CHAPTER NINE

# Portable Enzyme-Paper Biosensors Based on Redox-Active $CeO_2$ Nanoparticles

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## Contents



## Abstract

Portable, nanoparticle (NP)-enhanced enzyme sensors have emerged as powerful devices for qualitative and quantitative analysis of a variety of analytes for biomedicine, environmental applications, and pharmaceutical fields. This chapter describes a method for the fabrication of a portable, paper-based, inexpensive, robust enzyme biosensor for the detection of substrates of oxidase enzymes. The method utilizes redox-active NPs of cerium oxide ($CeO_2$) as a sensing platform which produces color in response to $H_2O_2$ generated by the action of oxidase enzymes on their corresponding substrates. This avoids the use of peroxidases which are routinely used in conjunction with glucose oxidase. The $CeO_2$ particles serve dual roles, as high surface area supports to anchor high

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loadings of the enzyme as well as a color generation reagent, and the particles are recycled multiple times for the reuse of the biosensor. These sensors are small, light, disposable, inexpensive, and they can be mass produced by standard, low-cost printing methods. All reagents needed for the analysis are embedded within the paper matrix, and sensors stored over extended periods of time without performance loss. This novel sensor is a general platform for the in-field detection of analytes that are substrates for oxidase enzymes in clinical, food, and environmental samples.

## ABBREVIATIONS

**APTS** aminopropyltrimethoxysilane
**$CeO_2$** cerium oxide
**GOX** glucose oxidase
**NPs** nanoparticles

Paper
Hydrogen peroxide

## 1. INTRODUCTION

Successful implementation of enzyme-based (bio)analytical systems and devices requires the development of functional enzyme-based materials and surfaces that retain the activity and function of the enzymes over substantial time periods, under ordinary conditions. In recent years there has been significant research on the use of nanostructured materials, nanoparticles (NPs) and nanocomposites to develop functional enzyme-based devices. Enzyme attachment and assembly of enzyme–NP conjugates are critical steps in the fabrication of enzyme-based biosensors. When used in conjunction with enzymes, nanomaterials can increase enzyme stability and enhance binding efficiency leading to improved performance of sensors. Enzyme–NP conjugates can be used as building blocks for the development of inexpensive, portable, and easy-to-use biosensing devices. The interesting feature of the current method is that the NPs serve as supports for the enzyme binding and also serve as the colorimetric reagent for analyte detection. The advances in the development, fabrication, characterization, and implementation of functional enzyme–NP conjugate systems for field-portable biosensors are described here. Due to their light weight and simple-to-use features, they are suitable for the on-site detection of a variety of analytes of interest for clinical diagnosis, environmental monitoring, public safety, and food quality control.

The demand for reliable, field-deployable and cost-effective devices that can provide rapid on-site assessment of the environment, water, air, or food chain continues to grow (Ding, Srinivasan, & Tung, 2015). The availability of affordable technology can enable local communities in resource limited and remote regions to improve health care, protect the environment, and maintain the safety of food. Such sensors can rapidly facilitate the World Health Organization's (WHO) goal for *Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment free, and Deliverable Sensors to end-users*, termed as "ASSURED" (Mabey, Peeling, Ustianowski, & Perkins, 2004). Enzyme-based methods have gained increasing acceptance in the biomedical field (Ispas, Crivat, & Andreescu, 2012; Wilson & Hu, 2000) and their use could be extended to other application areas as well. Enzyme-based devices combine the selective recognition capabilities of enzymes with the optoelectronic, catalytic, and mechanical properties of nanomaterials. Interfacing enzymes with nanostructured materials can generate functional enzyme–nanomaterial conjugates that can be used as active components in the development of practical enzyme-based assays for field monitoring. Nanomaterials have been shown to increase the stability and robustness, sensitivity, and shelf life of enzyme-based devices (Andreescu, Njagi, & Ispas, 2008). With the development of advanced nanomanufacturing techniques, enzyme-based devices that incorporate NPs can be miniaturized, mass produced, and multiplexed. They can be developed for real-time and point-of-care diagnostic testing (Ispas et al., 2012) and used in the field (Gabig-Ciminska, 2006).

