Chapter 7

ZnO for Probes in Diagnostics

Debjita Mukherjee¹, Ehsan Amel Zendehdel²#, Mojdeh Rahnana Ghahfarokhi³#, Minoo Alizadeh Pirposhte⁴#, Azadeh Jafarizadeh Dehaghani⁵#, Agnese Brangule⁶,⁷*, Dace Bandere⁶,⁷*, and Jhaleh Amirian⁶,⁷*

¹College of Medical, Veterinary and Life Sciences, University of Glasgow, University Ave, Glasgow G12 8QQ, United Kingdom
²The faculty of Art and Architecture, Eshragh Institute of Higher Education, Bojnord, Iran
³Department of Materials Engineering, Faculty of Materials Processing and Fabrication, Isfahan University of Technology, Isfahan, Iran
⁴Department of Materials Engineering, Faculty of advanced materials, Isfahan University of Technology, Isfahan, Iran
⁵Department of Materials Engineering, Faculty of Materials Processing and Fabrication, Isfahan University of Technology, Isfahan, Iran
⁶Department of Pharmaceutical Chemistry, Riga Stradiņš University, Dzirciema 16, LV-1007, Riga, Latvia
⁷Baltic Biomaterials Centre of Excellence, Headquarters at Riga Technical University, Kalku Street 1, LV-1658 Riga, Latvia

# These authors contributed same.
*Prof. Dace Bandere (dace.bandere@rsu.lv)
Dr. Agnese Brangule (agnese.brangule@rsu.lv)
Jhaleh Amirian (jalehamirian@gmail.com)

Abstract

Nanoparticles have revolutionized the field of diagnostics in recent years and ZnO nanoparticles (ZnO-NPs) have been one of the most commonly used ones. These easily synthesizable ZnO-NPs have a multitude of advantages over other metal-based nanoparticles owing to their biocompatibility, easy functionalization through their hydroxyl group-rich surface, and cost-effectiveness among several other benefits. Due to their inherent luminescence and fluorescent-tag functionalizing properties, ZnO-NPs have been useful as a probe in tumour and live cell bioimaging. ZnO-NPs have also been identified as probes in biosensors for the detection of various clinically important biochemical analytes like glucose and cholesterol, pathogens, drug molecules, and antibody-antigen based detection systems. In this chapter, several of the different
applications of ZnO as probes in diagnostics will be dealt with in detail. Also, the characteristics of ZnO nanoparticles useful for such applications and the way these devices and techniques are developed will be explained.

**Keywords**

ZnO Nanoparticles (ZnO-NPs), Biosensors, Diagnostics

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1. **Introduction**

Since the advent of nanomaterials in the late 1900s, several novel applications for these materials have been discovered. As technology progressed, healthcare systems have improved greatly with nanomaterials replacing bulk materials in most sectors ranging from the detection of diseases to their treatment. Like some of the other metal oxide nanomaterials, Zinc Oxide (ZnO) nanomaterials have found extensive applications in several fields due to numerous beneficial characteristics. This chapter focuses on the various applications of ZnO nanomaterials in diagnostics, describing in detail some of the recent and most interesting systems that have been researched.

1.1 **Nanomaterials in diagnostics**

To be useful as probes in diagnostics, the nanomaterials need to possess most or all of the following characteristics [1-2]:

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**ZnO Nanoparticles (ZnO-NPs), Biosensors, Diagnostics**
Large surface-to-volume ratio with small size ranging from 1 to 100 nm to be able to reach very small areas and/or detect minuscule structures
Ease of modifying the physical properties (composition, size, shape) of the nanomaterial based on application
Good target binding properties with the ability to bind a wide range of biologically relevant molecules
Robustness of the structures
Biocompatible
Does not interfere with the structural and/or functional integrity of the analyte molecule
Cost-effectiveness and simple production procedure

Not all of these characteristics are found in all nanomaterials hence making them unsuitable for biological applications. However, several features of the different ZnO nanomaterials turn out to be useful for their application as diagnostic probes. The noteworthy features of ZnO nanomaterials are as follows [3-4]:

These have dimensions like the bio-components which are used to form the bio-selective layer.
The large surface-to-volume ratios are very useful for increasing sensitivity of the analysis
Being a metal oxide material, ZnO is an n-type semiconductor with a wide band gap of 3.37 eV
It has a Bohr exciton radius of around 2.34 nm and a high dielectric constant.
ZnO nanomaterials possess unique electron transport ability and show multifunctionality based on electric conductance facilitating immobilization of various types of biomolecules
Functionalization of these materials is facilitated by their hydroxyl group-rich surface
These have a high isoelectric point (pH 9-9.5)
These materials have a broad photoluminescence (PL) range and can show highly intense photoluminescence at room temperature (with ultraviolet (UV), infrared, microwaves, radio waves, and even visible light).
When ZnO materials are irradiated with UV light, an electron/hole pair is created resulting in excitonic emission
They can be synthesized following simple steps and easily available raw materials and are therefore cost-effective
The ZnO nanomaterials have been tested for various diagnostics-based approaches. Materials such as nanorods (NRs), nanoparticles (NPs), nanowires (NWs), and quantum dots (QDs) are some of the most used nanomaterials of ZnO for diagnostics [5]. The commonly detected analytes belong to the following broad categories:

- Proteins (such as albumin, immunoglobulins, haemoglobin, etc.)
- Low molecular weight compounds (such as acetone, urea, riboflavin, dopamine, etc.)
- Nucleic acids (DNA or RNA)
- Cells (from tumour/cancer, somatic cells) and microbes (virus, bacteria, fungi, etc.)

