^{-7} M NE](image)

8.3.6 Concentration study

The luminescence intensity of AuNCs increased gradually with increasing concentrations of NE (Figure 8.6a). The relative fluorescence intensity, \(I/I_0\) was plotted as a function of concentration of NE (Figure 8.6b). A good linear relationship between \(I/I_0\) and concentration of NE was obtained over the range of 4.76 × 10^{-7} M to 4.98 × 10^{-8} M, with a detection limit of 8.55 × 10^{-9} M. The concentration dependence of relative fluorescence intensity follows the equation \(I/I_0 = 2.539C (\mu M) + 1.854\), \(R^2 = 0.977\).
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Figure 8.6a: Fluorescence spectra of AuNCs in the presence of various concentrations of NE

Figure 8.6b: Calibration curve for NE in the concentration range $4.76 \times 10^{-7} - 4.97 \times 10^{-8} \text{M}$

A comparison between other determination methods of NE and proposed method is shown in Table 8.1. As listed in table, the proposed assay is found to be superior in terms of lower limit of detection to that of the other quantification methods for NE. To study the precision of the developed
method, a series of 6 repetitive measurements were taken, and a RSD of 2.7 % was obtained, suggesting the good repeatability of the proposed method.

8.3.7 Mechanism

The luminescence observed in the AuNCs is attributed to the excitation of electrons into the $sp$-conduction band and subsequent radiative electron-hole recombination process. Gold colloids are a highly dispersed multiphase system with a distinct interfacial region which has an affinity to adsorb other substances to decrease the interfacial energy.

To study the interaction between NE and AuNCs@BSA UV-Vis absorption spectra and fluorescence spectra of nanoclusters were recorded before and after the addition of NE. Upon the addition of NE, the absorbance of AuNCs@BSA was decreased significantly (Figure 8.7) suggesting the surface state changes of AuNCs@BSA. Fluorescence spectra of AuNCs@BSA displayed a slightly red shifted emission peak at 666 nm instead of 653 nm in the presence of NE. The red shift and the enhancement of intensity of fluorescence emission peak of AuNCs by NE may be due to the interaction of NE either to the capping molecule (BSA) or the AuNCs core which led to the aggregation of AuNCs. The electron rich amine groups of NE have a strong charge transfer interaction with the aromatic nucleus of BSA on the surface of AuNCs. Also it was reported that electron-rich atoms (O, N) or functional groups can effectively enhance fluorescence via surface interactions. NE contains electron rich –NH$_2$ and -OH groups which can interact with AuNCs, thereby enhancing of luminescence intensity of BSA protected AuNCs.
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Such type of interactions decrease the interparticle distance between nanoclusters and causes aggregation. From TEM analysis it was confirmed that aggregation occurred for nanoclusters after the addition of NE (Figure 8.8).

Figure 8.7: UV-Vis absorption spectra of AuNCs in the presence of different concentrations of NE

Figure 8.8: TEM image of AuNCs after the addition of NE
Aggregation induced emission enhancement (AIEE) have attracted more attention since 2001 when Tang's group first reported that the luminescence of silose molecules was stronger in the aggregate state than that in the non-aggregated form. Restriction of intramolecular rotation and the prohibition of non radiative deactivation are the major causes of AIE effect.

Due to the large diameter of the AuNCs-NE aggregates, their vibration and rotation speed may decrease. The Brownian motion and collision probability also may be weakened which result in an increase of external energy transfer rate. When large diameter AuNCs-NE aggregates are formed, self-absorption decreased considerably, due to the increasing degree of conjugation (Figure 8.7). Because of the physical constraint in the aggregates, intramolecular rotations are restricted and new radiative decay channels are opened, that is electron-hole recombination increases. The above reasons might have led to the enhancement of fluorescence intensity of AuNCs.

FTIR spectra of AuNCs and AuNCs in the presence of NE are shown in Figure 8.9. From Figure 8.9 it is clear that in the presence of NE, there is a shift in the –OH stretching vibration frequency accompanied by an increase in intensity, which indicates improved H bonding in the presence of NE, resulting in the aggregation of AuNCs.
Selectivity

In order to study the selectivity of the protein stabilized fluorescent probe towards NE, the emission intensity of AuNCs@BSA was measured in the presence of $4.76 \times 10^{-7} \text{ M}$ concentration of biologically important substances such as NE, epinephrine, creatinine, 3,4-dihydroxy phenylalanine, uric acid and ascorbic acid. As shown in Figure 8.10a only NE induced a dramatic increase in the fluorescence intensity of AuNCs, whereas no obvious fluorescence changes were observed in the presence of other species. This revealed the selectivity of the AuNC based fluorescent probe towards the determination of NE.
Figure 8.10a: Selectivity of the sensor: Fluorescence emission changes of AuNCs in the presence of other biologically important substances.