The properties, fabrication, and characterization of enzyme–NP conjugates and their application for the development of field-deployable biosensors are described here. After a brief discussion of the current development status of NP-based enzyme biosensors, we describe a method that can be used as a general platform for the immobilization of enzymes and NPs on paper. An example of application for the immobilization of glucose oxidase (GOX) and redox-active NPs of $CeO_2$ to fabricate a fully integrated portable assay for the colorimetric detection of glucose using NPs as color-generating probes is given here.

## 2. NPs-BASED ENZYME BIOSENSORS

### 2.1 NPs for Biosensing Design

Metal and metal oxide NPs are the most common types of NPs used to design portable enzyme-based biosensing platforms. In a traditional

enzyme-based biosensor design, NPs can serve as: (i) signal amplifier to increase sensitivity; (ii) immobilization support to stabilize the enzyme; and (iii) as a sensor platform to increase surface area, enhance conductivity in electrochemical assays, or provide a colorimetric read-out in optical sensing schemes. The unique physicochemical properties of the NPs (i.e., catalysis, surface plasmon, magnetism, luminescence, conductivity, etc.) associated with their small size and tailored surface chemistry can generate powerful enzyme–NP conjugate systems that can be used as active components in biosensing devices.

Progress in nanotechnology has provided the ability to tailor the characteristics of NPs through synthetic design by controlling the particle size, surface properties, and chemical composition. Therefore, NPs with enhanced electronic, optical, or catalytic functions can be obtained (Baron, Willner, & Willner, 2007). The NP chemistry, its structure, surface charge, functionality, and composition have a critical role on the binding, biological activity, orientation, structure, and functionality of enzymes (Jo, Kim, Lee, & Kim, 2015; Kim, Grate, & Wang, 2006; Popat et al., 2011). Enzyme function can also be tuned by carefully selecting the characteristics of the medium such as pH, temperature, viscosity, and salt concentrations of the measurement environment (Czeslik & Winter, 2001; Jia, Zhu, & Wang, 2003). Functional enzyme–NP assemblies and biofunctionalized NP composites can find broad applicability in biosensing (Demin & Hall, 2009; Pingarrón, Yáñez-Sedeño, & González-Cortés, 2008), biocatalysis (Schoemaker, Mink, & Wubbolts, 2003), tissue engineering (Arruda et al., 2009), disease diagnosis (Arruda et al., 2009), biofuel cells (Naruse et al., 2011), and drug delivery (Ai, Jones, & Lvov, 2003).

## 2.2 Immobilization of Enzymes on NPs

Enzyme stabilization on sensor surfaces is an essential step for the development of reagentless and fully integrated field-portable biosensors. This process is governed by various interactions between the enzyme and the material interface. The composition and properties of nanoscale surfaces and interfaces control the functionality of these devices. The main challenge is to design and control the interfacial forces such that it will allow stable enzyme attachment while maintaining activity and function as close as possible to its native state. These materials should either possess the necessary functional groups needed for enzyme attachment or should be easily functionalized. Enzymes immobilized on NPs and NP-based composites

have demonstrated increased stability over free enzymes (Homaei, Sariri, Vianello, & Stevanato, 2013; Lata et al., 2015). The use of immobilized enzymes is preferred due to their ease of handling, prolonged availability, and robustness, increased resistance to environmental changes and reusability (Datta, Christena, & Rajaram, 2013).

The NP characteristics such as size and surface chemistry as well as the orientation and density of the immobilized enzymes all contribute to their catalytic performance. Enzyme configuration, orientation, and density can be controlled by changing the NPs surface chemistry (Ardao, Comenge, Benaiges, Álvaro, & Puntes, 2012). These can be tailored to promote electrostatic binding (surface charge) or by adding surface ligands for covalent (e.g., through glutaraldehyde or carbodiimide chemistry) or affinity (e.g., thiol) attachment. Enzyme entrapment within NP-based composite materials with biocompatible or conducting polymers is another strategy commonly used to create enzyme-based bioassays (Homaei et al., 2013). NPs can be used as enzyme carriers (Ding, Cargill, Medintz, & Claussen, 2015) to increase enzyme loading by virtue of their high surface area and reduce substrate diffusion limitations (Won, Aboagye, Jang, Jitianu, & Stanciu, 2010). In electrochemical assays, NPs can promote electronic communication between redox enzymes and electrode surfaces, which is essential for the development of electrochemical enzyme-based biosensors (Heller, 1990). Figure 1 shows a summary of the various NPs systems and their applications in enzyme-based biosensors.

