


Application of the Peroxidase-like Activity of Nanomaterials for the Detection of Pathogenic Bacteria and Viruses

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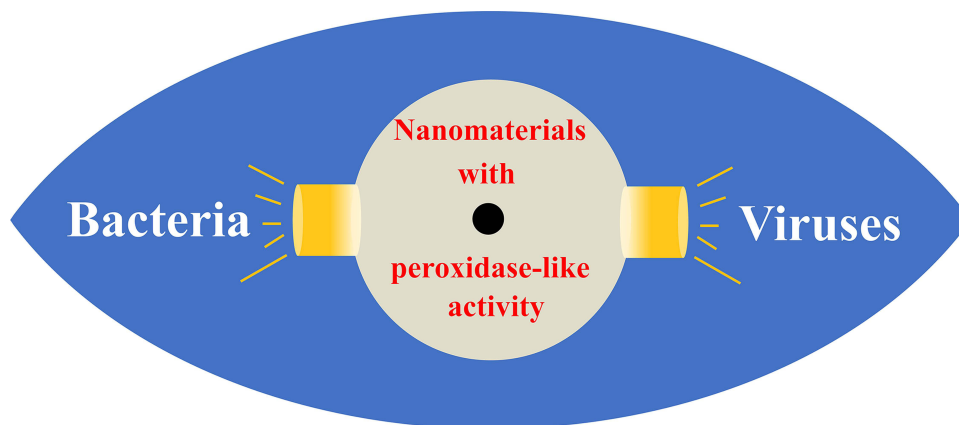
Abstract: Infectious diseases caused by pathogenic bacteria and viruses pose a significant threat to human life and well-being. The prompt identification of these pathogens, characterized by speed, accuracy, and efficiency, not only aids in the timely screening of infected individuals and the prevention of further transmission, but also facilitates the precise diagnosis and treatment of patients. Direct smear microscopy, microbial culture, nucleic acid-based polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA) based on microbial surface antigens or human serum antibodies, have made substantial contributions to the prevention and management of infectious diseases. Due to its shorter processing time, simple equipment requirements, and no need for professional and technical personnel, ELISA has inherent advantages over other methods for detecting pathogenic bacteria and viruses. Horseradish peroxidase mediated catalysis of substrate coloration is the key for the detection of target substances in ELISA. However, the variability, high cost, and environmental susceptibility of natural peroxidase greatly limit the application of ELISA in pathogen detection. Compared with natural enzymes, nanomaterials with enzyme-mimicking activity are inexpensive, highly environmentally stable, easy to store and mass producing, etc. Based on their peroxidase-like activities and unique physicochemical properties, nanomaterials can greatly improve the efficiency and ease of use of ELISA-like detection methods for pathogenic bacteria and viruses. This review introduces recent advances in the application of nanomaterials with peroxidase-like activity for the detection of pathogenic bacteria (both gram-negative bacteria and gram-positive bacteria) and viruses (both RNA viruses and DNA viruses). The emphasis is on the detection principle and the evaluation of effectiveness. The limitations and prospects for future translations are also discussed.

Keywords: nanomaterial, peroxidase-like activity, bacteria, virus, enzyme-linked immunosorbent assay

Introduction

Pathogenic bacterial and viral infections remain major threats to human health.¹ Efficient detection of pathogenic factors is helpful for controlling infection sources, cutting off transmission pathways and protecting susceptible people, as well as providing a diagnostic basis for rescuing infected individuals, which will ameliorate adverse consequences for human health and economic development. Detection methods for pathogenic bacteria and viruses include sputum smear microscopy, microbial culture, nucleic acid-based polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA) based on microbial surface antigens or human serum antibodies. These distinctive methods have made great contributions to the prevention and treatment of infectious diseases. However, the short resolution and sensitivity, different and difficult pathogen cultures, time-consuming detection and the need for specialized personnel, restrict the performance of the sputum smear microscopy method, microbial culture method, and PCR, respectively.^{2–7} Sputum smear microscopy has a relatively narrow application (mainly for *Mycobacterium tuberculosis*).⁸ Traditionally, the identification of pathogenic bacteria involves culturing microorganisms on agar plates followed by biochemical identification. However, this method has several drawbacks, including being time-consuming and laborious, having low sensitivity, and tending to produce false-negative results. PCR detects target bacterial DNA sequences, but cannot

Graphical Abstract



distinguish between live and dead bacteria. Although PCR is faster than microbial culture methods, it still requires expensive equipment and reagents and takes several hours.⁹ In contrast, ELISA is a more convenient and efficient method. Horseradish peroxidase (HRP) is an important component of ELISA, which catalyzes H_2O_2 oxidizing substrates with a color change for visualization of detection results. As a protein, HRP is expensive, and susceptible to denaturation, which leads to a loss of catalytic activity, greatly limiting the application of ELISA in pathogen detection.

Since 2007, a variety of inexpensive, environmentally stable, easily stored and mass-produced nanomaterials have been found to have catalytic properties similar to those of natural enzymes, such as peroxidase, which have thus been termed nanozymes.^{10,11} In addition, these nanozymes have unique physicochemical characteristics, such as photoelectric magnetism, large surface area, diverse morphology, and abundant surface groups. Therefore, as a substitute for horseradish peroxidase, nanozymes can greatly improve the effectiveness of ELISA-like methods for detecting pathogenic microorganisms.¹² The specific groups, antibodies and aptamers modified on the surface of nanozymes can recognize and capture intact individuals, antigens or DNA fragments of pathogenic bacteria or viruses, which brings about a correlation between the total catalytic activities of nanozymes and the numbers of pathogens. The peroxidase-like activities of nanozymes catalyze the generation of hydroxyl radicals ($\bullet\text{OH}$) from hydrogen peroxide (H_2O_2), which in turn oxidize the chromogenic substrate to produce a color change or chemiluminescence to characterize numbers of pathogenic bacteria and viruses.^{13,14} Common chromogenic substrates applied in nanozyme-mediated ELISA-like methods include 3,3',5,5'-tetramethyl benzidine (TMB), ophenylene-diamine (OPD), 2,2'-azidobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2'-diaminobenzidine (DAB), 10-acetyl-3,7-dihydroxyphenazine (also known as fluorescent red), terephthalic acid, and luminal substances.

