# Analytical Methods

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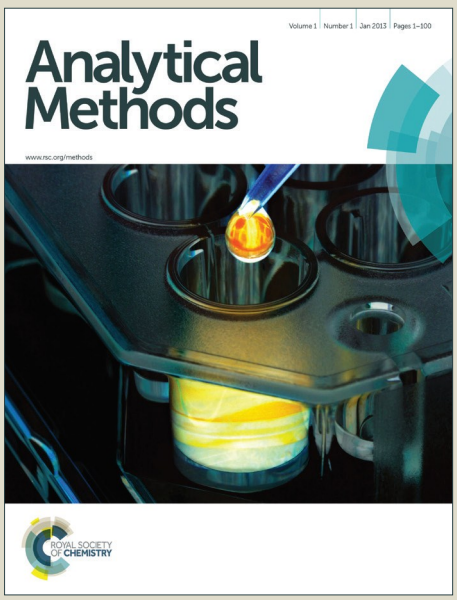


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# Non enzymatic colorimetric detection of glucose using cyanophenyl boronic acid included β-cyclodextrin stabilized gold nanoparticles.

**Priya A. Nair and K. Sreenivasan***

Laboratory for Polymer Analysis, Biomedical Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Poojapura, Thiruvananthapuram-695012, Kerala, India

*Tel.: +91 4712520248; fax: +91 4712341814

E-mail: sreeni@sctimst.ac.in (K. Sreenivasan)

**Abstract**

Selective recognition and estimation of glucose in biological fluids is important for the management of diabetes. Many non enzymatic methods have been emerged to address the limitations of in use enzymatic approaches. Still new techniques are designed considering the high demand for user friendly and cost effective methods. In this context, colorimetric assay by naked eye is widely appreciated as simple protocol adaptable to the rural population of third world countries. Herein we have adopted a "green" approach for the fabrication of a novel non-enzymatic glucose sensing method based on the inclusion of 4-cyanophenyl boronic acid (CPBA) into β-Cyclodextrin (β-CD) stabilized gold nanoparticles (GNP). CPBA though have a good selectivity towards glucose, its solubility is low in aqueous media and this issue is taken care of by β-CD. Three component system is synthesized by a tandem one pot method and used as such for the selective and sensitive detection of glucose in aqueous medium. We observed a gradual shift of plasmon absorption peak and an observable color change from red to blue with concentration of glucose. Concentration dependant color change was attributed to glucose mediated aggregration of the probes as revealed by TEM analysis. The applicability of the method to real biological matrix was evaluated by testing with human blood serum in the

concentration range 1 – 20 mM. This sensing methodology based on chemicals already available in the market dodging complex chemistry can be adapted for the routine glucose estimation.

**Key words:** 4-Cyanophenyl boronic acid; β-cyclodextrin; gold nanoparticles; glucose

## Introduction

Diabetes is spreading across the population like an epidemic. It is one of the most important health concerns of this century. According to world health organization the world wide prevalence of diabetics will be 366 million in year 2030 [1, 2]. India is slowly turning as a capital of diabetics. The latest figures released by the International Diabetics federation show that presently India has 62 million diabetics and nearly 52% of the population are not aware that they have high blood sugar level[3]. The most alarming factor is that many of the victims are children and young women.

To achieve good diabetic control frequent blood glucose monitoring is mandatory. Design and fabrication of methodologies to detect glucose is one of the most focused activities through all these years. In spite of the availability of numerous methods, it is still a hot area in the contemporary research. Over these years, several approaches have been emanated based on electrochemical, fluorescence and optical measurements. The devices in use are based on enzymatic reactions. The major limitations of these systems are said to be stability issues, need for mediators, factors against the enzyme activity and adverse influence on mass transport. Alternatively, massive efforts have been directed to develop chemical entities as glucose receptors. Boronic acid derivatives are emerged as excellent glucose binders and many methods[4, 5] have designed based on modified boronic acids[6-8]. Boronic acids are weak Lewis acid that can

interact with 1, 2 and 1, 3 diols to form a boronic acid diester group. This reaction is fast and reversible forming a stable boronate glucose complex[9, 10]. Selectivity is a major issue of these probes and attempts have been made to address these limitations through appropriate chemical amendments of boronic acid entities. Translation of many of these methods for the routine measurement of glucose is a challenge largely due to the complex chemistry involved in the synthesis and unaffordable cost. Diabetes often goes undetected particularly in the lower strata of the society largely due to unawarness and lack of facility. Simple approaches preferably a visibly observable color change based on widely and cheaply available chemicals can contribute effectively to address such a scanerio.