2. Types of detection mechanisms for ZnO probe-based analysis

There are various methods currently available for diagnostic techniques based on ZnO nanomaterials. The basic working principles of the most commonly used ones have been briefly described below:

2.1 Photoluminescence detection

Based on the inherent PL properties of ZnO, many biosensors made of ZnO nanomaterials use this technique for detecting the final analyte using spectroscopy or microscopy. In this technique, light (photons) in the ultraviolet (UV), visible, or near infrared (NIR) range are directed to a sample. The photons are absorbed by the sample and excite the sample’s electrons to higher excitation states (photoexcitation) [6]. The excited electron goes back to its equilibrium state after some time which results in the emission of the energy (photoemission) in the form of light. The resulting luminescence can be fluorescence, phosphorescence, or chemiluminescence depending on internal energy transitions, transition into a state with different spin multiplicity, or chemical reaction during transition (Figure 1). At a particular excitation wavelength, the maximum intensity of the emission can be observed for the sample which can help get a better resolution. Factors such as stability, quantum yield, energy transfer, and characteristics of excitation or absorption determine how suitable a nanomaterial is for PL applications based on its emission strength [7].
2.2 Diagnostic imaging techniques

The ZnO-based nanocomposites for bioimaging are commonly detected by Magnetic Resonance Imaging (MRI) and tomography (Computed/Positron emission/Single Photon emission computed) techniques. The working principle of MRI is based on the nuclear spin properties in the presence of a very strong external magnet [8]. The strong external magnetic field of the magnet results in parallel (low-energy state) or perpendicular (high-energy state) alignment of the nucleus with respect to the external field. A second radio frequency (RF) magnetic field (pulses) is applied perpendicular to the external field. This RF field results in the transition of the molecules/cells from a higher to a lower energy level after energy absorption. The energy absorbed is again emitted when relaxation occurs. The absorbed/emitted energy is detected by a coiled wire as voltages. The signals are amplified, and the results are obtained as Free Induction Decay (FID). Fourier transform analysis of the averaged FID can help understand the biochemical state of the tissues and can help distinguish between cancerous and healthy tissues. A basic setup of the MRI scan system has been shown in Figure 2. (A).

Nuclear medicine or tomography-based bioimaging works with the help of radiolabelled probe-tracing with gamma radiation trackers/detectors (cameras in most cases) [9]. The Single-photon emission computed tomography (SPECT) is based on the Compton effect, where a photon beam is emitted after interacting with electrons present in the tissues (Figure 2. (C)). The photons are either deflected away from the detector’s direction (attenuation) or are deflected towards the detector but with an altered direction of incidence to the detector (scattering). The photons emitted from the radiotracer are considered independent events to obtain specific information regarding the biochemical condition of
the tissues to form an imaging matrix. Positron-emission tomography (PET) involves positively charged electrons emitted due to the radioactive disintegration of the probe nucleus. The positron keeps losing kinetic energy and gets deflected from its original direction. It finally combines with an electron, annihilates, and finally emits two photons which are detected by the detector (Figure 2. (b)). The tomography techniques are capable of showing the function of tissues in a given region even before anatomical changes occur in these tissues.

Figure 2: Basic setup of common diagnostic techniques (A) MRI, (B) PET, and (C) SPECT.

2.3 Electrochemical detection

Most of the biochemical sensors are based on electrochemical or surface plasmon resonance (SPR) techniques because of high-sensitivity detections and ease of control of parameters affecting the detection. In electrochemical systems, two or three electrodes are commonly used [10]. A standard 3-electrode system contains the following:

(i) Working electrode: this is the main electrode which is mostly modified to improve functionality or sensitivity; it acts as a transducer for biochemical sensors
(ii) Counter electrode: this controls the flow of current to the working electrode
(iii) Reference electrode: it provides a stable potential to the working electrode

The biological element or target is detected by the working electrode through transduction and is then converted into electrical signals for analysis. This detection method is broadly classified into current-based (amperometric), charge, or potential-based (potentiometric such as voltammetry) and based on the variation in conductance of the medium
(conductometric). In most cases, the bio-recognition element (such as antibodies, antigens, or antibody fragments) is added as coatings on the working electrode for detecting analytes like specific microbes, cells, nucleic acids, or enzymes. Figure 3. (A) shows how electrochemical systems can be used in biosensing.

The other commonly used technique, SPR, is only observed at the metal-dielectric interface. In this method, a surface plasmon (electromagnetic plasma wave) propagating at the metal-dielectric interface, can be excited by an evanescent wave resulting in SPR [11]. These excited plasmons decay resulting in energy conversion to photons and a drastic decrease of the reflected wave. When this ray travels in a lower refractive index medium it undergoes total internal reflection (TIR) and the resonance angle (θ<sub>SPR</sub>) changes which is an indication of the absorption-desorption or association-dissociation phenomena occurring at the interface (Figure 3. (B)). In a standard SPR system, a Kretschmann configuration is followed. A prism is used in this configuration where the incident light propagates and reaches a gold film to which a testing cell is attached containing the solutions to be analysed. TIR occurs at this interface till the point where the angle of incident light is greater than the critical angle. At a particular angle, the evanescent waves can excite the plasmons resulting in SPR at the gold film interface. The incident angle of minimum reflectivity (or the SPR angle) can change due to changes in the refractive index of the test solution. Such changes in refractive index can be induced by modifying the mass and density of the solution which happens due to foreign bodies attaching to the gold film surface. The SPR angle (θ<sub>SPR</sub>) is given by the equation:

\[
\theta_{SPR} = \sin^{-1}\left(\frac{1}{n_1}\sqrt{\frac{(n_2n_g)^2}{n_2^2 + n_g^2}}\right)
\]

where, 

- \(n_1\) is refractive index of medium 1
- \(n_2\) is refractive index of medium 2 at interface (\(n_1 > n_2\))
- \(n_g\) is refractive index of gold film
Figure 3: (a) Electrochemical detection system as electrochemical cell (left) and as screen-printed electrode (right) for biosensors ; (b) Biosensing using SPR technique.