Detection of Pathogenic Bacteria

Pathogenic bacteria are one of the main factors causing food poisoning, wound infections, dental caries, pneumonia, sepsis, etc.¹⁵ The development of rapid and sensitive bacterial detection methods is of great significance for the rapid diagnosis of infectious diseases, food safety testing, etc.

Gram-Negative Bacteria

Escherichia coli O157:H7 (*Escherichia coli*, *E. coli*) belongs to the Enterobacteriaceae family and is one of the major pathogenic bacteria causing foodborne illnesses, which can cause hemorrhagic colitis and hemolytic uremic syndrome, etc.¹⁶ Gold@platinum nanoparticles functionalized with 4-mercaptophenylboronic acid (Au@Pt) rapidly adsorbed on the surface of *E. coli* to form *E. coli*-Au@Pt complexes through electrostatic adsorption and interaction between the boric acid moiety and lipopolysaccharide (LPS). Then, the *E. coli*-Au@Pt complexes were extracted by centrifugation for quantitative analysis of the number of bacteria. The peroxidase-like activities of Au@Pt nanozymes on the *E. coli*-Au@Pt complexes catalyze H_2O_2

to oxidize TMB to oxTMB (a blue product with a maximum absorption peak at 652 nm). Finally, the number of *E. coli* can be determined by oxTMB production, and the lower limit of detection (LOD) is as low as 7 CFU/mL (Figure 1).¹⁷ Nonspecific targeting of *E. coli* and the requirement for specialized techniques such as centrifugation, impede the promotion of the system. To overcome this shortcoming, anti-*E. coli* monoclonal antibodies but not LPS and immunochromatographic test strips but not centrifugation, were used for specific targeting and isolation of *E. coli*, respectively. In addition, PdPt nanozymes were chosen as HRP mimics for visual detection of target objects.¹⁸ Specifically, an anti-*E. coli* monoclonal antibody was attached to the surface of a PdPt nanozyme for targeted absorption with *E. coli*. *E. coli*-PdPt complexes formed when samples containing *E. coli* flowed through PdPt nanozymes in conjugate pads on lateral flow immunoassay paper. The rabbit anti-*E. coli* polyclonal antibody captured the *E. coli*-PdPt complexes when the *E. coli*-PdPt complexes passed through the test band (T) of the test paper. PdPt nanozymes in complex further catalyzed the oxidation of TMB by H₂O₂ to produce a blue color. The LOD for *E. coli* in milk determined by this method was 9.0×10^2 CFU/mL, which is only one thousandth of that of the conventional colloidal gold-based lateral flow immunochromatographic method.¹⁸ By replacing PdPt with PdRu, a nanozyme-based lateral flow immunochromatographic method was applied to detect *E. coli* in meat products.¹⁹

In addition to *E. coli*, other gram-negative bacteria can also be detected with nanozyme-mediated colorimetric detection systems based on sandwich immunoassays. Cheng et al²⁰ synthesized an anti-*Salmonella* antibody-modified ferric organic framework with peroxidase-like activity as a colorimetric detection system (Ab1-Fe MOF nanozyme) for *Salmonella* detection. The anti-*Salmonella* antibody Ab1 was used for recognition and binding to *Salmonella*, and the Fe MOF nanozyme was used for quantitative analysis of *Salmonella*. Additionally, anti-*Salmonella* antibody-modified magnetic beads (Ab2-magnetic beads) were introduced for the isolation of *Salmonella*. The assay system is much more efficient than the traditional HRP-based colorimetric detection system: the LOD of *Salmonella* was 34 CFU/mL with TMB as the chromogenic substrate according to this detection system, and the recoveries of *Salmonella* in milk were in the range of 94.7–124.0%. A similar assay system (nanozyme: silver nanoclusters; chromogenic substrate: OPD) has also been applied to the detection of *Listeria monocytogenes*, with detection limits as low as 10 CFU/mL.²¹ Combined with a microfluidic system and smartphone imaging, the LOD of foodborne *Salmonella* was 44 CFU/mL with MnO₂ nanosheets.²² With CuO nanozyme as a chromogenic system, *Salmonella* infection can be detected in serum diluted 800 times from typhoid patients, which facilitates the timely diagnosis of patients in the early stage of infection.²³

