Chapter - 3

Calix Protected Gold Nanobeacon as turn-off Fluorescent Sensor for Phenylalanine
Resume

A new calix platform has been used as reducing as well as stabilizing agent for the synthesis of gold nanoparticles (AuNps). The synthesised CRTH-AuNPs were characterized and analyzed by UV-vis spectroscopy, transmission electron microscopy (TEM) and energy dispersive X-ray analysis (EDX). CRTH-AuNps were checked for their stability at different pH and temperature. CRTH-AuNps having characteristic surface plasmon resonance and being fluorescent in nature were explored for their interaction behavior with different amino acids by UV-visible and fluorescence spectroscopy. Among various amino acids CRTH-AuNps were found to be meticulously selective and sensitive for phenylalanine (PHE) by means of fluorescence quenching. This assay allowed rapid and accurate determination of PHE in aqueous medium at room temperature with a linear range of detection from 100 nM to 820 nM. Furthermore, the CRTH-AuNps were also used for successful determination of PHE in human serum providing a scope of detection and determination of PHE in biological samples.
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1. Introduction

Nanotechnology, inextricably a multidisciplinary field, has an explosive growth in the past two decades due to its large applications in various fields of nanoscale electronics [1], optics [2], magnetics [3], energy [4], catalysis [5], nanomedicines [6], clothing [7], cosmetics [8], etc [9]. Because of their size dependent optical and electrical properties, inorganic nanoparticles are promising building blocks for assembly into nanostructured functional materials [10]. Plasmonic (noble metal) nanoparticles distinguish themselves from other nanoplasmonic forms such as semiconductor quantum dots, magnetic and polymeric nanoparticles by their unique surface plasmon resonance (SPR) [11].

The surface plasmon resonance band of gold nanoparticles is explicative resultant of size, shape and composition of the nanoparticles. Owing to the unique optoelectronic properties with their controlled size and morphology, gold nanoparticles as a new architectures have a great potential in the field of bio-nanotechnology as biomarkers, biosensors (amino acids, DNA), cancer diagnostic and vehicles for drug delivery in biological processes at nanoscale [12-14].

A number of synthetic procedures for obtaining gold nanoparticles in polar and nonpolar media are available in literature [15]. These AuNps are generally prepared by NaBH₄ or citrate reduction of HAuCl₄ in the presence of a stabilizer (e.g., small organic molecules, polymers and bio-macromolecules) containing at least one –NH₂ or –SH group in a nonpolar medium [10]. The presence of byproduct of these reducing agents may create complications when used in some bio-analytical applications. So the development of synthetic protocols for realizing a single entity as both the reducing as well as the protecting agent for
water dispersible gold nanoparticles becomes requisite. The explicit properties of calixarenes are already pioneered by many researchers. In literature calixarene hydrazides are reported to provide good stability to the nanoparticle in comparison to other group of stabilizer [16-21] but the property of using hydrazides derivatives of calixarene as reducing as well as stabilizing for the synthesis of metal nanoparticles is a very recent phenomenon [22-24]. Calix[4]resorcinarene provides a congenial platform for bringing appropriate functionalization and hence acts as useful molecular framework [18]. Such systems are expected to yield interesting nanostructural features in interaction with the guest species. Hence calix[4]resorcinarene tetra hydrazide (CRTH) was chosen in the same context as reducing and stabilizing agent.

Amino acids (AA), the key constituents of proteins, are small molecules with various functional side chain groups, which results in different roles of amino acids in physiological processes. D,L-phenylalanine (Phe) methyl ester is used as the starting compound in the bio-catalytic synthesis of aspartame, an artificial sweetener 200 times as sweet a sugar [25]. PHE, an essential amino acid, is a precursor of the amino acid L-tyrosine [26] and a whole series of neurotransmitters and hormones. The analysis of PHE is of interest in the early detection of phenylketonuria in neonates [27]. Untreated Phenylketonuria(PKU) lowers the IQ and executive abilities in children [28]. To date, several analytical methods have been developed for the determination of PHE [29, 30]. The Guthrie method, a bacterial inhibition assay, is generally used for screening neonatal PKU. However, the method is a semi-quantitative test and has disadvantages of low sensitivity and poor precision [31]. Other methods have been reported for the determination of PHE including high performance liquid chromatography
(HPLC), gas chromatography–mass spectrometry, electrochemical biosensor modified electrode method and enzymatic methods [27], [32]. However the fluorimetric method for the determination of PHE in serum was developed by McCaman and Robins as early as 1962. Very interestingly, the “chemical nose approach” was introduced for detection of proteins by functionalized gold nanoparticles by Rotello et al. [33]. However, most of these methods require the use of organic solvents, complicated extraction processes, expensive instruments, strictly controlled reaction conditions and time-consuming operating steps. Therefore, a simple, rapid, highly sensitive and selective method was developed for the determination of PHE by spectrofluorimetry.