3. Applications

3.1 ZnO-NPs in cancer diagnostics

Cancer or uncontrolled proliferation of cells at specific sites has become a common life-threatening disease in the modern world. Incidences of cancer increase globally every year. The International Agency for Research on Cancer (IARC) published statistics about the cancer situation in 2020 where around 19.3 million people were diagnosed with cancer and around 10 million deaths attributed to cancer [12]. Most of these deaths were due to late diagnosis of the condition which leaves very little time for treatment [13]. Several improved diagnostics are being developed currently to enable timely diagnosis of cancer with high specificity and sensitivity. Cancer diagnostics mainly involve one or all of the following [14]:

(i) *In vivo* bio-imaging of tumours using techniques such as upconversion luminescence (UCL), MRI, and such other.
(ii) Detection of cancer cells based on their surface functionalization
(iii) Detection of the moving tumour or vesicular cancerous cells
(iv) Biomarker-based detection

In the aspect of cancer diagnosis, ZnO nanomaterials are investigated both as single-material probes or in conjunction with other nanomaterials. The ZnO nanomaterials have inherent luminescence properties and can also easily get functionalized to the surfaces of
several fluorescence-emitters or other moieties useful in bioimaging in vivo thanks to their non-toxic properties [15]. Currently, ZnO nanocomposites are also being explored to enhance signal resolution. Nanostructures made of ZnO are also being implemented in biosensing elements for detecting cancer biomarkers due to factors such as the immobilization efficiency of antibodies to its surface, ease of fabrication, and high specificity [16].

The enzyme matrix metalloprotease 9 (MMP-9) has been identified as a biomarker for malignancy in tumours and cancer malignancy [17]. Normally, MMP-9 plays a significant role in the maintenance of the extracellular matrix (ECM) and the physiological processes of the central nervous system (CNS). The normal range for MMP-9 in blood serum ranges from 1-100 ng/mL (up to 500 ng/mL) [18]. In 2020, Shabani and colleagues developed a label-free biosensor where ZnO NPs were spin-coated and annealed over a gold (Au) electrode substrate and the ZnO nanorods were fabricated hydrothermally. Mouse monoclonal antibodies against MMP-9 were immobilized on these nanorods. Results were analysed using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) where linearity was observed for an MMP-9 concentration range of 1-1000 ng/mL which can cover the normal detection range for MMP-9. The biosensor had a detection limit of 0.15 ng/mL and showed a sensitivity of 32.5 μA/(decade x cm²). Compared to the traditional ELISA method, this biosensor produced results within a much shorter time (around 35 minutes) and with only an about 8% difference in accuracy.

Several kinds of research have been conducted to find techniques for using ZnO NPs in biomarker-based diagnostics. With PVA, ZnO NPs have been used for immobilizing biomolecules for identifying the epithelial cancer biomarker, EpCAM (Epithelial cell adhesion molecule) by Zhu and colleagues [19]. In another study, ZnO NRs were used by Wang and his group to coat a quartz crystal microbalance for label-free detection of CA 15.3 (breast cancer biomarker) [20]. Murugan and co-workers fabricated a gradient triple-layered core-shell NP with ZnO as one of the core components and silica as the shell for detecting the cancer biomarker, acetylcholine [21].

Some ELISA-based detection methods involving ZnO nanomaterials have also been used for antigen-based cancer diagnostics. In 2015, Pal and Bhand immobilized monoclonal antibodies for carcinoembryonic antigen (CEA) (capture antibodies) to ZnO NPs and added them to microwell plates [22]. A polyclonal antibody was used for detection while an HRP-labelled tertiary antibody was used for colorimetric detection. They observed a 3-fold increase in chemiluminescence compared to standard ELISA along with improvements in thermal stability. The system could detect CEA levels in blood serum from 1 pg/mL to 20 ng/mL.

Due to their inherent PL properties and semiconductor-like properties, ZnO nanomaterials have found extensive applications in the bioimaging of cancer cells and tumours. The chemiluminescence (CL) properties were applied for bioimaging of HeLa cells (cervical cancer cell-line) by Liu and co-workers in 2020 [23]. The CL activity is observed on addition of the compound bis (2,4,5-trichloro-6-carbopentoxyphenyl) oxalate (CPPO) and
H$_2$O$_2$ to the ZnO NPs to produce strong blue luminescence. The intermediate dioxetanedione intermediate produced during the reaction has high energy and can react with the interstitial zinc (Zn$_i$) to result in CL. To enhance the CL properties, silica (SiO$_2$) shell was added to the ZnO NPs which increases the quantum yield of the system from $6.2 \times 10^{-6}$ to $3.72 \times 10^{-4}$ E mol$^{-1}$. The ZnO@SiO$_2$ NPs allow dual-mode imaging of live cells as the ZnO NPs have inherent PL properties and yellow fluorescence can be observed when illuminated with 365 nm UV light. The ZnO@SiO$_2$ NPs show high specificity and a very low level of cytotoxicity (more than 90% viability) till a concentration of 500 μM.