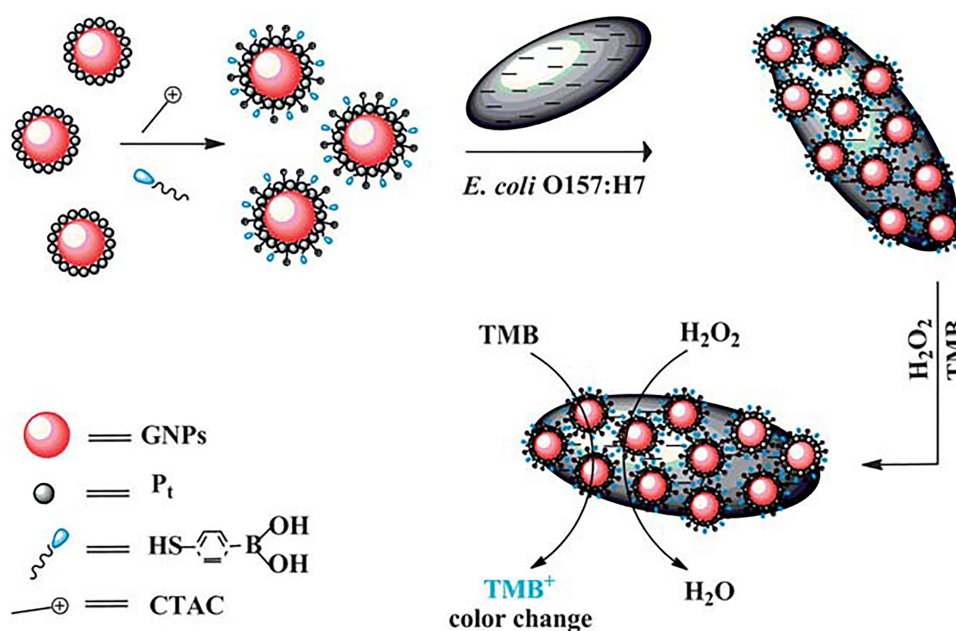


Figure 1 Schematic representation of the colorimetric detection of *E. coli* O157:H7 based on Au@Pt with peroxidase-like activities. Briefly, Au@Pt binding on *E. coli* O157:H7 catalyzes the oxidation of TMB by H₂O₂ to produce a blue color that can be identified by the naked eye or monitored by ultraviolet-visible spectrophotometry. Used with permission of Royal Society of Chemistry, from Su H, Zhao H, Qiao F, Chen L, Duan R, Ai S. Colorimetric detection of *Escherichia coli* O157:H7 using functionalized Au@Pt nanoparticles as peroxidase mimetics. *Analyst*. 2013;138(10):3026–3031;¹⁷ permission conveyed through Copyright Clearance Center, Inc.

The single-stranded DNA aptamer F23 specifically recognizes and binds to both *Pseudomonas aeruginosa* (*P. aeruginosa*) and AuNPs. Nevertheless, its affinity for *P. aeruginosa* is much greater than that for AuNPs. When only AuNPs are present in the assay system, F23 binds to the active site of the AuNPs and inhibits their peroxidase-like activity. When *P. aeruginosa* and AuNPs are present in the assay system together, F23 preferentially binds to *P. aeruginosa* but not to AuNPs. AuNPs are free and able to catalyze the oxidation of TMB by H_2O_2 to a blue product. The difference in color change in the presence or absence of *P. aeruginosa* can be used to evaluate infection status.²⁴ Taking the electrochemical activity of TMB into account, further improved the sensitivity of the assay system, and the LOD of *P. aeruginosa* was 60 CFU/mL.²⁴

In addition, Fe_3O_4 nanozymes, graphene quantum dot nanozymes, and PtPd nanoparticle functionalized porphyrin metal-organic framework nanozymes (PtPd@PCN-224) were also used to detect *Enterobacter sakazakii* (LOD: 10 CFU/mL),²⁵ *Yersinia enterocolitica* (LOD: 5 CFU/mL),²⁶ and *Burkholderia cepacia* (LOD: 12.8 aM),²⁷ respectively.

Gram-positive Bacteria

Staphylococcus aureus (*S. aureus*) is a foodborne pathogenic bacterium that produces enterotoxins, and often induces infectious diseases including pneumonia, pseudomembranous colitis, local suppurative infection, pericarditis, and sepsis.²⁸ Li et al²⁹ developed a nanozyme-based detection system for assessing *S. aureus* infection (LOD: 24 CFU/mL) (Figure 2). First, vancomycin was immobilized on 96-well microtiter plates to capture and hold *S. aureus* from the samples. Second, Au@FePPOP_{BFPB} modified with Ab2 antibody (bs-4582R) was introduced into the assay solution. The Ab2 antibody (bs-4582R) was capable of recognizing and binding the cell surface protein A of *S. aureus*, which promoted the formation of a vancomycin-*S. aureus*-Ab2-Au@FePPOP_{BFPB} sandwich immunocomplex. Au@FePPOP_{BFPB} is a porphyrin-based porous organic polymer (FePPOP_{BFPB}) crosslinked with Au nanoparticles (AuNPs) with peroxidase-like activity. Finally, Au@FePPOP_{BFPB} catalyzed the conversion of TMB via H_2O_2 to a blue product when H_2O_2 and TMB were added to the assay solution.²⁹ The magnetic properties of Fe_3O_4 ³⁰ and Co_3O_4 ³¹ nanoparticles further enhanced the sensitivity of the nanozyme based assay for *S. aureus* (LOD for the Fe_3O_4 nanozyme system: 10 CFU/mL; LOD for the Co_3O_4 nanozyme system: 8 CFU/mL). In addition to the bacterial body, marker proteins are also important targets for pathogenic bacteria screening. Penicillin binding protein 2 (PBP2a), a signature protein for methicillin resistant *Staphylococcus aureus* (MRSA) screening, can be recognized and bound by specific DNA aptamers (TTTTTAAGCCACGAACCYGTGCCXCACGAACAAWTGCCAGXCGYGCCATG). In addition, the DNA aptamer was also able to modulate the peroxidase-like activity of gold nanoparticle-supported graphene oxide (GO/Au) nanocomposites through the affinity between GO/Au and DNA. The LOD for PBP2a was as low as 20 nmol/L for the assay method involving the use of a DNA aptamer and GO/Au nanocomposites.³²