## 2.3 Fabrication of Low-Cost Enzyme Biosensors

Development of low-cost and portable devices for home diagnostics, screening and point-of-care testing requires fabrication and assembly of biofunctionalized surfaces on inexpensive substrates that have the appropriate detection chemistry, sensitivity, and selectivity. A key requirement in the production of enzyme-based bionanofunctionalized structures for portable and low-cost sensing is to achieve uniform deposition of NP–enzyme conjugates to ensure the required sensitivity and preserve the biological activity of the enzyme. In portable platforms, NPs are often embedded in polymeric films and are prone to aggregation, losing their nanoscale features. As a result, there is little control over the nanoassembly, and both NPs and enzymes are randomly distributed within the nanostructured network, which strongly affect the sensitivity and reproducibility of sensing devices. Many enzyme biosensors reported in literature involve complicated multistep procedures,

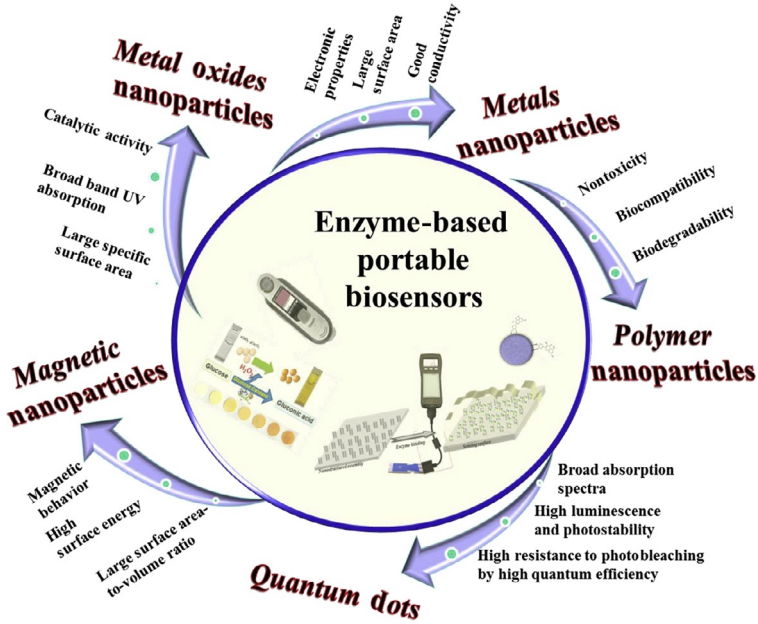


**Figure 1** Overview of the various NPs systems and their properties for enzyme-based portable biosensors.

expensive substrates and reagents, most of which are carried out manually, and require special conditions to conserve bioactivity. In many cases reagents needed for detection or signal amplification (including bioreagents, colorimetric dyes, and nanoelements) are added postfabrication, adding additional cost, and inconvenience for analysis. Automatic deposition procedures such as inkjet and 3D printing enable scalable production with increased reproducibility (Abe, Suzuki, & Citterio, 2008; Gowers et al., 2015).

Low-cost biosensing devices have been developed recently on paper, thread, or plastic as alternatives to conventional instrumentation for field analysis (Martinez, Phillips, Butte, & Whitesides, 2007; Metters, Houssein, Kampouris, & Banks, 2013; Riccardi et al., 2016). Applications include point-of-care medical diagnosis, environmental monitoring, and food quality control (Martinez et al., 2007; Martinez, Phillips, Whitesides, & Carrilho, 2010; Pelton, 2009; Zhao, Ali, Aguirre, Brook, & Li, 2008). These platforms have been integrated with colorimetric (Dungchai, Chailapakul, & Henry, 2010; Martinez et al., 2007) and electrochemical (Dungchai, Chailapakul, & Henry, 2009; Nie et al., 2010; Reches