For *in vivo* bioimaging of tumours, ZnO NPs have been used as part of the probes in conjunction with other materials to enable multi-modal imaging to obtain better specificity and detailed information about the tumour. A very interesting system was developed by Hong and colleagues in 2015 for multi-modal tumour imaging in mice [24]. The ZnO NPs showed red fluorescence and allowed detection by Positron emission tomography (PET) thereby enabling dual-mode imaging. The ZnO NPs were synthesized by calcination, and they were PEGylated after thiolation. The ZnO-PEG-NH$_2$ (1 part) was reacted with S-2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) (10 parts). The NOTA-ZnO-PEG-NH$_2$ (1 part) and succinimidyl carboxymethyl PEG maleimide (SCM-PEG-Mal) (30 parts) to obtain the intermediate NOTA-ZnO-PEG-Mal. Both of these reactions occur at pH 8.5. A biomarker, CD 105 is commonly detected in proliferative tumour cells which were targeted using the TRC-105 antibody which was conjugated to the NOTA-ZnO-PEG-Mal intermediate to form the NOTA-ZnO-TRC105. Finally, radio-labelled copper ($^{64}$Cu) was added to the antibody-conjugated system to form the final PET-detection probe which was shown to detect 4T1 tumour in mice. Addition of the TRC105 antibody to the nanocomposite improved accumulation (almost by 3-fold) of the composites specifically at the tumour site. Figure 4 shows the step-by-step synthesis of the ZnO NP-based probe and PET scan results in mice using the synthesized probe.

Most of the bioimaging techniques that have been developed for cancer diagnostics focus on serving more than the purpose of just bioimaging. These bioimaging techniques also target therapy or drug delivery applications and thereby act as theranostic agents. This topic has been covered in detail in the last section of this chapter (Section 2.4).
Figure 4: The stepwise synthesis of the ZnO NP-based probe and in vivo 4T1 tumour analysis in mice using PET scan with the synthesized probe.

(Reprinted (adapted) with permission from {Hong et al. (2015). Red fluorescent zinc oxide nanoparticle: a novel platform for cancer targeting. ACS Appl. Mater. Interfaces 7, 3373-3381.}. Copyright © {2015} American Chemical Society)

3.2 ZnO-NPs in microbial disease diagnosis

Microbial diseases are currently the cause of mortality in a large part of the global population. Over the past few years, several viral diseases such as Ebola, swine flu, SARS, and several bacterial diseases such as pneumonia, tuberculosis, cholera, and meningitis, have significantly affected life expectancy [25]. Detecting these diseases is a challenge not only because of the microscopic size of the causative agents but also the time needed for obtaining the results for diagnosis and treatment. Some of the most commonly available techniques for microbial disease diagnosis include immunoassays such as ELISA, RT-PCR, bacterial culture analysis, viral isolation in chicken embryos, and a few other techniques which involve complex steps or time-consuming techniques [26]. In several cases, these techniques have problems with sensitivity or problems with a precise analysis of the bacterial/viral load. Recently, the COVID-19 pandemic showed how important fast diagnosis of such microbial diseases can help to control the spread and severity of the disease [27]. Nanostructures, due to their excellent surface-to-volume ratio and small size, can play an important role in identifying the microbes in a highly efficient manner within the sample received from the infected persons.
Bacterial infections can be treated efficiently if diagnosed early and accurately. Detection of bacteria is easier based on the various bacterial enzymes and by-products which have been widely studied. A device was developed by Vasudevan and colleagues (2020) for quorum sensing of the signalling molecules known as N-Acyl-Homoserine Lactones (AHLs) from gram-negative bacteria *Pseudomonas aeruginosa* to diagnose urinary tract infection (UTI) [28]. The device is based on photoluminescence (PL) and has probes made of ZnO NPs functionalized with cysteamine which acts as a linker (ZnO-Cys). The ZnO NPs were synthesized using a microwave and following calcination, the cysteamine was immobilized on the NPs in ethanol. The biosensor showed a maximum sensitivity of 97% and a linear detection range of 10-120 nM in Artificial Urine Media (AUM) samples during the lag phase of the bacteria. A significant achievement of this biosensor was that it was able to show the PL signature peak of 468 nm even in the presence of interference factors in AUM.