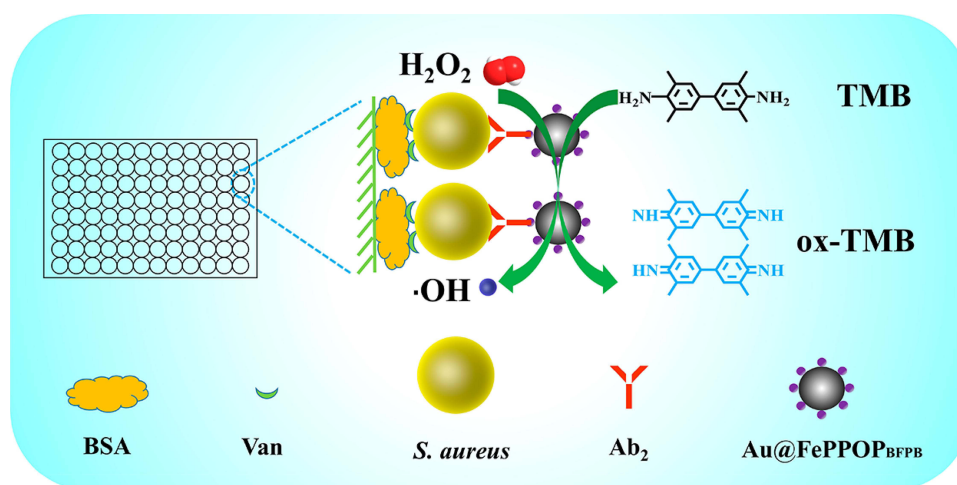


Figure 2 Schematic representation of the colorimetric detection of *S. aureus* based on Au@FePPOP_{BFPB} with peroxidase-like activities. After capture by vancomycin (Van) on a 96-well microplate, *S. aureus* combines specifically with bs-4582R on Au@FePPOP_{BFPB}. Then, H_2O_2 oxidizes colorless TMB to blue ox-TMB by the peroxidase-like activities of Au@FePPOP_{BFPB}. The degree of color change is related to the concentration of *S. aureus*. Reprinted with permission from Li D, Fang Y, Zhang X. Bacterial detection and elimination using a dual-functional porphyrin-based porous organic polymer with peroxidase-like and high near-infrared-light-enhanced antibacterial activity. ACS Appl Mater Interfaces. 2020;12(8):8989–8999. Copyright 2020, American Chemical Society.²⁹

Similarly, G-quadruplex deoxyribonuclease immobilization enhanced peroxidase-like activity as well as affinity for *Streptococcus mutans* of Fe₃O₄ nanozymes, which shows great promise in the clinical prevention and diagnosis of dental diseases (LOD for *Streptococcus mutans*: 12 CFU/mL).³³

Detection of Viruses

Due to their rapid spread, and high morbidity and mortality rates, viruses pose a major threat to human health.³⁴ Rapid detection of the virus is necessary for the timely diagnosis and treatment of infected individuals, as well as for preventing cross infections between healthcare workers and blocking community epidemics. Positive results from nucleic acid-based PCR are currently the most common gold standard for the early detection of confirmed cases. However, this method usually requires professional biosafety laboratories, sampling personnel, testing personnel, and sample transportation systems. The high-intensity workload of large-scale centralized sampling in the community not only causes extreme physical and mental fatigue and even work-related injuries among healthcare personnel, but also poses a potential risk of cross-infection among the sampled population. Moreover, this approach is time-consuming and costly (routine sampling: 4–6 h; large-scale sampling: 24–48 h), seriously weakening the immediate reliability of nucleic acid test results and causing great inconvenience in people to travel. Based on the ELISA-like antigen-antibody reaction mechanism, the use of an antigen self-assessment kit greatly improves the disadvantages of nucleic acid detection, such as not needing professional guidance, and reducing the detection time to 15–30 min. Most of the antigen detection kits available on the market use colloidal gold as a tracer, which has limited sensitivity and a narrow window of detection.

RNA Viruses

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has had a devastating and indelible impact on global economic and social development and people's health.³⁵ Rapid and sensitive detection of SARS CoV-2-positive individuals is essential for preventing the spread of the disease. Combining iron disulfide nanosheets (FeS₂) with high peroxidase-like activity and recombinase polymerase amplification (RPA) can shorten the time required for the detection of SARS-CoV-2.³⁶ Based on the principles of the double antibody sandwich-lateral flow immunoassay and Co-Fe@hemin nanozymes as chemiluminescent tracers, the detection of SARS-CoV-2 can be more rapid and sensitive. S-dAb antibodies against the receptor binding domain of the SARS CoV-2 spike protein (S-RBD) were coupled with Co-Fe@hemin nanozymes as detection and visualization components in a conjugate pad. The paired S-RBD capture antibody (S-cAb) and anti-human IgG antibody were immobilized on the test strip as a test band and a control band, respectively. The luminal substrate was chosen as the chemiluminescent agent for signal production. The signal can be captured by a smartphone camera or charge-coupled device and analyzed with Image-Pro Plus software. The entire assay was rapid (16 min), sensitive (LOD: 360 TCID₅₀ /mL), and specific (no cross-reactivity with influenza A subtypes, including H1N1, H3N2, H5N1, H1N1, and H7N9).³⁷ CD147 was modified on the surface of metal-organic framework nanozymes (MIL-101(CuFe)) for rapid (within 30 min) and highly sensitive (LOD: 3 PFU/mL) visual diagnosis of SARS-CoV-2 infection. SARS-CoV-2 will cover the surface of MIL-101(CuFe) due to its immunoaffinity with CD147, resulting in a decrease in the binding ability of MIL-101(CuFe) to substrates (H₂O₂ and TMB). The decrease in the catalytic activity of MIL-101(CuFe) was proportional to the amount of SARS-CoV-2 in the sample.³⁸