et al., 2010) signal transduction systems. Such devices can be miniaturized, they are disposable and can be used for on-site analysis. Some examples of paper-based bioassays include: patterned-paper fabricated by photolithography for the detection of glucose and bovine serum albumin (Martinez et al., 2007), sol–gel bioinks for the detection of neurotoxins (Hossain et al., 2009), and aptamer—NP-based lateral flow devices for the detection of DNA sequences (Liu, Mazumdar, & Lu, 2006). Several NP-based bioassays have been reported; few of these have been adapted for implementation with paper platforms (Zhao, Brook, & Li, 2008). Traditionally, these assays are based on NP aggregation or dispersion of colloidal gold in the presence of the analyte (Elghanian, Storhoff, Mucic, Letsinger, & Mirkin, 1997; Zhao, Brook, et al., 2008). An example is the detection of glucose in urine using colloidal Au NPs functionalized with GOX (Radhakumary, 2011). These assays utilize presynthesized NPs, and in vast majority of the cases, these are used in colloidal dispersions. Methods that enable large scale fabrication of bionanofunctionalized surfaces that conserve both the nanoscale properties and bioactivity are of great interest for the manufacturing of inexpensive, portable sensors. Current research focuses on the use of 3D and inkjet technology for enzyme printing. These methods can potentially be used for the automatic fabrication of the entire biosensing device.

An enzyme-based NP system that takes advantage of the catalytic, optical, and redox properties of redox-active $CeO_2$ NPs is described here. We couple the optical properties of these NPs with the versatile and inexpensive nature of paper and the catalytic activities of redox enzymes to design a portable enzyme-based biosensor.

## 3. $CeO_2$ NPs FOR ENZYME IMMOBILIZATION AND ENZYME-BASED BIOSENSORS

$CeO_2$ NPs have demonstrated interesting properties for sensing, biomedical, and drug delivery applications (Andreescu et al., 2014). These NPs are unique because of the presence of cerium in dual oxidation states ($Ce^{3+}$/$Ce^{4+}$) at their surface, which enable them to be used as both oxidation and reduction catalysts (Pirmohamed et al., 2010). $CeO_2$ NPs have high oxygen mobility at their surface (Preda et al., 2011; Zhang, Wang, Wang, & Li, 2006) and a large oxygen diffusion coefficient, which facilitates the conversion between valance states $Ce^{3+}/Ce^{4+}$ and allows oxygen to be released or stored in the crystal structure (Dutta et al., 2006; Wang et al., 2011;

Xu et al., 2010). Their catalytic, oxygen rich properties, rich functional groups, and low toxicity (Das et al., 2007) make these NPs an excellent immobilization material for enzymes (Ansari, Solanki, & Malhotra, 2009; Ornatska, Sharpe, Andreescu, & Andreescu, 2011). $CeO_2$ NPs have unique optical properties derived from the redox conversion between the two oxidation states and can form charge transfer complexes of unique colors with $H_2O_2$ and with phenol-type compounds. The optical and redox properties of these NPs enable them to be used as colorimetric indicators to replace commonly used soluble dyes (Ornatska et al., 2011; Sharpe et al., 2014; Sharpe, Frasco, Andreescu, & Andreescu, 2013) which provide exciting opportunities for the rational design of portable and robust enzyme/NP/biosensors.

The use of $CeO_2$ NPs in contact or proximity with oxidase enzymes can eliminate or minimize problems associated with low oxygen levels for enzymes that require oxygen as a cosubstrate, enabling these biosensors to work in hypoxic environments. The catalytic and oxygen storage/release capacity of $CeO_2$ NPs were used to fabricate "oxygen rich" electrochemical biosensors (Ozel, Ispas, Ganesana, Leiter, & Andreescu, 2014). $CeO_2$ also facilitates the stabilization of the enzyme through strong electrostatic interactions, thereby enhancing the operational and storage stability of enzyme sensors based on these materials (Khan & Dhayal, 2008; Li et al., 2006; Njagi, Ispas, & Andreescu, 2008; Topoglidis, Cass, O'Regan, & Durrant, 2001). Coimmobilization of oxidase enzymes with these particles greatly improved performance of *in vivo* biosensors under hypoxic conditions for real-time measurements of dopamine with tyrosinase (Njagi, Chernov, Leiter, & Andreescu, 2010), lactate with lactate oxidase (Sardesai, Ganesana, Karimi, Leiter, & Andreescu, 2015), and glutamate with glutamate oxidase (Ozel et al., 2014). Enhanced capabilities were achieved by doping the $CeO_2$ particles with Pt to amplify the catalytic activity and imparting conductivity, which significantly increased the detection sensitivity. For example, a Pt-$CeO_2$-based biosensor fabricated by coimmobilizing the particles with GOX in a porous chitosan matrix enabled sensitive detection of physiologically relevant glucose concentrations in conditions of severe oxygen depletion, with a detection limit of 37.4 $\mu M$ and of lactate in implantable conditions (Sardesai et al., 2015; Sardesai, Karimi, & Andreescu, 2014). In vitro characterization of the biosensor demonstrated high selectivity against physiological levels of ascorbic acid, a storage stability of 3 weeks, a response time of 6 s, and linear sensitivity over a wide concentration range. Other applications of this system include implantable enzymatic biofuel cells and wearable devices.