Point-of-care (POC) devices have been in trend in diagnostics due to several factors like portability, simple operation, low time to result, and ease of handling without the need for professional intervention [29]. For improving the performance of such POC devices, the incorporation of ZnO nanostructures in them is being explored. In 2019, a POC device was developed by Xia and colleagues, for virus detection using ZnO nanorods [30]. The microfluidic device was made of PDMS on a SU-8 mold. The PDMS channels were immersed in ZnO growth media after activation with KMnO₄ to allow the synthesis of the ZnO nanorods on the surface. Mouse antibodies were introduced to these channels after silanization. The nanorods aid in improving the surface area of the detection system (the PDMS channels in this case) by providing a 3-D surface for attachment of the detection probes (the antibodies). The virus sample can now be introduced to this surface and is captured by the antibodies. Another antibody conjugated with gold nanoparticles (AuNPs) is added to develop a sandwich immunoassay. After adding a silver enhancer to this system, the viral load can be estimated by colorimetric analysis using a smartphone-integrated system (Figure 5). The imaging system is developed with a lens, an LED, and two polarizers, together with the smartphone camera. The mean grayscale value of the images was used for analysis. The device has a detection limit of $2.7 \times 10^4$ EID₅₀/mL with naked eyes and $8 \times 10^3$ EID₅₀/mL with the smartphone-based imaging system which is one order of magnitude and three times better than the conventional fluorescence-based ELISA, respectively. The time to result from the virus capture process is only 1.5 hours. The device was used for Avian Influenza virus (AIV) analysis in this study, but it has been mentioned to be usable for other viral detections as well.
DNA isolation from bacteria or fungi has been a common technique for microbial diagnostics. The conventional commercial DNA extraction kits available currently involve disruption of the cell wall of the microbe, extraction of DNA in sodium dodecyl sulphate (SDS), removal of unnecessary proteins, followed by the precipitation of DNA using isopropanol [31]. In the case of fungal DNA extraction, an added step of lyophilization of mycelia is done before the above-mentioned steps. Qiao and colleagues have explored ZnO NPs as an alternative to the standard lysis buffer used in the isolation kits due to their inhibitory effects on microbes. The ZnO NPs was found to be useful as a lysis buffer for bacterial and eukaryotic cell but had limitations of application in fungal DNA isolation due to their cell wall. However, in 2020, Qiao and colleagues found a combined form of ZnO (ZnO-S-300) which could overcome this limitation in the fungi Aspergillus. The resulting assay not only makes the process much less cumbersome but also reduces the use of bulky, expensive equipment. Unlike normal ZnO NPs, the ZnO-S-300 surface is positively charged leading to easy binding of this material to the spores.

Biosensors with ZnO-based nanomaterials have been developed for detecting bacteria and virus infections. One such device was made by Huang and co-workers in 2020 for acid-responsive and bimodal detection of the common pathogenic bacteria Salmonella which
spreads through contaminated food/water and from animals and can cause severe food poisoning and diseases such as typhoid [32]. In this system, ZnO is used as a capping agent for a conjugated system with mesoporous silica nanoparticles (MSNs) and Curcumin (CUR) [33]. The CUR acts as a dual-signal reporter on acid stimulation with acetic acid (HAc) which is unique among the existing Salmonella biosensors. The microfluidics-based system used three inlets for 3 different things: (i) amine-modified MSNs were incubated with CUR to form NPs followed by capping with amino-modified ZnO NPs to form MSN@CUR@ZnO NPs (MCZ NPs) and finally polyclonal antibodies against Salmonella were functionalized with tetrazine and trans-cyclooctene, (ii) magnetic NPs with monoclonal antibodies against Salmonella, and (iii) the Salmonella sample to be detected. After mixing in a Koch fractal structured chamber, a sandwich immunoassay is established. The HAc is then introduced to this system which allows dissolution of CUR which is then collected for fluorescence and absorbance measurements. The biosensor worked for 102 to 107 CFU/mL of the Salmonella bacteria and showed results within 1.5 h. The lower limit of detected colonies was 63 CFU/mL and 40 CFU/mL for colorimetric and fluorescent measurements, respectively with high recovery percentages.

Among other uses of ZnO NPs in microbial diagnosis, they have been used for improving diagnostics using techniques such as PCR. In 2020, Upadhyay and colleagues presented a method of using a relatively newer type of ZnO nanomaterial, nano-flowers, for developing a nano-PCR technique to diagnose canine vector-borne disease (CVBD)-causing pathogens [34]. Hydrothermally prepared ZnO nanoflowers were incorporated with the pathogenic samples in a concentration of 1 mM which showed significant enhancement of obtained results. The pathogenic strains of Babesia canis vogeli and Hepatozoon canis were diagnosed successfully using the nano-PCR method. The adsorption of the ZnO nanoflowers to the isolated DNA samples thereby improving the sensitivity of the PCR and simultaneously helping reduce the turnaround time of the process without affecting the amplification process in any way. Figure 5 shows the intricate structure of the nanoflowers and the confirmation of the nano-PCR diagnosis of Babesia canis vogeli and Hepatozoon canis at 619 bp and 666 bp respectively in the agarose gel electrophoresis analysis.

ZnO nanomaterials are being investigated to find novel applications in microbial diagnostics. Only a few of such works have been discussed in detail above. Other than the works mentioned in this section, there have been several such research involving ZnO nanomaterials as components in probes or sensor devices for microbial analyte detection. The next section deals with biosensors based on ZnO nanomaterials.
3.3 ZnO-NPs in biochemical biosensors

The ZnO nanomaterials do not only find application in detecting tumours or cancers, and microbial nucleotides but can also be used for the detection of important chemicals and biomolecules such as biomarkers and macro or micro-nutrients [35]. This makes ZnO NPs important as materials of choice for biosensors. Some electrochemical detection-based biosensors have been developed using ZnO NPs.

Detection of chemicals such as glucose is particularly important for diagnosing and monitoring the common disorder, Diabetes, which affects a significant part of the population [36]. Diabetes (Type 1 and Type 2) occur due to the improper functioning or absence of the hormone insulin which breaks down the glucose in the blood to be converted into glycogen which is accepted into the cells for metabolic activities. Glucose levels of 3.9mM (70mg/dl) indicates hypoglycaemia (low blood glucose) and levels greater than 10mM (180mg/dl) indicates hyperglycaemia (high blood glucose) both conditions being potentially lethal for a human if left untreated [37]. Mutuchamy and co-workers (2018) developed a high-performance electrochemical biosensor with Nitrogen-doped Carbon sheets (NDCS) embedded with ZnO NPs for estimating blood glucose levels [38]. Interestingly, a green approach was followed for synthesizing the ZnO@NDCS using a hydrothermal method with zinc powder (for ZnO), aqueous ammonia (for nitrogen), and peach fruit (for carbon sheets) as precursors. The sensor was fabricated on a glassy carbon
electrode (GCE) with glucose oxidase (GOx). The GCE/ZnO@NDCS/GOx biosensor showed a linear detection range from 0.2 to 12 mM which nicely covers the clinical range of normal blood glucose levels. The system showed the lowest detection limit of 6.3 μM, a sensitivity of 231.7 μA mM$^{-1}$ cm$^{-2}$ and an extremely low time of 3 seconds for amperometric current-based detection from human blood serum. The synthesis method and mechanism of glucose detection are depicted in the image below (Figure 7).

