Tannic Acid Capped Copper Nanoclusters as a Cost-Effective Fluorescence Probe for Hemoglobin Determination

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For the first time, we report on a copper nanoclusters based fluorescence sensor for hemoglobin (Hgb). The aggregation-induced quenching of tannic acid capped copper nanoclusters’ (TACuNCs) fluorescence by a Hgb-H2O2 mixture that mimics the Fenton’s reagent is used here for the selective determination of Hgb. It is possible to effectively determine Hgb using this sensitive and cost-effective sensor in the linear range of 5.0 × 10⁻⁵ to 4.0 × 10⁻⁴ M with a detection limit of 5.6 × 10⁻¹⁰ M. The practical utility of the sensor is evident from the good recovery values obtained from Hgb spiked with artificial blood serum.

Keywords Hemoglobin, tannic acid, copper nanoclusters, fenton’s reagent, fluorescence

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Introduction

Hemoglobin (Hgb) is a globular protein⁰ which acts as an oxygen transporter in vertebrates. The presence of four heme units in a single Hgb molecule is responsible for its efficient oxygen transport. The normal Hgb level in human males is 13.0 - 18.0 g/dL and in females is 12.0 - 16.0 g/dL. An abnormality in the concentration of Hgb in blood can cause serious health effects such as anaemia and polycythaemia. While anaemia is a condition due to a lack of enough red blood cells (RBC’s), polycythaemia is a kind of cancer where the bone marrow produces unusually high RBC’s. Both of these conditions can be diagnosed by effective monitoring of the Hgb level in blood. Thus a number of analytical methods such as liquid chromatography, electrochemistry, fluorimetry, colorimetry and spectrophotometry have been developed for the determination of Hgb. As most of these assays involve time-consuming procedures, expensive and hazardous precursors or tedious synthesis, the need for a facile and cost-effective method is always a necessity for Hgb determination.

Fluorimetry is a rapidly advancing and widely used analytical technique in the chemical sensor development of biological molecules owing to its simplicity and good sensitivity. Among the various fluorescent materials, metal nanoparticles are very much preferred in sensor development because of their ease of synthesis, robustness, tunable fluorescence, good biocompatibility and excellent photostability. Metal nanoclusters comprised of several to hundreds of atoms are in the forefront of fluorescent probes. Even though Au, Ag, Pt and Pd nanoparticles have been used in developing chemical sensors, Cu nanoparticles are the most favoured because of their very facile and cost-effective strategy of synthesis compared to the former ones. Various capping agents are employed for improving the stability and fluorescence property, which very much depends upon the morphology of these clusters. Polyphenols, like tannic acid, can easily passivate on copper nanoclusters with the aid of hydroxyl groups. They are also capable of forming intra and intermolecular hydrogen bonds, and thereby create supramolecular structures which can protect the nanoclusters from oxidation and aggregation. The literature clearly shows biological sensing platforms in which copper nanoclusters are utilized to determine neurotransmitters, vitamins, enzymes as well as in bioimaging.

Fenton’s reagent is a combination of iron (Fe²⁺) and hydrogen peroxide (H₂O₂) which can generate hydroxyl (HO•) and hydroperoxyl (HOO•) radicals that are very powerful oxidising agents. This capability of Fenton’s reagent has been extensively utilized for oxidative degradation, and thereby the removal of many organic pollutants, like tannic acid. Herein, we have adopted this reaction for the sensitive determination of Hgb using tannic acid capped copper nanoclusters (TACuNCs). An amplification in the quenching effect of H₂O₂ on the fluorescence of TACuNCs upon the addition Hgb is the basis of this sensor. To the best of our knowledge, no fluorescence sensor based on copper nanoclusters has been reported for Hgb.

Experimental

Chemicals

Tannic acid, hemoglobin, myoglobin, hemin, Human Serum Albumin (HSA) and L-cysteine were purchased from Sigma Aldrich, USA. Ascorbic acid, Zinc sulphate and calcium chloride were obtained from S. D. Fine Chem. Pvt. Ltd., India. Cholesterol, glucose and tyrosine were procured from SRL. Pvt. Ltd., India. Hydrogen peroxide, KCl and NaCl were from Merck lifescience Pvt. Ltd., India. Histidine was from Spectrochem. Pvt. Ltd., India and phenyl alanine and valine were obtained from HiMedia Laboratories Pvt. Ltd., India. CuSO₄·5H₂O was purchased from Spectrum Chemicals, India.
CoSO₄ was from Universal Laboratories, India and Rhodamine 6G was procured from Loba Chemie Pvt. Ltd., India.

**Instrumentation and characterization**

Fluorescence spectroscopic studies were carried out using a Fluoromax-4 spectrofluorometer of Horiba Scientific. UV-visible spectra and Fourier Transfer Infrared spectra (FTIR) were recorded using a Spectra-UV-Visible double-beam UVD-3500 and a JASCO-4100 FTIR spectrometer, respectively. Transmission electron microscopy (TEM) images were taken using JEM-2100 HRTEM and Zeta sizerNano ZS series Malvern instruments of Horiba Scientific were used for Dynamic Light Scattering (DLS) analysis.