Human immunodeficiency virus (HIV) infects CD4⁺ T cells and destroys the immune system, resulting in acquired immune deficiency syndrome (AIDS), which cannot be effectively cured. Early detection of HIV infection is crucial for the prevention, diagnosis and treatment of AIDS. The complementary strands of HIV DNA, CHIV1 or CHIV2, were adsorbed onto the surface of Au@Pt or magnetite nanoparticles (MNPs) to form CHIV1-Au@Pt or CHIV2-MNPs, respectively. CHIV1-Au@Pt/HIV/CHIV2-MNP sandwich complexes were formed when HIV DNA was added to the system. MNPs were used for magnetic separation and enrichment of the complex, while the excellent peroxidase-like activity of the Au@Pt nanozymes can catalyze the oxidation of fluorescent red by H₂O₂ for the detection of HIV infections. The method was highly sensitive (LOD: 5 pmol/L DNA) and specific. Moreover, DNA mutants with base mismatches can also be identified.³⁹ After specifically binding with DNA probes on the surface of NiFe₂O₄@UiO-66 nanozymes, HIV-1 DNA prevents substrates (eg, H₂O₂ and terephthalic acid) from entering the activity center of the

nanozymes, which leads to a loss of catalytic activity. There was a linear relationship between the concentration of HIV-1 DNA and the degree of inhibition of $\text{NiFe}_2\text{O}_4@\text{UiO}-66$ activity (LOD: 1 fmol/L).⁴⁰

Patients infected with Ebola virus (EBOV) have a sudden, severe and rapidly progressive illness with a high mortality rate. Despite the high rate of EBOV transmission, patients are generally not infectious during the incubation period.⁴¹ Therefore, rapid diagnosis of this disease is essential to contain the EboV epidemic. Duan et al⁴² prepared a test strip capable of rapid screening for EBOV infection with iron oxide nanozymes as a visual system (DAB as a chromatic substrate) based on a double antibody sandwich lateral flow immunoassay. Specifically, the anti-EBOV antibody 4G7 was crosslinked on nanozymes, and the EBOV capture antibody 1H3 in the test line (T) was used to capture EBOV in the samples. The quality control of the test paper was evaluated by the use of a goat anti-mouse IgG antibody in the control line (C). The percentage of positive cells was greater for this test strip than for the traditional ELISA method (LOD: 240 PFU/mL (only 1/10 of the colloidal gold method)), which can be used to screen patients with EBOV in the incubation period, even detecting other infectious viruses with corresponding virus-capture antibodies.

Approximately 3–5 million cases of seasonal influenza virus occur each year, approximately 290,000–650,000 of which die from respiratory disorders caused by the disease.⁴³ Ahmed et al⁴⁴ proposed a simple, highly sensitive, specific and economical colorimetric immunoassay for the detection of avian influenza viruses. An anti-avian influenza virus-specific antibody was conjugated with gold ions (Au^{3+}) forming bioconjugates. The bioconjugates bind to the surface of avian influenza viruses via interactions between the virus and the antibody, and are subsequently reduced by TMB to AuNPs with a blue–green color. Due to the peroxidase-like activity of AuNPs, the color of the solution changes from blue–green to more intense dark blue in the presence of TMB- H_2O_2 solution. The LOD for H5N1 in this assay system is as low as 1.11 pg/mL (only 1/1000th of commercial ELISA-based kits). By selecting the appropriate antibody conjugated with Au^{3+} , the LODs of this method for avian influenza A (H4N6) and avian influenza A (H9N2) viruses in blood samples were 0.0269 HAU and 0.0331 HAU, respectively. On this basis, Oh et al⁴⁵ further developed a magnetic nanozyme-linked immunosorbent assay (MagLISA) (Figure 3) for the specific and highly sensitive detection of H1N1. MagLISA consists of a capture and separation probe (MagNB-Abs), and a detection probe (Au NZ-Abs). Fe_3O_4 magnetic nanoclusters were coated with a SiO_2 shell, after which the anti-influenza virus monoclonal antibody Ab1 was absorbed, forming MagNB-Abs. The SiO_2 shell not only blocks the enzyme-like activity of Fe_3O_4 (avoiding interference with that of Au nanozymes) but also provides the positive charge required for the adsorption and capture of Ab1 antibody. Au NZ-Abs were prepared by the anti-influenza virus polyclonal antibodies Ab2 immobilized on positively charged Au particles via electrostatic attraction. When influenza virus present in specimens, MagNB-Abs, influenza virus, and Au NZ-Abs will form a sandwich immunocomplex via antigen-antibody interactions. After magnetic collection, the peroxidase-like activity of AuNZs on the sandwich-like immune complex catalyzes the oxidation of TMB by H_2O_2 to generate colorimetric signals, thus determining the viral content. The LOD of this method for clinically isolated human serum samples is 2.6 PFU/mL, which is as low as 1/400 or 1/2000 of that of conventional ELISA or commercial kits, respectively.