Literature depict quite a large number of work for non-enzymatic glucose detection. Huang et al have developed a non-enzymatic gluocse sensor based on gold nanoparticle with 3-aminophenyl boronic acid as the glucose sensing group [11]. It was shown by Strano et al that among the different phenyl boronic acid derivatives, 4-cyanophenyl boronic aicd (CPBA) shows good selectivity to gluocse [12]. Accordingly in the present study we have used CPBA as the glucose sensing moiety. The solubility of this molecule in aqeous media is, however, poor. Cyclodextrins (CD) are water soluble cyclic oligosaccharide that acts as host molecule to various hydrophobic guests thereby allowing its solubilization and stabilization. This specific feature makes them suitable for applications like biosensing[13], and drug delivery[14]. β-Cyclodextrins (β-CD), a member of CD family contains seven glucose molecules in a toroid structure and having an outer hydrophilic ring and inner hydrophobic cavity[15]. Glucose molecule having two cis diol moiety simultaneously bind with two boronic acid groups thus requires a diboronic acid molecule for its detection. But usually the synthesis of diboronic acid takes tedious procedure. One facile way to obtain multivalent binders from mono boronic acid is to position them in well

controlled manner in some assembly. Thus they behave as a diboronic aicd moiety and can detect glucose. Herein, we attempted to design the sugar responsive construct by complexing β-CD and CPBA. Shinkai et al have reported the design of multivalent receptor by placing two boronic acid groups into an anthracene ring[16]. Subsequently Jiang et al and Hayashita et al have explored supramolecular assembly of monoboronic acid - cyclodextrin for the detection of glucose[17, 18]. In these reports fluorescence of receptor molecule was monitored for the measurement.

Colorimetric changes detectable by naked eye have been advocated as effective and simple testing approach. The major task is the inclusion of an appropriate reagent with the CD-boronic acid system to translate the binding of glucose into a detectable color change. Wine red colored Gold nanoparticles (GNPs) are an interesting platform and in fact have been used widely in the detection of different targets including glucose. Analyte mediated aggregration leads to observable color change[19]. The choice of reducing agent and the capping agent used for nanoparticle synthesis depends on size range and morphology required for nanoparticle applications. In this work we have used CD as the reducing agent which also acts as the capping agent for the nanoparticle. Thereafter the GNP was modified with CPBA by a one pot method, in which the boronic acid derivative form inclusion complex with as prepared β-CD stabilized gold nanoparticles by non-covalent interaction. As far as we know it is the first time CD capped GNP act as a template for monoboronic acid. The change in local surface plasmon resonance of the assembly upon interaction with glucose is used for its estimation.

## Experimental section

### Materials

Gold (III) chloric acid, α-D-glucose, 4-cyanophenyl boronic acid, β–cyclodextrin, fructose, galactose, lactose and sodium hydroxide (NaOH) were obtained from Sigma- Aldrich (Bangalore, India). All the reagents were of analytical grade. The human serum samples were obtained from a local clinical laboratory.

**Synthesis of β -CD capped gold nanoparticles (CDGNP)**

The β -CD-GNP was synthesized as per an earlier reported procedure[20] with slight modifications. Briefly 10 mL of 2 mM β-CD solution was mixed with 10 mL of 1mM gold solution at $30^0$C to get a clear solution. To this added 250 μL of 1M NaOH was added. The solution is boiled in a hot plate till a light red color of GNP appears. The whole reaction was complete within 5 min. The solution is cooled and kept in a refrigerator till use. The final concentration of CDGNP is 1mM.

**Complexation of CPBA on to CDGNP**

CPBA (0.03 mM) was added to as such prepared CDGNP solution and was vortexed at RT ( $30^0$C) till all the added particles got dissolved. The resultant solution had a concentration of 2 mM. The clear supernatant solution was taken and used for further studies.

**Synthesis of inclusion complex of β–CD and CPBA**

An inclusion complex of CD and CPBA was prepared by kneading method as per an earlier reported method[21]. Briefly excess amounts of CD and CPBA were taken and grinded thoroughly in a mortar for 30 min with the addition of excess ethanol. The precipitate formed was collected, dried and kept in desiccators. The approach for determining the stoichiometry of the complex is detailed in supporting information.