In this chapter the synthesis of calix protected AuNps by a simple one-pot process have been reported. Effective and selective interaction of PHE, with the AuNps was quantitatively studied among other amino acids (alanine, aminobutyric acid, arginine, aspartic acid, cysteine, dopamine, glutamic acid, glycine, histidine, isoleusine, leucine, methionine, serine, threonine, tyrosine, tryptophan and valine) and its application was attributed for sensing of PHE in human serum as real sample. The method applicability was validated by spiking known amount of PHE in human serum samples. The present approach is simple, highly selective and reproducible. Therefore, the proposed method has a great potential in diagnosis of disease associated with PHE.

2. **Materials and method**

2.1. **Reagents**

All metal salts including gold chloride trihydrate (H\text{AuCl}_3 \cdot 3\text{H}_2\text{O}) and human serum were purchased from Sigma-Aldrich. Amino acid kit was purchased from
SRL. Other reagents and solvents of analytical grade were purchased from commercial sources and used without further purification. All aqueous solutions were prepared from Millipore water (resistivity, 18 MΩ; Millipore Systems). TLC plates (F-2009) were obtained from the Merck. The pH was adjusted with NaOH or HCl. 2.0 mM. Stock solutions of amino acids were freshly prepared by dissolving their commercial crystals in deionized water and stocked at 20°C and their working solutions of were prepared by appropriate dilution of the stock solution.

2.2. Instruments

Inductively coupled plasma-atomic emission spectrophotometer (ICP-AES) JY 2000-2 model was used to check the absence of Au in centrifugate. Absorption spectra were recorded at room temperature on a Jasco V-570 UV-Vis spectrophotometer. Fluorescence spectra were recorded on Jasco FP-6500 spectrofluorometer. pH of the solutions was measured using pH analyzer Li 614-Elico. Samples were sonicated by using PCI 1.5L (H) sonicator. The Malvern Zetasizer (Model; ZEN3600) was used for the zeta potential measurements by laser Doppler electrophoresis as such without dilution. TEM images were recorded in MACK/model JEOL, JEM 2100 at an accelerated voltage of 200 kV. The TEM specimens were prepared by depositing a drop of dilute solution of an appropriate amount of gold nanoparticles in water on carbon coated copper grids and was dried in vacuum and directly observed under TEM.
3. Experimental


All glassware for preparation of gold nanoparticles were thoroughly cleaned with freshly prepared aqua regia, rinsed extensively with deionized water, and then dried in an oven for 2–3 h. The gold nanoparticles were prepared by the reported procedure in the literature [23]. In brief 50 mL (0.1 mM) solution of HAuCl₄ was added to a 250 mL of conical flask, then 50 mL (0.1 mM) aqueous solution of CRTH was added rapidly under vigorous stirring at maintained temperature (40°C) for 10 min. CRTH stabilized gold colloids (CRTH-AuNps) were obtained by vigorous stirring which was continued for 1 hr to ensure complete homogenization. The colour of the solution turned from yellow to ruby red which indicates the successful formation of gold nanoparticles (Scheme 1). This CRTH-AuNps solution was then subjected to centrifugation on REMI (R-8C) laboratory centrifuge at 14000 RPM. Absence of free gold ions and CRTH in supernatant liquid was ascertained by ICP-AES and UV-Visible spectrophotometry. The residue was redispersed in water to get 0.00098 % solution of CRTH-AuNps for further studies. The gold colloidal solution was stored in a refrigerator.