![Figure 7: Green synthesis of ZnO NPs embedded- Nitrogen doped carbon nanosheets and the reactions occurring at the modified electrode for glucose detection. (Adapted with permission from {Muthuchamy et al. (2018). High-performance glucose biosensor based on green synthesized zinc oxide nanoparticle embedded nitrogen-doped carbon sheet. J of Electro. chemistry, 816, pp.195-204.). Copyright © {2018} American Chemical Society)](image)

In 2011, Devi and colleagues developed a ZnO NP-based biosensor for detecting the purine base compound, xanthine in fish meat [39]. In previous studies, it has been indicated that xanthine metabolizes in the body to produce uric acid (UA) which can be dangerous if not excreted out of the system correctly. Uric acid accumulation can lead to diseases such as diabetes, gout, fatty liver disease, and even cardiovascular diseases [40]. Certain purine-rich foods such as fish and meat can lead to accumulation of UA triggering such diseases, based on their freshness leading to an increase in xanthine levels. This makes it important for detecting xanthine levels of such foods before determining their consumption period and sending them to market [41]. The biosensor developed by Devi and colleagues involved making a nanocomposite film on a platinum (Pt) electrode composed of ZnO NPs and pyrrole (ZnO/polyPyrrole/Pt) by electro-polymerization followed by physisorption of
xanthine oxidase (XOD) to form the working electrode. With Ag/AgCl as the reference electrode and a Pt wire as auxiliary electrode, the xanthine biosensor was constructed which showed linear detection levels for xanthine from 0.8 μM to 40 μM within just 5 seconds. The biosensor was found to lose just 40% of its efficiency over 100 days of use (200 tests) which proves the coating to be quite stable [39].

The very commonly available sterol in the human body, cholesterol, is produced mainly in the liver and is present as low-density and high-density lipoprotein (LDL and HDL). The cholesterol serves as a biomarker for several cardiovascular diseases (CVD), stroke, cardiac arrest, and even can indicate Type II diabetes [42-43]. Recently, Agrawal and colleagues developed a biosensor for detecting cholesterol levels based on Localized Surface Plasmon Resonance (LSPR) unlike the traditionally used amperometric electrochemical sensors [44]. They used Optical Fiber Sensors (OFSs) such as multimode-photosensitive-multimode (MPM) and single-photosensitive-single (SPS) (together known as core-mismatch Fibers) due to several advantages such as robustness, ease of fabrication, low cost, and a broad range of measurements. For immobilization, AuNPs of varied sizes (10 nm and 30 nm) were used for making 3 probes and coated with ZnO NP and combined with the OFSs. The 10 nm probe with MPM showed the best sensitivity of around 0.6898 nm/mM. Linear results were observed for a wide range of 0.1-10 mM which included the clinical level of around 5.17 mM of Cholesterol in blood serum. A limit of detection as low as 0.6161 mM was observed.

Another important compound in the human body is hydrogen peroxide (H₂O₂) which has found significance as a biomarker for diseases such as Alzheimer’s disease (AD), inflammatory diseases, CVD, and cancer [45]. When H₂O₂ levels increase in the body beyond normal levels, the above-mentioned diseases can be triggered. But under normal conditions, H₂O₂ helps in physiological activities such as cell signalling, vascular development, and immune cell activation. In 2018, Sekar and his group developed a biosensor for detecting H₂O₂ levels based on ZnO-PVA nanocomposite synthesized by electrospinning [46]. Bioelectrodes were made with this nanocomposite in conjugation with the enzyme catalase (CAT) which is a heme protein-based enzyme that allows the conversion of H₂O₂ into oxygen and water and enhances the specificity and sensitivity of the biosensor. A thin membrane of chitosan was added further to the electrode surface which provides a smooth and stable morphology to the electrode surface for better sensing. The nanocomposite enhanced electrical conductivity, improved mechanical strength, and better immobilization of CAT. Analysis was done with cyclic voltammetry and the Au/ZnO-PVA/CAT/Chitosan bio-electrode showed linearity for the range of 1 μM to 17 μM with a very low limit of detection of 9.13 nM. A high sensitivity of 210 μA μM⁻¹ cm⁻² was observed with a time to result of even lesser than a second.

Biomarker detection has been an especially important field of application where ZnO nanomaterials have been extensively used. Many other groups have researched the development of ZnO-based biosensors for glucose [47-50], cholesterol [51-54], xanthine [55-57] and hydrogen peroxide [58-61]. Some of the other significant studies include the metal-organic framework-derived cobalt-doped ZnO NR gas biosensor for acetone
detection as a diabetes biomarker [62]. A similar copper-doped ZnO NP was developed for
the CVD biomarker myoglobin [63]. Based on the semiconductor-like properties of ZnO
NPs, a field effect transistor system was developed for the CVD biomarker Troponin I for
enhanced modulation of current and antibody-based biomarker molecule capture [64]. A
self-assembly based fabrication strategy was used to produce ZnO NP- rGO electrodes for
dopamine detection [65].