**Characterization techniques**

UV-Visible absorption spectroscopy was carried out in a Varian, Cary-Win Bio UV spectrophotometer (Melbourne, Australia). For UV –Visible studies 800 µL of probe solution mixed with 200 µL of different concentration of glucose (1 mM to 20 mM) and the mixture was vortexed at $30^0$C and absorption spectra were measured. For the analysis of human blood serum samples, 200 µL of fresh human blood serum obtained from clinical laboratory was added to the probe solution. The FTIR spectra of the samples were recorded on a Nicolet 5700 FT-IR (Madison, USA) spectrometer using Diamond ATR accessory. The transmission electron microscopy (TEM) images of the samples are obtained on a Hitachi-H 7650 (Tokyo, Japan). The TEM samples were prepared by dropping an aqueous dispersion of sample onto a 200 mesh copper grid and the solvent evaporated in air.

**Results and discussion**

During the last decade tremendous efforts have been directed in the design and development of boronic acid derivatives as glucose sensing probes. β-CD with its large and hydrophobic cavity can hold boronic acid moieties resulting in multivalent scaffold that can bind with glucose. Another advantage of using β-CD is that it improves the solubility of otherwise hydrophobic CPBA receptor. An inclusion complex of boronic acid and cyclodextrin capped GNP was obtained simply by vortexing CPBA with GNP solution. As prepared CDGNP is well dispersed in aqueous media and the solution had a red wine color. The solution formed after adding CPBA retained red wine color without any observable shift in absorption peak reflecting the lack of any aggregration mediated through intermolecular hydrogen bonding between boronic acid groups and hydroxyl groups in CD. The absorption peak at 525 nm of CDGNP before and after

complexation with CPBA is shown in Fig 1. The formation of the inclusion complex (CPBA-CDGNP) was further confirmed by FTIR spectroscopy. The FTIR spectra of CDGNP and CPBA-CDGNP are shown in Fig 2. The CD stabilized GNP showed peaks at 1653 $cm^{-1}$, 1368 $cm^{-1,}$ and 1301 $cm^{-1}$ characteristics of the cyclodextrin. The spectrum of CPBA-CDGNP showed an additional peak at 2226 $cm^{-1}$ corresponding to the cyano group in CPBA. Spectral studies suggest the formation of an assembly consisting of CPBA and CDGNP.

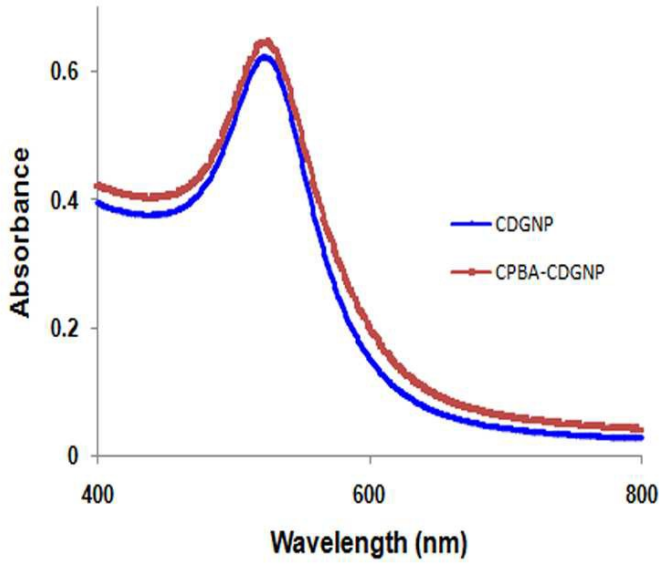



Figure1: Absorption spectra of CDGNP and after its complexation with CPBA (CPBA-CDGNP)