## 4. DESIGN OF A $CeO_2$-BASED COLORIMETRIC ENZYME BIOSENSOR

### 4.1 Method Principle and Main Characteristics

This method takes advantage of the color changes resulting from the alterations in the redox state and composition of NPs in response to $H_2O_2$, the product of the enzymatic reaction of most oxidase enzymes. $CeO_2$ NPs and the enzyme are both incorporated into a paper-based format. Ceria NPs have been found to possess oxidase and peroxidase-like activity (Asati, Santra, Kaittanis, Nath, & Perez, 2009), suggesting that they could potentially replace oxidase and peroxidase enzymes (Ornatska et al., 2011). The coexistence of two oxidation states imparts unique surface chemistry, redox, and catalytic activity (Davis & Thompson, 2002; Zhang, Wang, Koberstein, Khalid, & Chan, 2004) which make these particles interesting candidates for biosensing applications. The method described here is demonstrated for the detection of glucose using GOX as a model system (Ornatska et al., 2011) but the same approach can be used to fabricate other oxidase enzyme-based biosensors. To fabricate the biosensor, $CeO_2$ NPs, and GOX (binds specifically to β-D-glucopyranose and does not act on α-D-glucose) are coimmobilized onto filter paper using a silanization procedure. In the presence of glucose, the enzymatically generated $H_2O_2$ induces a visual color change of the $CeO_2$ NPs, immobilized onto the modified paper surface, from white-yellowish to dark-orange, in a concentration-dependent manner. The visible color change is due to the change in the oxidation state and formation of surface complexes (Babko, 1954; Yu, Hayes, O'Keefe, O'Keefe, & Stoffer, 2006). Figure 2 shows the biosensor concept and

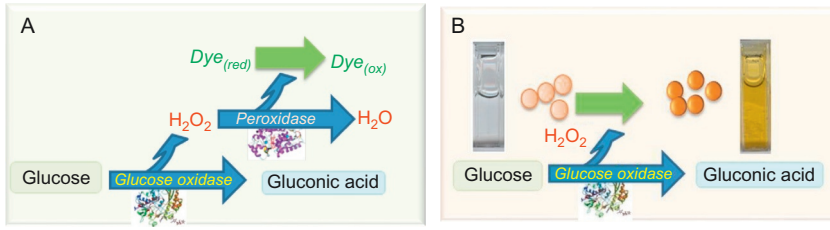


**Figure 2** Schematic representation of the working principle of colorimetric enzyme-based assays for the detection of glucose: (A) conventional assay involving the use of HRP and an organic dye. (B) $CeO_2$ NPs-based assay for the detection of glucose. This principle has been demonstrated on paper surfaces with both the enzyme and the NPs stabilized on paper to create a reagentless enzyme assay (Ornatska et al., 2011).

detection mechanism by comparison with a conventional colorimetric assay involving the use of a soluble redox dye and the peroxidase enzyme to quantity the enzymatically generated $H_2O_2$. In the $CeO_2$ biosensor, the $CeO_2$ particles replace both the peroxidase and the corresponding organic dye, significantly increasing the stability and robustness of the assay, reducing the price, while also serving as a robust support for the enzyme.

The method enabled a detection limit of 0.5 m*M* glucose with a linear range up to 100 m*M* and a reproducibility of 4.3%. The $CeO_2$ NPs paper sensor is fully reversible and can be reused for at least 10 consecutive measurement cycles. The only analysis step needed is the addition of the substrate. The biosensor does not require external reagents, as all the sensing components (enzyme- and color-generating reagent—$CeO_2$) are fixed onto the paper platform. The sensor can be stored for at least 79 days at room temperature while maintaining the same analytical performance over this time.