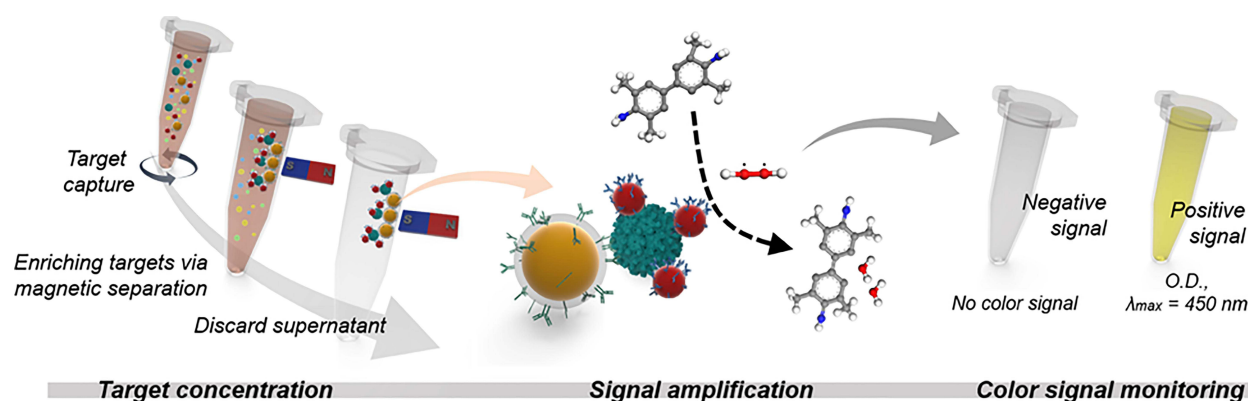


Figure 3 Schematic illustration of the magnetic nanobead-based nanozyme-linked immunosorbent assay (MagLISA) for influenza virus quantification. Three components of the MagLISA, biomarker recognizer, sample enrichment, and peroxidase-like artificial catalysts, are responsible for the recognition, separation, and visualization of influenza viruses, respectively, and constitute a robust and essential sensing platform for influenza virus infection detection. Reprinted with permission from Oh S, Kim J, Tran VT, et al. Magnetic nanozyme-linked immunosorbent assay for ultrasensitive influenza a virus detection. *ACS Appl Mater Interfaces*. 2018;10(15):12534–12543. Copyright 2018, American Chemical Society.⁴⁵

Norovirus (NoV), with its positive-sense single-stranded RNA (ssRNA) genome, is the leading cause of acute viral gastroenteritis worldwide, and is commonly referred to as “winter vomiting disease”. NoV is responsible for 267 million infections and more than 200,000 deaths worldwide each year. Weerathunge et al⁴⁶ developed a colorimetric sensor for the ultrasensitive and highly specific detection of NoV particles, which takes advantage of the greater affinity of the AG3 aptamer (GCTAGCGAATTCCGTACGAAGGGCGAATTCCACATTGGGCTGCAGCCCGGGGGATCC) toward the NoV capsid than does AuNPs, and the inherent peroxidase-like activity of AuNPs (Figure 4). When NoV is not present in the specimens, the AG3 aptamer binds to the surface of the AuNPs and blocks the peroxidase-like activity of the AuNPs; when NoV is present in the analyte, the AG3 aptamer binds to the NoV and undergoes a conformational change. The AG3 aptamer detached from the AuNPs, resulting in exposure of the active catalytic site of the AuNPs. Then, the AuNPs oxidize TMB to a blue product. In contrast, the AG3 aptamer will not bind to nonspecific targets, such as gram-positive bacteria (*S. aureus*), gram-negative bacteria (*E. coli*), or the bacteriophage MS2; therefore, the catalytic activity of AuNPs cannot be restored, thus ensuring the specificity of the assay. The detection system for NoV is rapid (10 min) and ultrasensitive (LOD: 30 viruses/mL).

With anti-zika virus antibody-modified Au-Pt nanozymes as signal amplifiers, H₂O₂/TMB solution for visualization, and a microplate reader or smartphone as a signal recorder and processor, zika virus in blood could be identified at concentrations as low as 1 pg/mL.⁴⁷ When more stable and robust antigen-labeled Au-Pt nanorods are used as a colorimetric probe substituting for antigen-labeled HRP, the nanozyme system can be used for the detection of antibodies in human serum due to rubella virus,⁴⁸ measles virus,⁴⁹ and mumps virus⁵⁰ infections, and the LODs can reach 10 ng/mL. Zhan et al⁵¹ developed a gold nanoparticle/graphene oxide hybrid-based colorimetric immunoassay for respiratory syncytial virus. Owing to its Hg²⁺-enhanced peroxidase-like activity, the LOD was as low as 0.04 pg/mL (only 1/50 of that of commercial ELISA kits (2 pg/mL)), which was favorable for the early diagnosis of respiratory syncytial virus infection.

DNA Viruses

The peroxidase-like activity of gold nanomaterials can also be used for the detection of DNA viruses. Khoris et al⁵² constructed Au@Ag nanocomposites with enhanced peroxidase-like activity by depositing Ag shell layers on the surface of AuNPs. When Au@Ag nanocomposites were used as a colorimetric probe in a sandwich immunoassay, the sensitivity of the assay was improved for the detection of hepatitis E virus (HEV) in fecal samples from HEV-infected monkeys (LOD of HEV-like particles: 10 pg/mL). Wu et al⁵³ integrated rabbit anti-pig IgG antibody-modified Au-Pt/SiO₂ nanozymes and H₂O₂/HAuCl₄ chromogenic substrate as a detection system. This system detected a 1:10⁷ dilution of pig circovirus type 2 antibodies in the positive pig serum samples, which is a much greater sensitivity than that of traditional ELISA (maximal dilution ratio: 5:10⁵).

In addition, coupling the high peroxidase-like activity of FeS₂ nanosheets with RPA technology can also be used for the rapid detection of human papillomavirus infections.³⁶