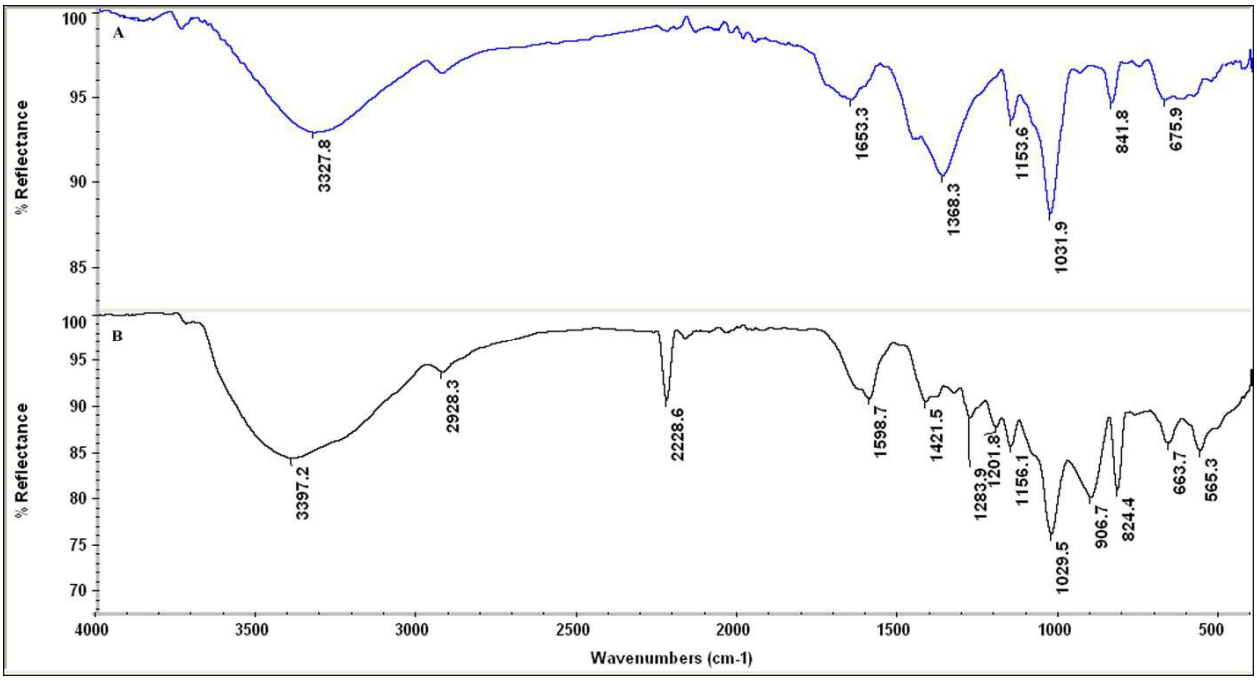



Figure 2: FTIR spectra of (A) CDGNP and (B) CPBA-CDGNP

This view was further substantiated by TEM analysis. TEM micrograph depicted in Fig 3(A) shows that the average diameter of the CDGNP is 13.5±1.7 nm. The average size of CPBA-CDGNP particles as revealed by TEM is 14.5±1.5 nm (Fig 3(B)). Nearly the identical sizes of the particles favors that CPBA is stabilized by inclusion complex formation with β-CD.

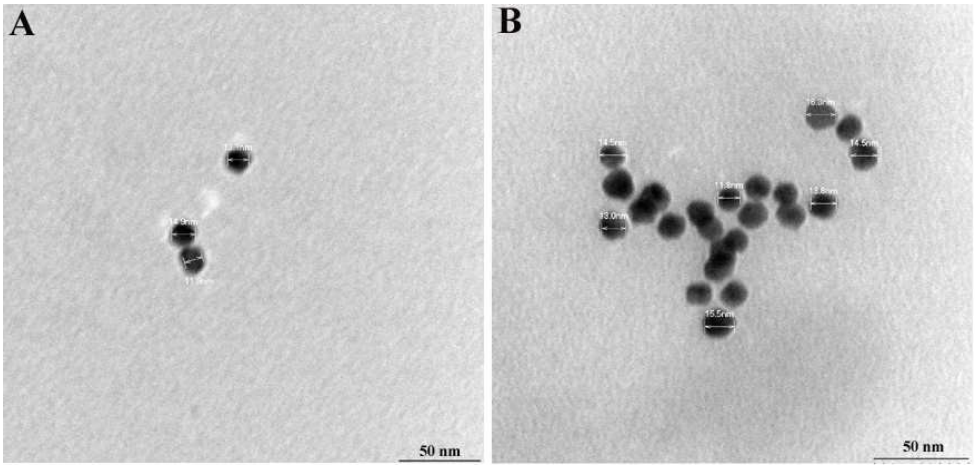


Figure 3: TEM of (A) CDGNP, (B) CDGNP-CPBA

**Glucose detection studies**

As the nanoparticle preparation was carried out in alkaline medium the resultant solution had a pH around 8. Basic condition is known to favor the reaction between boronic acid groups and glucose to form boronate ester by transforming boronic acid to boronate anion[22]. Thus the CPBA incorporated solution can be directly used for glucose detection studies without any further steps.