Robustness, ease-of-use, high reproducibility, and stability of the assay set these sensors apart among other assays that involve the use of sensitive dyes and enzymes. Sensors based on these particles are portable and operate similar to a small sensor patch, allowing for rapid and sensitive detection of target analytes in various environments including food, clinical, and environmental matrices. Thus, replacement of peroxidase and color-generating reagent is no longer required with this strategy. This improvement is significant and can be used as a general platform in the assays of substrates that use oxidases and generate hydrogen peroxide as the product.

## 4.2 Materials

### *4.2.1 Sensing Elements*

Filter paper (Whatman filter paper #1) used as the paper platform for all sensors was purchased from VWR, Philadelphia, PA (supplier No. 1001-110) and VWR Catalog #28450-106 and the $CeO_2$ NPs from Sigma Aldrich, St. Louis, MO (Catalog #289744).

### *4.2.2 $CeO_2$ NP Sensors*

$CeO_2$ NPs used for paper sensor fabrication are of <20 nm in diameter and they are purchased from a variety of suppliers. The example demonstrated here was developed with cerium (IV) oxide NPs (20 wt.% colloidal dispersion in 2.5% acetic acid, 10–20 nm average particle size) from Sigma Aldrich (Catalog #289744). $CeO_2$ NPs from other suppliers or synthesized in the lab can also be substituted. Different particles may show different color intensities and some may aggregate and may not be uniformly deposited on the paper surface. In general, the particles in aqueous dispersions provided

superior characteristics over dry $CeO_2$ powders for this type of assays (Sharpe et al., 2013).

### 4.2.3 Enzyme and Other Reagents

GOX (G6641), glucose (G5767), aminopropyltriethoxysilane (APTS), chitosan, glutaraldehyde, $H_2O_2$, and sodium phosphate were purchased from Sigma Aldrich.

## 4.3 Methods

### 4.3.1 Fabrication of $CeO_2$-Enzyme Paper

The sensing paper is comprised of $CeO_2$ NPs coimmobilized with GOX on cellulose paper (Ornatska et al., 2011). First $CeO_2$ modified filter paper was made by immersing 11 cm diameter filter paper rounds into baths of 3% $CeO_2$ NP dispersion for 10 min and dried for several hours at 75°C. The NP dispersion was sonicated before use. After drying, the $CeO_2$ NP paper was dip-soaked in 5% APTS in ethanol for 10 min to stabilize the NPs. The paper with stabilized NPs was then dried for 10 min at 100°C. The APTS stabilization procedure enables the formation of siloxane bridges between the OH-rich surface of the cellulosic fibers and the hydroxylated ceria through hydrogen bonding (Salon, Abdelmouleh, Boufi, Belgacem, & Gandini, 2005), effectively attaching the particles to the paper matrix. $CeO_2$ NPs prepared by this method show good uniformity and surface coverage. The amino-functional groups of the APTS are further used for the grafting of GOX. We note that the $CeO_2$ NPs maintained their color changing properties in response to $H_2O_2$ after silanization, which demonstrates that the chemistry used here is compatible with NPs.

### 4.3.2 Enzyme Immobilization on the $CeO_2$-Silane Paper

To immobilize the enzyme on the NP-coated, modified cellulose matrix, the silane-$CeO_2$ NP paper disks were soaked in 1% chitosan solution (in 0.5% succinic acid) for 10 min and air dried for 5–10 min. The biopolymer chitosan coating on the silica paper increases enzyme stability while providing access to the enzyme active site. The paper was then treated with 5% glutaraldehyde for 1 min for crosslinking the enzyme and various components, dipped in water and air dried for 5–10 min. A solution of 20 μL of 9 mg/mL GOX (190.8 U/mL) was applied onto the $CeO_2$ paper to load the enzyme and modified paper sensors were then rinsed with phosphate buffer at pH 7.4 and air dried.