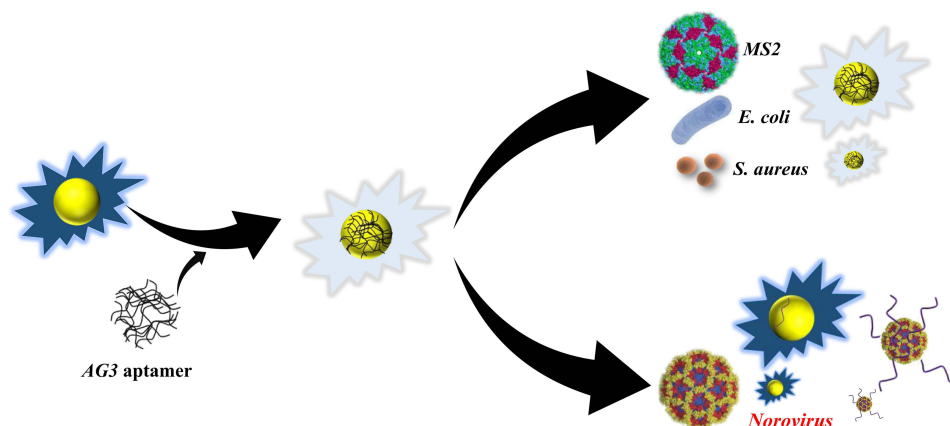


Figure 4 Schematic illustration of a norovirus nanozyme aptasensor. By interacting with AG3 aptamers on the surface of Au nanoparticles, Norovirus, but not other pathogenic microorganisms, induces aptamer desorption to expose the Au nanoparticle surface, which leads to recovery of nanozyme activity to characterize the amount of norovirus present in the sample. Reprinted with permission from Weerathunge TV. Ultrasensitive colorimetric detection of murine norovirus using nanozyme aptasensor. *Anal Chem.* 2019;91(5):3270–3276. Copyright 2019, American Chemical Society.⁴⁶

Summary and Perspective

Considering the significant advantages of ELISA over sputum smear microscopy, microbial culture and PCR, ELISA is a better option for detecting pathogenic bacteria and viruses. The chromogenic reaction catalyzed by natural enzymes is a critical step for successful completion of ELISA. Nanozymes not only exhibit inherent and enhanced peroxidase-like activity, but also have unique advantages compared to natural enzymes. Therefore, nanozymes are an excellent alternative to natural enzymes for ELISA because natural enzymes are expensive and prone to inactivation. A large number of efficient, sensitive, and specific nanozyme-based ELISA-like assays have been developed for the detection of pathogenic bacteria (both gram-negative bacteria and gram-positive bacteria) and viruses (both RNA viruses and DNA viruses) (Table 1). For this application, nanomaterials with peroxidase-like activity mainly consist of pristine, composite, or

Table 1 Application of the Peroxidase-Like Activity of Nanomaterials in the Detection of Pathogenic Bacteria and Viruses

Pathogenic Microorganisms			Nanozymes	Substrates	Linear Range	
Bacterium	Gram-negative	<i>Escherichia coli</i>	Au@Pt-MPBA ¹⁷ Pd-Pt ¹⁸ PdRu ¹⁹	TMB TMB TMB	7–6×10 ⁶ CFU/mL 9.0×10 ² –1×10 ⁶ CFU/mL –	
		<i>Salmonella Enteritidis</i>	Fe-MOF ²⁰ MnO ₂ NFs ²²	TMB TMB	34–1×10 ⁷ CFU/mL 44–4.4 × 10 ⁶ CFU/mL	
		<i>Listeria monocytogenes</i>	IgY-BSA-Ag NCs ²¹	OPD	10–10 ⁶ CFU/mL	
		<i>Pseudomonas aeruginosa</i>	GNPs ²⁴	TMB	60–6×10 ⁷ CFU/mL	
		<i>Enterobacter sakazakii</i>	Fe ₃ O ₄ NPs ²⁵	TMB	10–1×10 ⁵ CFU/mL	
		<i>Yersinia enterocolitica</i>	GQDs ²⁶	TMB	5–1×10 ⁷ CFU/mL	
		<i>Burkholderia pseudomallei</i>	PtPd@PCN- 224 ²⁷	TMB	–	
		Gram-positive	<i>Staphylococcus aureus</i>	Au@FePPOP _{BFPB} ²⁹ IgY-Fe ₃ O ₄ /Au ³⁰ Co ₃ O ₄ MNE@fusion-pVIII ³¹ GO/Au ³²	TMB TMB ABTS TMB	10 ² –10 ⁷ CFU/mL 10–10 ⁶ CFU/mL 10–10 ⁴ CFU/mL 20–300 nmol/L
	<i>Streptococcus mutans</i>		DNA-Fe ₃ O ₄ NPs ³³	TMB	12–1×10 ⁹ CFU/mL	
	Virus	RNA Virus	SARS-CoV-2		Co-Fe@hemin ³⁷ MIL-101 (CuFe)-CD147 ³⁸ FeS ₂ ³⁶	Luminol TMB TMB
HIV			CHIV ₁ -PtAuNps CHIV ₂ -MNP ³⁹	Amplex Red	5–500 pmol/L	
EboV			NiFe ₂ O ₄ @UiO-66 ⁴⁰ Fe ₃ O ₄ MNP ⁴²	Terephthalic acid DAB	0.05–300 pmol/L 0.1–1000 ng/mL	
Avian influenza A			H5N1 H4N6 H9N2 H1N1	Au NPs ⁴⁴ Au NPs ⁴⁴ Au NPs ⁴⁴ MagNBs-Au ⁴⁵	TMB TMB TMB TMB	1.11×10 ⁻⁶ –10 µg/mL 100–0.01 HAU 100–0.01 HAU 44.2×10 ⁻¹⁵ –5×10 ⁻⁶ g/mL
NoV			Au NPs ⁴⁶	TMB	20–33,000 particles/mL	
Zika virus			Pt@Au NPs ⁴⁷	TMB	0.001–1 ng/mL	
Rubella virus			Au@Pt@ SiO ₂ ⁴⁸	TMB	10–10 ⁵ ng/mL	
Measles virus			Au@Pt NRs ⁴⁹	TMB	10–10 ⁴ ng/mL	
Mumps virus			AgS-APMSN ⁵⁰	TMB	10–10 ⁵ ng/mL	
Respiratory syncytial virus			AuNPs-GO ⁵¹	TMB	0.04–10 pg/mL	
DNA Virus		Hepatitis E virus		Au NPs@Ag ⁵²	TMB	0.010–10 ng/mL
		Porcine circovirus type 2		Au-Pt/SiO ₂ ⁵³	TMB	0.050–10 ng/mL
		HPV		FeS ₂ ³⁶	TMB	–