The UV-Visible absorption spectra of CDGNP- CPBA before and after addition of varied concentrations of glucose are shown in Fig 4. On adding varying amount of glucose ranging from 1 mM to 20 mM the absorption spectra showed an upward shift in the region between 650 nm and 550 nm. This up shift in absorption is a clear optical signature of the formation of aggregates. The integrated absorbance between these wavelengths was used as a spectroscopic parameter of aggregation formation. For quantitative analysis an empirical measurement of the aggregation process called aggregation parameter was then determined, which measures the variation of the integrated absorption between 550 and 650 nm. The aggregation parameter then determined from the equation (A-A0)/A0, where A is the integrated absorbance between 550 and 650 nm for the glucose added system and A0 is the integrated absorbance between 550 and 650 nm of the blank solution. Similar type of indicator has been used to analyze the aggregation of gold and silver particle induced aggregation in presence of different analytes[23, 24]. The aggregation parameter increased linearly with increase in the concentration of glucose. The detection limit as estimated from change in aggregation parameter is 1mM. The calibration plot of aggregation parameter with concentration of glucose was shown in Fig 5. No incubation time was required for the coupling of glucose to boronic acid as the response was instant. Upon

addition of higher concentration of glucose the color turned blue, thus this method can be used as a qualitative colorimetric method for glucose detection without the use of any sophisticated instruments. When the glucose concentration exceeds 8 mM (144 mg/dL), color change can be observable by naked eye. This information appears to be helpful for an individual to get an idea about his category in terms of glucose concentration.

The shift in the absorption peak and the gradual color change with the increase in concentration of glucose was attributed to glucose mediated aggregration of the probes. Analyte induced inter plasmon coupling of gold nanoparticles leading to aggregation is widely known[25, 26]. Wu et al have designed glucose sensing probe by glucose mediated assembly of phenyl boronic acid modified quantum dots[27]. The change in fluorescence was used for the quantification of glucose. Our view was further confirmed by TEM analysis. Glucose mediated aggregration of the probes is apparent from the micrograph (Fig 6).

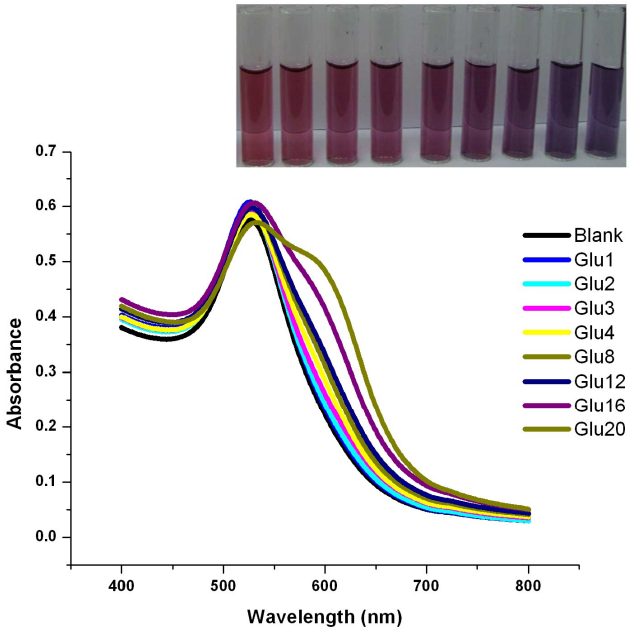


Figure 4: The absorption of spectra of CPBA-CDGNP before and after adding varied concentrations (0 mM - 20 mM) of glucose. (inset is the photographic image of respective solutions)

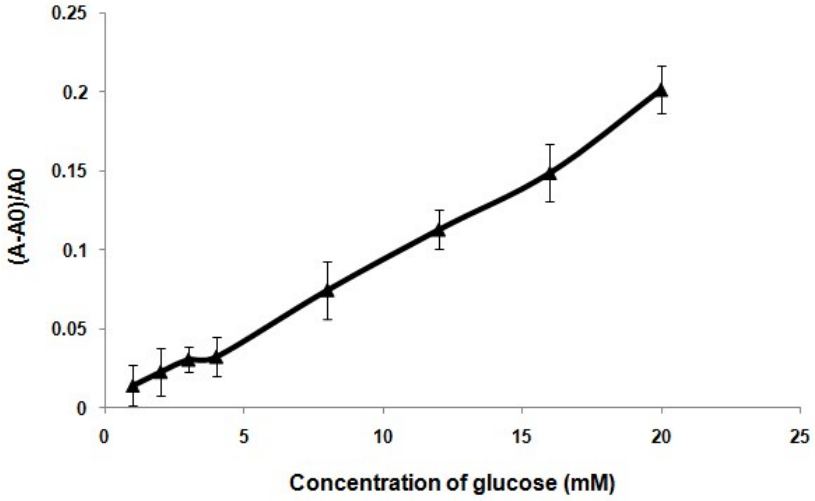



Figure 5: Calibration plot of aggregation parameter with concentration of glucose.