## 4.4 Detection and Measurement Procedure

Glucose detection was achieved with the $CeO_2$–GOX modified paper immersed in glucose solutions of concentrations ranging from 0.5–500 m*M* in 0.05 *M* phosphate buffer pH 7.4. Alternatively, samples containing glucose solutions can be added to each $CeO_2$–GOX testing disk. A standard calibration curve was created with increasing glucose concentrations, displaying the relationship of color intensity to the concentration of glucose. Typical calibration curves are linear but logarithmic relationship can also be obtained under specific conditions. Calibration curves are intended for use in quantitative analysis of samples. Color responses can be read once sensors are fabricated and dried.

The $CeO_2$ sensor displays an immediate dark-orange color upon the addition of $H_2O_2$ in the concentration range from 2.5 to 100 m*M*. When the $CeO_2$ NPs were coimmobilized with GOX on paper, addition of glucose generates $H_2O_2$ which reacts with the immobilized NPs, generating the dark-orange color in a concentration-dependent manner. No change in color was observed in the absence of the enzyme, which demonstrates that the color formed is due to the enzymatically generated $H_2O_2$ and that the sensor is selective for glucose. The color is evenly distributed on the surface of the biosensor indicating uniform distribution and surface coverage of the NPs and enzyme onto the paper. This biosensor can be used to detect glucose at concentrations ranging between 0.5 and 100 m*M* (Figure 3).

The biosensors are reproducible with a standard deviation below 5%. For example, when 11 biosensors were tested, the average color intensity after addition of 100 m*M* glucose was 102.2 (±4.4). The biosensors can be stored for at least 79 days at room temperature. Stability was evaluated by periodic testing of the response of GOX biosensors from the same lot (e.g., prepared at the same time) with the addition of 100 m*M* glucose. The sensors stored in the refrigerator showed no decrease in response after 79 days over this time period. The response of biosensors stored at room temperature decreased by 10% after 50 days and by 15% after 79 days of storage. This also demonstrates effective immobilization of the enzyme on the paper disk and enhancement of enzyme stability in the sensing matrix.

For quantification of the color observed with the glucose solutions, the $CeO_2$–GOX paper sensors were scanned with a conventional office scanner. Images were analyzed using Adobe Photoshop or the Image J software. The colors of the paper were assessed using the eyedropper tool on Photoshop, which took an average of 31 × 31 pixels (or 961 pixels) close to the center of

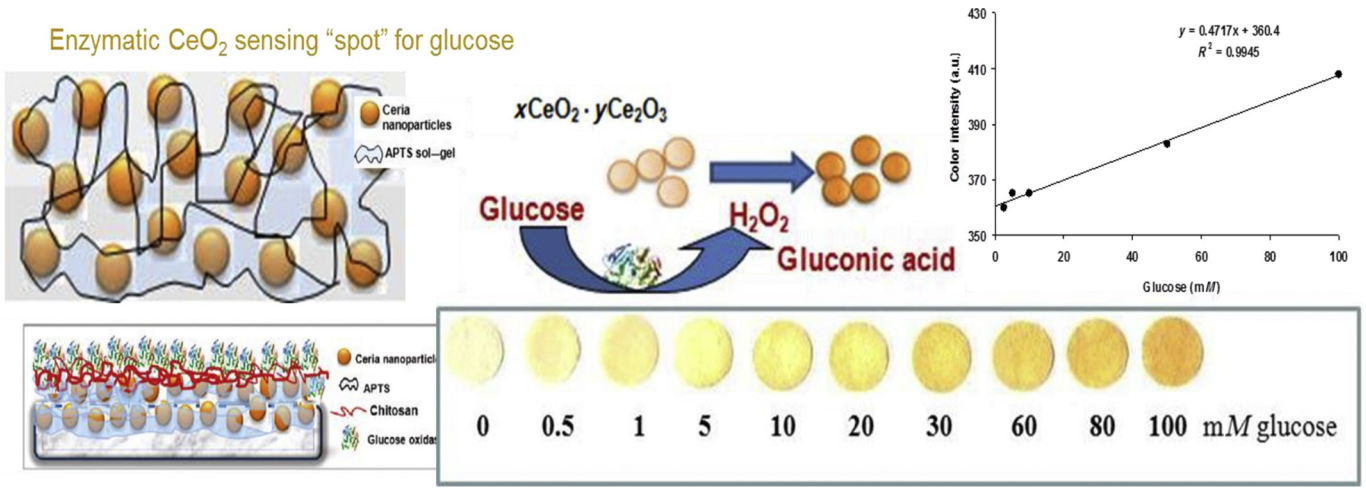


**Figure 3** $CeO_2$–GOX paper-based biosensor and colorimetric response to glucose concentrations ranging from 0 to 100 m*M* glucose.

the paper sensors. The blue color intensity was found to be the most sensitive option as blue is the complementary color to yellow/orange. The intensity of the color was used to construct the calibration curve and characterize the performance of the assay. Alternatively, the Pantone CapSure® handheld color analysis device may be used for the creation of a color database due to its ability to generate unique color ID codes and RGB values that can be referenced by all users of the same device.