3.2. General procedure for the UV-visible and fluorescence measurements

Stock solution of the CRTH-AuNps and various amino acids were prepared in deionised water. 2.5 mL of CRTH-AuNps (0.00098%) and 2.5 mL (200 µM for UV-visible measurements and 2 µM for spectrofluorimetric measurements) of each
amino acids were taken in a 5 mL volumetric flask. The interaction behaviour of various amino acids with CRTH-AuNps was recorded on absorption spectrophotometer and spectrofluorimeter.

3.3. **Temperature, pH and time dependent stability study of CRTH-AuNps by UV-visible and fluorescence measurements**

It has been found that pH has a great effect on the stability of nanoparticles. Therefore, the stability of CRTH-AuNps was determined at different pH by measuring SPR and emission spectra. The pH of AuNps dispersion was adjusted using 0.1N hydrochloric acid and 0.1M sodium hydroxide solution (pH 4, 5, 6, 7, 8, 9 and 10) using calibrated pH meter. Also the change in SPR of the CRTH-AuNps was recorded up to 120 days using UV/Visible spectroscopy. The stability was also determined at different temperature (10-40°C) by measuring SPR.

4. **Results and Discussion**

4.1. **Synthesis and mechanism of formation of gold nanoparticles (CRTH-AuNps)**

Water soluble calix[4]resorcinarene tetrahydrazide (CRTH) which has been used to act as reducing as well as stabilizing agent functions in two ways, first the hydrazide group on periphery reduces the metal ions to metallic gold which further nucleates to form the nanoparticles. Second, the web type structure of the cyclic ligand (CRTH) restrains the growth of nanoparticles by engulfing it in the inherent hollow cavity so effectively that they do not aggregate and retain their size and stability for months together. Reduction of HAuCl₄ by calix[4]resorcinarene tetrahydrazide occurs via oxidation of the amino group i.e.,
transfer of electrons from the amine of calix[4]resorcinarene tetrahydrazide to the Au\(^{3+}\) ions [22].

\[
\text{HAuCl}_4 + 3\text{NR}_3 \rightarrow \text{Au}^{0} + 3\text{NR}_3^{+} + \text{H}^{+} + 4\text{Cl}^{-}
\]

CRTH stabilizes nanoparticles by passivating at the surface of gold nanoparticles and owing to the coordination of nitrogen atoms of hydrazide (-NH-NH\(_2\)) group with Au atoms at the surface of gold nanoparticles (Scheme 2). Further metallic gold nucleates to form gold nanoparticles and stabilizes it electrostatically [34-36]. The high stability of CRTH-AuNPs may be due to the fit between the cavity of the cyclic ligand and the guest nanoparticle [37].

4.2. Characterization of CRTH-AuNps

AuNPs exhibit strong surface plasmon resonance (SPR) that depends on the size and shape of AuNPs. Here, the SPR band of CRTH-AuNps was observed at 526 nm (Fig. 1a). The morphology and particles size of CRTH-AuNps as shown in (Fig. 1b) revealed that the particles are roughly spherical in shape as well as well dispersed with an average particles size of 11±2 nm (Fig. 1c). Further zeta potential (Fig. II) value, which indicates the overall charge on synthesized gold nanoparticles (CRTH-AuNps) was found to be 10.7 MeV. This higher positive value supports successfully introduction of hydrazide groups onto the surface of nanoparticles. The CRTH-AuNps were found to be highly stable for 120 days at neutral pH.

4.3. Effect of pH, temperature and time on stability of CRTH-AuNps

The stability of CRTH-AuNps at different pH (4.0 to 10.0) was studied by UV-VIS spectrophotometry (Fig. IIIa) and spectrofluorimetry (Fig. IIIb). The red shift in SPR is an explicit parameter for determining the increases in size (aggregation) of the nanoparticles. It was observed that there is no change in SPR band but a
trivial change in its absorbance and intensity. However, further experiments on CRTH-AuNps were performed at pH 7.0 (being neutral pH). There was no change in wavelength and absorbance up to 120 days (**Fig. IIIc**). Likewise the stability of CRTH-AuNps was checked at different temperatures. It was noticed that CRTH-AuNps remain stable only between (10-40°C), evidenced by negligible change in absorbance (**Fig. IV**) and beyond this range the particles tends to agglomerate.