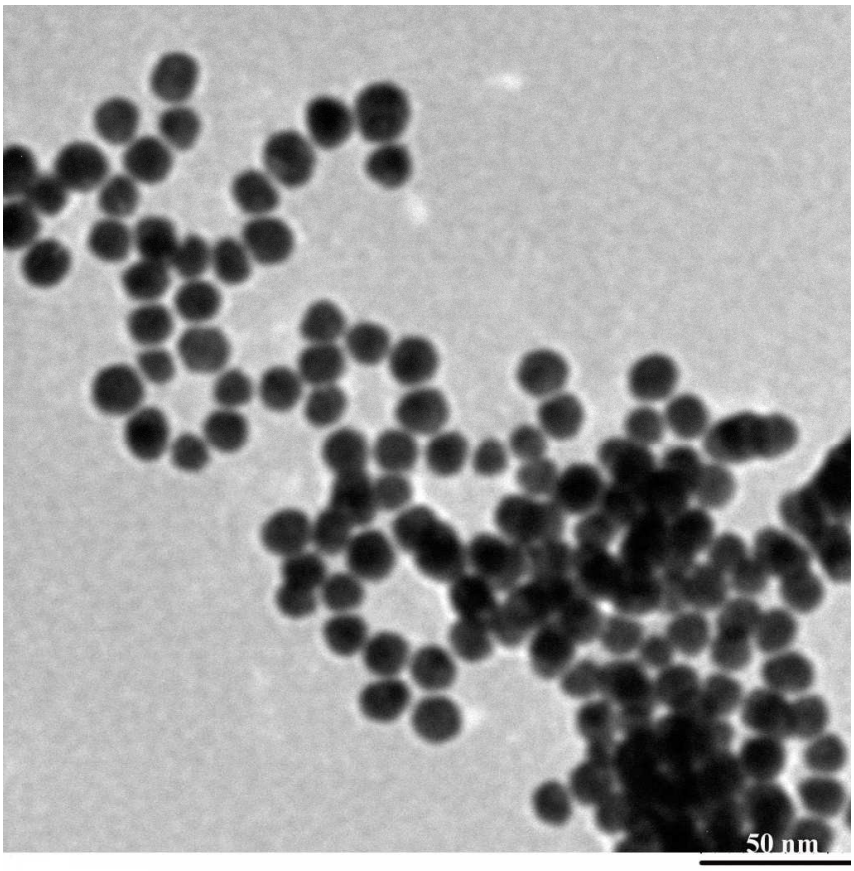
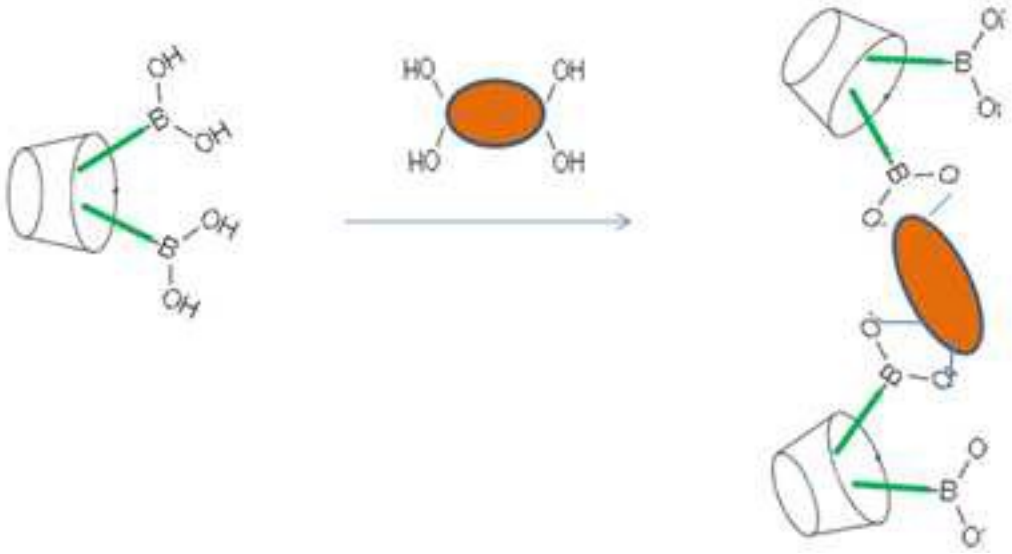