**Synthesis of tannic acid capped copper nanoclusters**

Tannic acid-capped copper nanoclusters were synthesized as per the reported procedure. To 200 μL of a 1.0 × 10⁻³ M CuSO₄·5H₂O solution, 100 μL of a 1.0 × 10⁻³ M tannic acid solution was added and made up to 20 mL with deionized water. The mixture was then stirred at room temperature for 5 min. Then, 200 μL of a 1.0 M ascorbic acid solution was added to it and again stirred for 6 h at 50°C to obtain pale-yellow colored TACuNCs, which were stored in a refrigerator at 4°C before use.

**Analytical procedure**

After 40 μL of a TACuNCs solution and 5.0 × 10⁻³ M of a H₂O₂ solution were mixed, to it various concentrations of Hgb were added and made up to 2 mL. Each of these mixtures was incubated for 30 min, and then fluorescence spectra were recorded by exciting at 360 nm. The quenching of fluorescence was then measured as the relative fluorescence intensity (I/I₀), where I is the intensity of the probe in the presence of H₂O₂ and I₀ is that in the presence of both H₂O₂ and Hgb. The linear correlation between I/I₀ and the concentration of Hgb was then plotted.

Artificial blood serum was prepared as per the reported procedure. To the TACuNCs solution, 5.0 × 10⁻³ M H₂O₂ and the required amount of Hgb was added and diluted to 2 mL with deionized water after spiking with artificial blood serum. The fluorescence intensities were measured at a 360 nm excitation wavelength after 30 min of incubation, and then the recoveries were calculated.

**Results and Discussion**

**Characterization of synthesized TACuNCs**

The synthesized TACuNCs were characterized by TEM, DLS, FTIR, UV-Visible spectroscopy and fluorescence spectroscopy. TEM images showed that the copper nanoclusters were well dispersed, spherical in shape with an average diameter of 2.1 nm. The mean hydrodynamic diameter found from DLS was also 2.3 nm, which is in accordance with a report. (Fig. 1) The effective capping of tannic acid over the CuNCs was determined using FTIR spectroscopy. By comparing the FTIR spectrum (Fig. S1 (Supporting Information)) of TA and TACuNCs, it is clear that the characteristic peaks of tannic acid are present in the latter with a narrowing of the O-H stretching peak. This implies a decrease in the intermolecular hydrogen bonds among tannic acid molecules, since the hydroxyl groups are employed for passivation over CuNCs.

The UV-Visible spectrum of the TACuNCs shows two weak absorption peaks at 255 and 288 nm in accordance of a report. Furthermore the absence of any localized surface plasmon peaks corresponding to copper nanoparticles implies that only pure CuNCs are formed. The TACuNCs have a fluorescence excitation maxima and emission maxima (λmax) at 360 and 430 nm, respectively (Fig. S2 (Supporting Information)) with a quantum yield (QY) of 28.3%. The QY was measured using Rhodamine-6G as the standard.

**Optimization of experimental parameters**

Various experimental parameters such as the time of incubation and the concentration of H₂O₂, have been optimized for the best performance of the sensor. It was found that the relative fluorescence intensity of TACuNCs in presence of Hgb-H₂O₂ mixture remained constant only after about 30 min of incubation, which might be due to the involvement of Fenton’s reaction. The optimum concentration of H₂O₂, which gives the maximum quenching of the TACuNCs, was found to be 5.0 × 10⁻⁵ M (Fig. S3 (Supporting Information)). Hence, further studies were carried out under these conditions.

**Performance of the sensor**

**Effect of concentration**

It was found that both H₂O₂ and hemoglobin have a negligible quenching effect on TACuNCs individually, and it became amplified by more than 7 fold when a Hgb-H₂O₂ mixture was added (Fig. S4 (Supporting Information)). The fluorescence intensity decreased gradually with increasing concentration of the Hgb upto micromolar range.

Upon plotting the relative fluorescence intensity against the concentration of Hgb in the range of 5.0 × 10⁻⁸ to 4 × 10⁻⁶ M, a linear graph ((R² = 0.9971) which follows the Stern-Volmer equation (I/I₀ = Ksv [Q] + 1, Ksv – Stern-Volmer constant, [Q]– concentration of quencher) was obtained (Fig. 2). Furthermore,
the proposed sensor exhibits an excellent limit of detection of $5.6 \times 10^{-10}$ M, which was calculated by taking the ratio $3S/M$, where $S$ is the standard deviation of the lowest point and $M$ is the slope of the calibration plot.

A comparison of the proposed sensor with the existing sensors for Hgb (Table S1 (Supporting Information)) shows that the former has a comparable performance with them in terms of its sensitivity and selectivity. Moreover, the sensor is much more cost-effective than the other reported ones for the determination of Hgb.