For analysis of unknown concentrations of the glucose samples, sample was added to the $CeO_2$–GOX paper and the intensity of the color measured. An example of the application of the as-prepared sensing paper is for the detection of glucose in serum, in the presence of a variety of biological materials which are potential interfering substances. Glucose concentration in serum was determined with these biosensors (3.71 m*M*), as deduced from the calibration curve using the standard addition method.

## 5. COMMENTS ON THE METHOD

### 5.1 NP Choice

The surface characteristics of NPs and their chemical nature dictate the interaction and performance of the enzyme biosensor. The surface functionality can affect enzyme activity and binding characteristics (Sheldon, 2007). Surface reactivity also varies with the synthetic procedure and additives/surface coatings used to stabilize the particles (Sharpe et al., 2013) and can increase by addition of dopants such as Pt (Sardesai et al., 2014). One outstanding question is whether engineered NPs can be incorporated in a biologically relevant environment while conserving their useful properties for sensing. In their vast majority, synthesized $CeO_2$ NPs contain a surface coating to increase stability and dispersibility. The functional group of the surface ligand is used to attach enzymes and it can vary among particles. In principle, we recommend particles with hydroxyl, amino, or carboxylic terminal groups. The color changing properties and reactivity of these particles can vary greatly from one particle type to another. NPs should be tested for color formation with $H_2O_2$ before their use in the fabrication of the biosensors. Size, uniformity, and homogeneity of the NPs can affect their optical properties as well as the overall performance of biosensor. $CeO_2$ NPs should first be tested to verify uniform deposition and stability on paper. Uniform coating of the NPs in the paper can be tested by the addition of $H_2O_2$ to the $CeO_2$-modified paper and examining the color distribution.

## 5.2 Enzyme and NP Immobilization

Both the enzyme and the NP can be printed on the paper surface to enable scalable fabrication of these devices. Natural biopolymers, chitosan, and alginate can serve as printing inks. These polymers are excellent materials for the immobilization and stabilization of enzymes and they form transparent films. A recently developed colorimetric paper-based portable enzyme biosensor with the enzyme attached to deposited layers of chitosan and alginate, provided extended stability and functionality of these sensors over 250 days at room temperature (Alkasir, Rossner, & Andreescu, 2015). Similarly, enzyme–polymer conjugates were interlocked in the fibers of paper for enhanced stability and activity of GOX (Riccardi et al., 2016). We used printing conditions to mass-produce $CeO_2$–GOX assay and $CeO_2$ NPs are also synthesized in situ by printing to improve stability and uniformity of the NPs. With suitable optimization of printing ink formulations, the entire sensing assembly, including the enzyme and particle attachment, could be fabricated by inkjet or by 3D printing.

## 5.3 Testing for Interferences

The effect of interfering compounds should be tested for all sensing applications. Control experiments with paper sensors in the absence of enzyme are recommended to check for potential interferences from the sample, in particular, from compounds that may nonspecifically react or alter the color changing properties of $CeO_2$ NPs.

Some compounds that have phenolic structures such as dopamine or phenol can generate a color change by forming charge transfer complexes at the $CeO_2$ NP surface but these changes are only observed if the compounds are present at millimolar concentrations. Interferences can be rejected by the addition of permselective or electrostatic membranes. For example, chitosan has electrostatic rejection properties against ascorbic acid and uric acid (Ozel, Wallace, & Andreescu, 2011) and it can be deposited on the sensing surface to prevent access of these compounds to the active NP platform. Thus, advantages and limitations of these NPs are to be kept in mind in the rational design of biosensors based on $CeO_2$ NPs.

## 5.4 Color Analysis

Color analysis of sensor responses can be achieved via smartphone and tablets with the use of an appropriate color analysis application.

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