Figure 6: TEM image of CPBA-CDGNP added with 100 mM of glucose

The two cis diol moiety in glucose can simultaneously bind to two boronic acid groups; therefore the selective detection of glucose can be achieved by using diboronic acid groups. We reasoned that the hollow cavity of CD can hold two boronic acid groups thus creating a diboronic acid moiety enabling the binding of two cis diol groups of glucose. To ascertain the formation of 1:2 inclusion complex of CD and CPBA, we prepared inclusion complex and its composition was analyzed. From the composition, we confirmed the stoichiometry of the complex as 1:2. The details are shown in the supporting information. Hayashita et al have also shown that two molecules of phenyl boronic acid azo probe can be included into the cavity of γ-cyclodextrin and the assembly exhibited a selective response to D-glucose by forming a 2:1 inclusion complex [28]. In another study 2:2 inclusion complex of stillbene boronic acid and γ–CD has been used for the

selective and sensitive detection of glucose[19]. The aggregation of nanoparticle upon addition of glucose is attributed to the formation of a complex of CPBA with glucose molecule as shown in scheme 1.



Scheme 1: Formation of CPBA glucose complex

**Selectivity of the assay**

Recognizing glucose among different sugars is important and to certain extent it is difficult as all the sugars possess the same hydroxyl functionalities with only difference being in their stereochemistry[29, 30]. In this study different sugars like galactose, fructose, mannose and lactose were tested to evaluate the selectivity of the method in sensing glucose. The concentrations of all the sugars were taken as 20 mM. From the VU-Visible spectra it was clear that with other sugars only a minor change in the absorption was occurred. But with glucose a marked shift in the absorption spectrum was observed. Thus the developed sensor exhibited good selectivity to

glucose over other molecules having similar molecular weight and functionality. The results are shown in Fig 7.

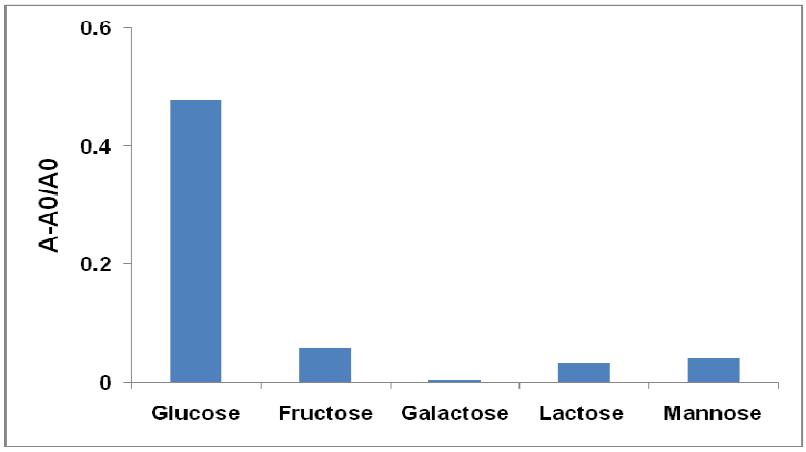



Figure 7: Relative absorbance of different sugars.

**Blood glucose analysis**

The potential of the method to measure glucose concentration in real samples was illustrated by estimating glucose levels in blood serum collected from clinical laboratory. The blood glucose level obtained by this developed method was comparable to that of the data obtained from the clinical lab. This data validate the potential of the newly developed method for the detection of glucose in biological fluids. The results are shown in Table 1.

Table 1: Determination of glucose in blood serum

| Blood serum sample | Concentration of glucose (mM)[a] | Concentration of glucose (mM)[b] |
|---|---|---|
| 1 | 6.66 | 6.50±0.133 |
| 2 | 7.00 | 7.20±0.258 |
| 3 | 12.00 | 11.80±0.052 |

a. Value from clinical laboratory (average of three measurements)

b. Concentration obtained by GNP-CPBA probe (average of three measurements)

## Conclusions

In summary, we have designed a simple, enzyme free new strategy for the colorimetric detection of glucose. Glucose binding probe was generated by mixing CPBA with GNPs stabilized by β-CD. Our results indicated that glucose mediates the aggregration of the probes resulting peak shift and color change. The chemo sensor has good analytical performance and can estimate glucose in 1 to 20 mM range. The notable feature of the method is its adaptability to rural set up in the sense that it can be generated by using available chemicals without complex synthetic routes. The color change with concentration is the added advantage and the method seems to have potential to translate for the cost effective determination of glucose.