### 4.4. Interaction of various amino acids with CRTH-AuNps by spectrophotometry and spectrofluorimetry measurements

The absorption spectra (**Fig. V**) and the fluorescence spectra (**Fig. VI**) of the CRTH-AuNps in the presence of different amino acids (alanine, aminobutyric acid, arginine, aspartic acid, cysteine, dopamine, glutamic acid, glycine, histidine, isoleusine, leucine, methionine, serine, threonine, tyrosine, tryptophan and valine), were studied at pH 7.0 [38]. An addition of 1 ml of 200 μM PHE solution to 1 mL of 0.00098% of CRTH-AuNps leads to a bathochromic shift of 16 nm in the absorption spectra whereas addition of 1 ml of 2 μM PHE solution to 1 ml of 0.00098% of CRTH-AuNps leads to quenching up to 85%. No noticeable change was observed either in absorption spectra as well as in emission spectra in presence of other amino acids. The mechanism of sensing is based on the interaction between CRTH-AuNps and PHE, which is a wavelength change-based method [39]. After establishing the selectivity of CRTH-AuNps for PHE among various amino acids (**Fig. VII**), its sensitivity was determined by performing emission titration with 20 nM to 1.0 μM concentration of PHE (**Fig. VIII**). The gradual decrease in quenching was observed with increase in concentration of PHE. The minimum, maximum and linear range of detection was observed as 20
nM, 1.0 µM and 100 nM to 820 nM, respectively. The inset (Fig. VIII), showed the Stern–Volmer plots of CRTH-AuNps for PHE. The plot exhibited a linearity of 0.9942 with respect to quenching towards the analyte. Therefore these nanoparticles can be used as a highly selective and sensitive fluorescence ‘‘turn-off’’ sensor for PHE without any further modification. This highly selective and sensitive sensing by means of fluorescence quenching may result from the aggregation of CRTH-AuNps induced by the cross-link complexation between CRTH-AuNps and PHE [40, 41].

4.5. Real sample

The level of PHE in human serum was detected without any pretreatment at room temperature. As the linear range of detection of PHE by CRTH-AuNps is from 100 nM to 820 nM, therefore we spiked the human serum sample with 200, 400 and 600 nM of standard sample of PHE. Since quenching of intensity is linear with the successive addition of analyte ions. The difference in fluorescence intensity was measured from the stern-volmer plot (Fig. IX) to determine the quantity of analyte concentration in the human serum sample (Table 1).

Conclusion

A new calix platform i.e. calix[4]resorcinarene tetrahydrazide (CRTH) has been used for the preparation of the AuNps where hydrazide groups on the periphery are used for reduction and inherent hollow cavity with web type structure provides excellent stabilization to AuNps. Preparation of the AuNps was carried out in a simple one-pot process, and the particles obtained were roughly spherical in shape as well as well dispersed with an average particles size of 11±2 nm. Effective binding of PHE, to the AuNps was studied quantitatively
among other amino acids and its application was attributed for sensing of PHE (amino acid) in human serum sample. High selectivity of CRTH-AuNps for PHE has a great potential in diagnosis of disease associated with PHE e.g. Phenylketonuria

**Scheme**

**Scheme 1.** Schematic representation showing the preparation of gold nanoparticles (CRTH-AuNps)
Scheme 2. Mechanism of formation of gold nanoparticles (CRTH-AuNps)
Figure 1a. UV-Vis spectra of CRTH stabilized AuNps

Figure 1b. TEM image of gold nanoparticles (CRTH-AuNps)
Figure 1c. EDX and Particle size distribution graph of CRTH-AuNps
Figure 3a. Effect of pH on surface plasmon resonance of CRTH-AuNps
Figure 3b. Effect of pH on fluorescence intensity/wavelength of CRTH-AuNps
Figure 3c. Stability of gold nanoparticles (CRTH-AuNps) with respect to time on wavelength/absorbance.

Figure 4. Effect of temperature on wavelength/absorbance of CRTH-AuNps
Figure 5. UV-Vis spectra of CRTH-AuNps after the addition of various amino acids at pH 7.0
Figure 6. Fluorescence spectra of CRTH-AuNps on addition various amino acids (2 µM) at pH 7.0

Figure 7. Effect of interference on the detection of PHE
Figure 8. Spectrofluorimetric titration of CRTH-AuNps on addition of PHE (1 nM to 0.8 µM). The inset shows a Stern-Volmer plot of relative intensity versus concentration of PHE.
Figure 9. Graph shows the concentration of PHE found in human serum samples.
References