Other than biomarker detections, ZnO NPs can be applied in biosensors for estimating drug
concentrations as well which can indicate the dosages and efficacy of administered drugs
for instance in chemotherapy. In a very recent study, Karimi-Maleh and co-workers
developed a DNA-based biosensor for estimating levels of the chemotherapeutic drug,
idarubicin (IDR). The drug, IDR, is used to treat several types of cancers with leukaemia
and lymphomas being the common ones. IDR can intercalate into the major groove of the
DNA double-helix and binds through the guanine and cytosine bases [66]. In the biosensor
device, the ZnO NPs were incorporated with Platinum (Pt) and Palladium (Pd), and these
NPs were added to the single-walled carbon nanotubes (SWCNTs) and were obtained by
the combination of chemical precipitation and one-pot method, to apply as a modification
to GCE [67]. A double-stranded DNA (dS-DNA) from calf thymus was added, using a
layer-by-layer deposition on the nanocomposite, as a biological recognition element to
improve the sensitivity and efficiency of the system. The signal from the guanine base of
the dS-DNA was measured and a range of detection from 1.0 nM to 65 μM was obtained
with a low limit of detection of 0.8 nM. A recovery of ~98% to ~105% was observed for
this system. The device could be used for both, diagnosis of drug levels and as a support
for docking analysis of the drug molecule. A similar application was done for ZnO NPs
after doping with Calcium to detect concentrations of an anti-viral drug named Acyclovir
[68].

The biosensors involving ZnO NPs are huge in number. A few of the recent and interesting
ones were discussed in this part. Several groups around the world are synthesising novel
combinatorial materials with ZnO NPs to develop new diagnostic platforms for biosensor
development.

3.4 ZnO for theranostic applications

The ZnO nanomaterials have several advantages over other materials one of which is their
multi-functionality. They not only find application in diagnostics but also the same
materials can fulfil therapeutic actions in the patient’s system. A lot of ZnO-based
nanomaterials have been used for theranostic applications such as immunotherapy, drug
delivery, or cytotoxic destruction for treating diagnosed tumours/cancers [69]. The ZnO
NPs show certain advantages like low toxicity and high stability under normal biological
conditions. As described in previous sections of this chapter, ZnO NPs are widely
applicable in bioimaging due to PL and semiconductor-like properties [70]. They are also
quite cost-effective and have an eco-friendly manufacturing process unlike the non-
biocompatible cadmium (Cd)-based nanocomposites (NCs) which had highly polluting
production processes [71]. The ZnO quantum dots (QDs) have shown significant
applications in theranostics due to their quantum-level properties such as broad absorption and narrow emission bands, low self-absorption, tunable emission wavelengths, large Stokes shifts and stable both photochemically and metabolically [72].

On irradiation of ZnO crystals with UV, when in aqueous suspension, electron/hole pair generation takes place which is a characteristic of semiconductors [73]. In ZnO nanomaterials, this leads to photochemical reactions being triggered in the system which leads to the generation of reactive oxygen species (ROS). The valence band holes present on the surface of these materials react with the hydroxyl ions present in water generating hydroxyl radicals (OH•), while oxygen is also reduced to generate the superoxide anion (O₂⁻). The ROS production is also enhanced by any pro-inflammatory response of the cell against these nanomaterials. This can be extremely useful for local heat-based ablation of cancer cells as in photodynamic therapy (PDT) where a photosensitive material is excited at a particular wavelength for ROS production inducing localized photodamage in tumour/cancer cells [74]. The figure below (Figure 8) shows how a theranostic NP helps in PDT.

![Figure 8: The mechanism of action of nanoparticles as theranostic agents for PDT [101].](image)


A drawback of these heat-based techniques is that the cells acquire resistance to thermal stress quite easily thereby negating the efficiency of the process. Chen and colleagues (in 2014) developed an integrated nanoassembly-based platform using ZnO NPs along with
reduced graphene oxide (rGO) and Hyaluronic Acid (HA) [75]. The photoactivity and ROS-producing capacity of ZnO; the graphene acts as an enhancer for the ZnO by preventing the recombination of the electron-hole pairs generated. Graphene also has high optical absorption at the nano-infrared (NIR) range. The nanoassembly allows sequential irradiation with NIR light which acts as a stimulus for the hybrid ZnO-rGO to generate ROS. The HA is combined with deoxycholic acid (DA) to provide colloidal stability to the nanoassembly. The FITC labelled HA-DA breaks down with the help of the hyaluronidase-1 (Hyal-1) enzyme (abundant in tumour microenvironment) to produce high fluorescence signals with the rGO for target bioimaging of cancer cells [76]. The platform allowed highly selective apoptosis induction in the cancer cells by both PDT and Photothermic therapy (PTT) and allowed fluorescence-based detection of the cancer cells.

Another technique that has been commonly used in cancer theranostics is the use of imaging agents for immunotherapies. In immunotherapy, molecules that play a significant role in immunity such as antigens, antibodies, and cytokines (also adjuvants), are delivered to the cancer tissues. These molecules then trigger the host’s immune response against these cancerous cells and induce apoptosis [77]. In 2011, Cho and colleagues used ZnO-based NPs for dendritic cells (DCs) to trigger an immune response against tumour cells. The NPs had a core-shell structure with the core made up of superparamagnetic iron oxide (SPIO) and the shell made up of ZnO with ZnO-binding peptide (ZBP) which carries tumour antigens which they demonstrated with CEA in their research paper [78]. The SPIO acts as a high-contrast MRI agent whereas the photonic ZnO allows PL-based detection while simultaneously acting as a carrier for the antigens. The NP-antigen complex was delivered to the DCs which then accepted these complexes without any transfection agents. The loaded DCs triggered a good T-cell response against the tumour cells which was confirmed after in vivo analysis in mice where these complex-loaded DCs showed a reduction in tumour growth and greater survivability. For bioimaging, analysis was done using a confocal microscope for in vitro samples and with MRI for the in vivo detection of the tumours in mice.