## Acknowledgements

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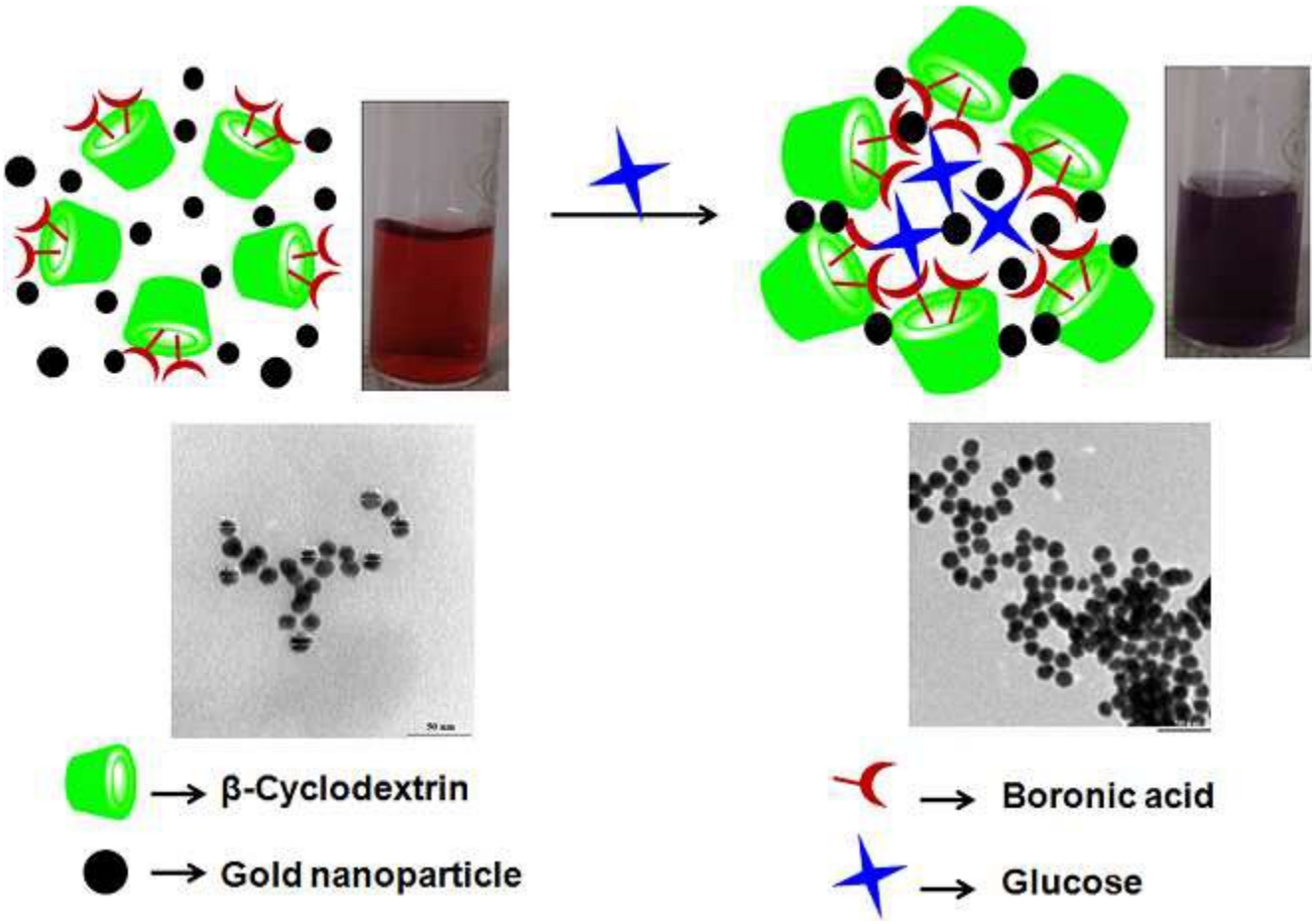


***Gold nanoparticles generated and stabilized by β-cyclodextrin-cyano phenyl boronic acid complex enables colorimetric detection of glucose***