Abbreviations: CFU, colony-forming units; TMB, 3,3',5,5'-tetramethyl benzidine; TMB; OPD, ophenylenediamine; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DAB, 2,2'-diaminobenzidine; AR, Amplex Red; TA, terephthalic acid; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; HIV, human immunodeficiency virus; EboV, Ebola virus; NoV, Norwalk viruses or Norovirus; HAU, hemagglutination unit; HPV, human papilloma virus.

derivative forms of Ag NCs, Au NPs, Co_3O_4 NPs, Fe_3O_4 NPs, FeS_2 NPs, GQDs, MnO_2 NFs, and metal-organic frameworks. The chromogenic substrates included TMB, OPD, ABTS, DAB, fluorescent red, terephthalic acid, and luminal substances. The tested bacteria included *Escherichia coli*, *Salmonella Enteritidis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Enterobacter sakazakii*, *Yersinia enterocolitica*, and *Burkholderia pseudomallei*, which are gram-negative, as well as *Staphylococcus aureus*, and *Streptococcus mutans*, which are gram-positive. The tested viruses included SARS-CoV-2, HIV, EboV, avian influenza A, NoV, Zika virus, Rubella virus, measles virus, mumps virus, and respiratory syncytial virus, all of which are RNA viruses. Additionally, the DNA viruses tested were hepatitis E virus, porcine circovirus type 2, and HPV. Different nanozymes can detect various targets with different detection limits and linear ranges. Overall, these methods demonstrate excellent potential for detecting pathogenic microorganisms and are expected to become the next generation of detection techniques.

However, the current research on the detection of pathogens by nanozymes is still in the initial stage. There are significant improvements needed to advance the practical applications of these materials.

(1) The catalytic activities of nanozymes are still lower than those of natural enzymes. Fortunately, the properties of nanozymes can be tuned by the physicochemical characteristics of the nanomaterial (such as size, shape, surface charge, chemical composition), addition of small molecules (such as ATP), irritation (light, ultrasonic), and other environmental factors (pH, temperature, etc.).⁵⁴ New techniques such as machine learning and deep neural networks can be used to optimally design nanozymes based on the catalytic properties of enzyme-like activities.

(2) Further investigation is required to reconcile the disparity between the catalytic activity and the substrate selectivity of nanozymes. Crosslinking recognition molecules on the surfaces of the nanozymes is necessary to improve substrate selectivity. Nanozymes primarily catalyze reactions through surface electron transfer, while natural enzymes catalyze reactions via specific amino acid residues within a hydrophobic pocket. Surface modifications could lead to a decrease in catalytic activity.

(3) The influence of the sample microenvironment on detection must be considered. Nanozymes can efficiently and stably catalyze reactions under various harsh environmental conditions, including high salinity and wide pH. To determine sensitivity and specificity, some studies of nanozymes rely on the addition of structural analogs to the detection system. However, interferences from a variety of other unrelated substances, such as proteins and metal ions, that may be present in real samples have been ignored. False-positive or false-negative results may occur because these substances may adsorb to the surface of the nanozymes and interfere with detection. Moreover, additional real patient sample test reports with nanozymes are needed to assess the potential of these nanomaterials for real-world applications.

(4) Compared to multiple enzyme-mimicking activities, nanomaterials with only one unique enzyme-mimicking activity are essential for improving sensitivity and accuracy. For example, iron oxide nanomaterials possess peroxidase-like and catalase-like activities. These activities can decompose the same substrate H_2O_2 into different products ($\bullet\text{OH}$ and H_2O , respectively), inducing a reduction in production of $\bullet\text{OH}$ and a decrease in peroxidase-like activity. Notably, nanomaterials may have other enzyme-like activities that are not yet fully understood. These activities could interfere with or destroy biomarkers of pathogenic bacteria and viruses, possibly resulting in inaccurate detection.

(5) As a necessary substrate for the application of peroxidase mimics, H_2O_2 decomposes easily and is harmful to human health and the environment. In contrast, O_2 is more stable, safer and readily available. Thus, nanomaterials with oxidase-like activity rather than peroxidase-like activity are a better alternative to natural enzymes when nanozymes are applied in the detection of pathogenic microorganisms.

(6) Large-scale applications of nanozymes are still enormous challenges for their promotion and application in real-world scenarios, although nanozymes have shown impressive performance in laboratory-based research and development.

(7) Potential risks to the environment and human health are important considerations that must be taken seriously. First, pathogenic microorganisms can be detected in vitro using nanozymes without requiring direct human contact. Nonetheless, there remains a possibility of contact during usage. Second, the preparation of nanozymes may involve toxic and harmful reagents. Third, nanozymes are different from natural enzymes due to their high stability, which allows them to remain stable in the natural environment. Accordingly, the biocompatibility and biodegradability of nanozymes

are crucial factors in assessing their potential for practical applications. Green synthesis strategies and comprehensive biosafety assessments are important considerations.

(8) The detection of pathogenic microbes can be facilitated by integrating the enzyme-like activity of nanomaterials with their magnetic, thermal, and optical properties, along with microfluidic technology, lateral flow strips, and digital imaging and analysis technology for smartphones, which will accelerate the application of nanozymes for practical use.

In conclusion, the application of nanomaterials with peroxidase-like activity shows promise for detecting pathogenic microorganisms. Achieving industrial application of nanozymes and solving practical problems will require collaborative efforts from all parties involved.

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Disclosure

The authors report no conflicts of interest in this work.

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