Mechanism of quenching

The catalytic conversion of hydrogen peroxide by Fe$^{2+}$ ions into powerful oxidants, such as hydroxyl and hydroperoxyl free radicals, is known as Fenton’s reaction, and the combination $(\text{H}_2\text{O}_2 – \text{Fe}^{2+})$ as Fenton’s reagent:

$$\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{HO}^\bullet + \text{OH}^– + \text{Fe}^{3+},$$

$$\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{HOO}^\bullet + \text{H}^+ + \text{Fe}^{2+}.$$

It has been reported that the HO$^\bullet$ and HOO$^\bullet$ radicals formed by the photo Fenton’s reaction and the photodecomposition of H$_2$O$_2$ can be used for the degradation of tannic acid. In hemoglobin, iron is present in the +2 oxidation state, which in the presence of H$_2$O$_2$ can mimic the Fenton’s reagent and generate the radicals. When it is added to TACuNCs, tannic acid molecules which are capped over the CuNCs become oxidatively degraded to CO$_2$ and H$_2$O by the HO$^\bullet$ and HOO$^\bullet$ radicals.

Thus CuNCs loses their stability and become aggregated which results in its fluorescence quenching. This radical mechanism of quenching was confirmed based on the following evidence. In 2014, Cao et al. reported a fluorescence turn-off sensor for Fe$^{3+}$ ions based on TACuNCs, where $\lambda_{max}$ of the probe remained constant and the corresponding mechanism was the electron transfer from tannic acid towards Fe$^{3+}$ ions. But in the proposed sensor, a gradual red shift in $\lambda_{max}$ was observed (Fig. 2a) and a similar shift was also noticed for TACuNCs in the presence of ferrous ion-H$_2$O$_2$ and ferric ion-H$_2$O$_2$ mixtures (Fig. S4 (Supporting Information)). Since hydroxyl and hydroperoxyl radicals are the common entities present in these cases, it can be concluded that the quenching of fluorescence is not due to an electron-transfer mechanism, but may be due to the oxidative degradation of tannic acid by these radicals. This was further confirmed by adding ethanol as a radical scavenger, which decreased the quenching efficiency of a Hgb-H$_2$O$_2$ mixture (Fig. S5 (Supporting Information)) on
The aggregated nature of the probe in the presence of a Hgb-H₂O₂ mixture is clear from TEM images and DLS analysis shows an increase in the particle size caused by aggregation (Fig. 3). In addition to that, the lifetime of the probe (1.1 ns) remained constant upon the addition of Hgb-H₂O₂ mixture (1.0 ns). All of this evidence clearly suggests that the proposed sensor follows a static quenching mechanism.

**Selectivity of the sensor**

The selectivity of the sensor towards Hgb was assessed by comparing its response (quenching) towards other heme containing biomolecules, such as myoglobin and hemin. It was found that the quenching of TACuNCs by myoglobin and hemin was only 40 and 1.4%, respectively, whereas by the same concentration of Hgb it was 85% (Fig. 4), which points towards the remarkable selectivity of the sensor towards Hgb.

The effects of various coexisting species such as glucose, cholesterol, ascorbic acid, phenylalanine, valine, tyrosine, histidine, cysteine, Ca²⁺, Na⁺, K⁺ in the determination of Hgb were analyzed. It shows that the signal changes caused by glucose, histidine, valine, phenylalanine and Na⁺ were within the tolerance limit of 5%, even when present at a 100 fold excess concentration than Hgb. At the same time, tyrosine, cholesterol and K⁺ did not interfere in the determination of Hgb when present at a 10-fold excess concentration, but interfered at higher concentrations. However, ascorbic acid, cysteine and Ca²⁺ showed interference in the analysis at a 1:10 concentration ratio and HSA, Cu²⁺, Co²⁺ and Zn²⁺ showed interference at a 1:1 concentration ratio with Hgb (Table 1).

**Application studies**

The application of presently developed sensor has been investigated in artificial blood serum using spike recovery analysis. The results are given in Table 2. For five measurements in the spiked blood serum, a mean recovery of 101% and an RSD value of 4.4% were obtained. Comparable recovery and RSD values between the proposed and classic spectrophotometric method (Wu’s method) justify the practical utility of the proposed sensor in real sample analysis.

**Conclusions**

A turn-off fluorescence sensor for hemoglobin has been developed based on TACuNCs based on the reaction of Fenton’s reagent on tannic acid in which an amplified fluorescence quenching was achieved upon the addition of a Hgb-H₂O₂ mixture. The sensor is effective in the determination of Hgb in the linear range of 4.0 × 10⁻⁹ to 5.0 × 10⁻⁸ M with a limit of detection of 5.6 × 10⁻¹⁰ M. The mechanism behind the sensor has been clearly investigated. The practical utility of the proposed method has been analyzed in spiked artificial blood serum and good recovery values reveal the reliability of the sensor. The excellent sensitivity, selectivity and cost-effectiveness
of the sensor makes it a cut above the existing ones.

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Supporting Information

An additional table contains comparisons with other sensors and figures in the characterization of TACuNCs as well as evidence for the mechanism. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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