Figure 8.10b: Fluorescence behaviour of AuNCs in the presence of $4.76 \times 10^{-7}$ M NE with the coexistence of other substances at 100-fold excess concentrations.
Additionally, the fluorescence intensity of AuNCs was measured in the presence of NE \((4.31 \times 10^{-7} \text{ M})\) and various concentrations (1:1, 1:10 and 1:100) of the above mentioned foreign species. All the compounds tested except epinephrine did not interfere the determination of NE, and they can be tolerated up to 100 fold molar excess concentrations (Figure 8.10b). Epinephrine, when present at a concentration greater than 40 fold of NE, a signal change above 5% was observed and interfere the determination of NE severely (Table 8.2).

8.3.9 Application study

The practical application of the developed assay for the analysis of NE was tested by spiking the urine sample with a known amount of standard NE solutions and determining its recovery. The results were summarized in Table 8.3. Recoveries were obtained from 99% to 102%, which showed the reliability of the present method. A good agreement was observed between proposed method and HPLC method,\(^{351}\) indicating that the present method can be used for the determination of NE in real samples. These results demonstrated that this novel sensing system has great potential for quantitative analysis of NE levels in urine samples.

8.4 Conclusions

A simple of fluorescent platform was developed for the sensing of NE based on the fluorescence enhancement of BSA templated AuNCs. Hydrogen bonding induced aggregation and the resulting restricted rotation of AuNCs was responsible for the observed fluorescence enhancement of nanoclusters in the presence of NE. Clear evidence for the aggregation of
AuNCs by the addition of NE was obtained from HRTEM analysis. The AuNCs based sensing system exhibited a very low limit of detection as low as $8.55 \times 10^{-9}$ M. The developed turn on fluorescence sensor is simple, sensitive and was applied for the real sample analysis with satisfactory results.

Table 8.1: Comparison with other determination methods for NE

<table>
<thead>
<tr>
<th>Method</th>
<th>Linear range (M)</th>
<th>LOD (M)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectrophotometry</td>
<td>$1.18 \times 10^{-4}$ to $5.91 \times 10^{-6}$</td>
<td>-----------</td>
<td>355</td>
</tr>
<tr>
<td>Capillary electrophoresis</td>
<td>$1.00 \times 10^{-4}$ to $1.00 \times 10^{-6}$</td>
<td>$0.85 \times 10^{-6}$</td>
<td>356</td>
</tr>
<tr>
<td>Ion chromatography</td>
<td>$0.295 \times 10^{-4}$ to $5.91 \times 10^{-8}$</td>
<td>$5.91 \times 10^{-9}$</td>
<td>358</td>
</tr>
<tr>
<td>HPLC-MS</td>
<td>$2.36 \times 10^{-5}$ to $5.91 \times 10^{-6}$</td>
<td>$5.67 \times 10^{-8}$</td>
<td>359</td>
</tr>
<tr>
<td>Voltammetry</td>
<td>$7.55 \times 10^{-6}$ to $8.49 \times 10^{-7}$</td>
<td>$5.91 \times 10^{-8}$</td>
<td>360</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>$4.76 \times 10^{-7}$ to $4.98 \times 10^{-8}$</td>
<td>$8.55 \times 10^{-9}$</td>
<td>Proposed method</td>
</tr>
</tbody>
</table>

Table 8.2: Effect of foreign species on the determination of NE ($4.31 \times 10^{-7}$ M)

<table>
<thead>
<tr>
<th>Foreign Species</th>
<th>Concentration (M)</th>
<th>Signal change %</th>
</tr>
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<tbody>
<tr>
<td>L-DOPA</td>
<td>$4.31 \times 10^{-5}$</td>
<td>4.23</td>
</tr>
<tr>
<td>Catechol</td>
<td>$4.31 \times 10^{-5}$</td>
<td>3.22</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>$4.31 \times 10^{-5}$</td>
<td>3.12</td>
</tr>
<tr>
<td>Uric acid</td>
<td>$4.31 \times 10^{-5}$</td>
<td>3.45</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>$1.72 \times 10^{-5}$</td>
<td>4.53</td>
</tr>
<tr>
<td>Creatinine</td>
<td>$4.31 \times 10^{-5}$</td>
<td>4.32</td>
</tr>
</tbody>
</table>

L-DOPA: 3,4-dihydroxyphenylalanine
### Table 8.3: Determination of NE in urine sample

<table>
<thead>
<tr>
<th>Added (M)</th>
<th>Proposed method</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found (M) *</td>
<td>RSD</td>
<td>Recovery %</td>
<td>Found (M) *</td>
</tr>
<tr>
<td>9.90 \times 10^{-8}</td>
<td>9.91 \times 10^{-8}</td>
<td>0.07</td>
<td>100.1</td>
<td>9.99 \times 10^{-8}</td>
</tr>
<tr>
<td>2.44 \times 10^{-7}</td>
<td>2.47 \times 10^{-7}</td>
<td>0.09</td>
<td>101.2</td>
<td>2.38 \times 10^{-7}</td>
</tr>
<tr>
<td>4.31 \times 10^{-7}</td>
<td>4.29 \times 10^{-7}</td>
<td>0.03</td>
<td>99.5</td>
<td>4.36 \times 10^{-7}</td>
</tr>
</tbody>
</table>

*average of five replicate measurements*