ZnO NPs have also been identified as a good drug delivery agent which has been another approach commonly used for cancer theranostics involving ZnO nanomaterials. The nanomaterial-based drug delivery systems (DDSs) serve as strategies for increasing the bioavailability and solubility of drugs, controlling the release of drugs at the specific site to prevent cytotoxicity to healthy cells, and providing protection to the drugs from untimely degradation [79-80]. The ZnO nanomaterials have been found to be good pH-responsive DDSs which allow the formation of linkers between the host and the foreign molecules carrying the drugs and can also generate polymeric micelles which carry the drugs inside which are sensitive to pH [71]. The ZnO NPs can also act as pH-responsive “caps” or “gatekeepers” for other drug-carrying nanomaterials such as MSNs to cover the pores present in them thereby preventing leakage of the carried drug [81]. A similar system has been described in Section 2.2 of this chapter for a Salmonella biosensor.

A very innovative multi-functional ZnO-based nanoplatform was developed by Wang and co-workers in 2014 based on the above-mentioned “gatekeeping” role [82]. The system
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uses lanthanide-doped upconverting nanoparticles (UCNPs) for fabricating the core which was developed by doping with different rare earth metals without the need for any further functional modification. The UCNP core (with NaYF₄: 20%Yb³⁺, 2%Er³⁺/ NaGdF₄: 2%Yb³⁺) is surrounded by the MSN shell which carries the chemotherapeutic drug Doxorubicin (DOX) and the pores in this MSN shell are sealed by amino-functionalized ZnO QDs. The UCNP core allows trimodal imaging for high-resolution imaging of the cancer tissues with upconversion luminescence (UCL), computed tomography (CT) scan, and MRI, to get more information about the cancer cells as compared to single-modal imaging. The acidic environment at the tumour site triggers the pH-dependent sustained release of the drug from the MSN capped with ZnO which ensures the drug is not released until the platform reaches the tumour site preventing cytotoxicity to the normal cells. The core allows highly enhanced signals for CT and MRI imaging of tumours in mice which are more durable than standard contrast agents. ZnO QDs dissolve on reaching the tumour site which is found by characterisation using TEM and EDX and quenching of green fluorescence of the ZnO QDs (due to PL) on entering the tumour site.

DOX-carrying or adsorbed diagnostic probes are very popular in ZnO QD-based research and have been extensively studied in various forms and different combinations for either in vitro or both in vivo and in vitro theranostic applications [83-86]. These nanosystems were mostly stimulated by pH alterations or by radiation. ZnO NP-based theranostic agents were also developed for other carrying other drugs such as Paclitaxel and even some antibiotics [87-89]. Based on the inherent properties of ZnO nanomaterials, the effect of the delivered drug gets enhanced [90].

Theranostic applications of ZnO-based nanomaterials are not restricted to cancer/tumour diagnostics and treatment. Several studies have been performed to use the antiseptic properties of ZnO for anti-microbial (anti-bacterial and anti-fungal) therapy post-diagnosis [91]. Some of the common mechanisms for such anti-microbial activity include ROS production, accumulation of NPs inside bacterial cells and liberation of Zn²⁺ ions, and disruption of the bacterial cell wall by electrostatic interactions. Chen and colleagues synthesised a fluorescent nano-probe with BSA-conjugated ZnO QDs (ZnO@BSA) for both diagnosis of bacterial infection and destroying the detected pathogens which in this case are Methicillin-resistant S. aureas (MRSA) [92]. The human antimicrobial peptide (UBI29-41) and the NIR dye, hydrophilic indocyanine green (ICG) derivative, or MPA are covalently functionalized to ZnO@BSA. This system could properly identify bacterial infection compared to cancer or inflammation-induced infections. They also doped the antibiotic Vancomycin, which works as a last-resort antibiotic against MRSA, on the ZnO@BSA-MPA system. The conjugated system showed better drug activity compared to isolated Vancomycin. Also, Methicillin was conjugated to the system instead of Vancomycin and it was surprisingly found to act upon the resistant MRSA strains by making pores on the cell wall of the bacteria due to the conjugated structure. The conjugated ZnO@BSA molecules enable non-invasive diagnosis of bacterial infection and simultaneously plays the role of DDSs for the antibiotics Vancomycin and Methicillin.
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improving their inhibitory effects. Similarly, ZnO-based nanomaterials have been used for treating bacterial infections *in vitro* and *in vivo* [93-95].

Among other applications of ZnO nanomaterials in theranostics include anti-fungal infection treatment [96], anti-inflammatory therapy [97], wound healing [98-99], and treatment for diseases such as diabetes [100].

**Conclusion**

This chapter focused on several interesting applications of the ZnO nanomaterials in the field of diagnostics. Research is in progress for improving these materials to diversify their applications and develop novel devices for improving diagnostics in the current scenario where diseases such as cancer and microbial infections are steadily increasing. The upcoming chapters will deal with other applications of ZnO nanomaterials in other chemical and biological fields.

**References**


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