INNOVATIVE AND MULTIDISCIPLINARY APPROACHES IN DETECTING BIOLOGICAL AGENTS USING CONTEMPORARY TECHNOLOGIES

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Introduction

Biological agents (BA), encompassing bacteria, viruses, and toxins, pose severe threats to both human and animal health, capable of causing serious illnesses and even fatalities (Janik et al. 2020). Consequently, BA detection holds paramount significance across diverse domains, including public health, food safety, and biodefense, and it assumes critical importance in promptly identifying and mitigating outbreaks, as well as advancing biodefense initiatives (Mir et al., 2022; Zhang et al., 2022).

Although conventional techniques such as Enzyme-Linked Immunosorbent Assay (ELISA), Polymerase Chain Reaction (PCR), and Mass Spectrometry (MS) still find extensive use in a variety of scientific and clinical approaches for the qualitative and quantitative characterization of biomolecules (Grigorov et al., 2023; Drakulić et al., 2013; Popović et al., 2019), these methods face an array of limitations with respect to BA detection. They often involve prolonged durations, necessitate specialized equipment and expertise, and may lack the requisite sensitivity to detect trace amounts of BA, particularly in the context of field-based detection scenarios (Kabiraz et al., 2023).

Consequently, a persistent demand emerges for advanced and multidisciplinary methodologies to enhance BA detection. Efforts are continuously being made to develop detection technologies that overcome the limitations of traditional methods (Janik-Karpinska et al., 2022). The argument for supporting innovative and multidisciplinary approaches, including biosensors, nanotechnology, and genomics, emanates from their potential to overcome the limitations of conventional detection techniques. By fusing perceptions from various fields such as biology, chemistry, physics, and engineering, these approaches have the potential to yield detection technologies with enhanced sensitivity, specificity, and rapidity (Janik-Karpinska et al., 2022; Walper et al., 2018).

Mass Spectrometry Based Methods

Mass spectrometry (MS) is an effective analytical technique used to measure the mass-to-charge (m/z) ratio of ions in a sample and to determine the mass of particles or molecules. It provides information regarding the structure of molecules, their overall atomic mass, molecular fragments, and individual atoms. It is currently a widely exploited tool in molecular biology, enabling the examination of large molecules and their interactions and alterations, organelles and cell lysates, intact cells and cell lines,

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tissues, and even organisms to characterize the protein structure under normal or pathological conditions (Jones, 2020).

The basic principle of MS involves ionizing a sample and subjecting ions to an electric and/or magnetic field to separate them based on their m/z. The resulting data is represented as a mass spectrum, which shows the intensity of ions at different m/z. MS-based methods typically use only microgram (μg) quantities of biological materials for examining diverse proteins, precise identification of protein isoforms, and their post-translational modifications (PTMs) (Kaur et al., 2019). The ultrahigh sensitivity, specificity, and low turnaround time in molecular detection make this technology highly powerful in the detection of a wide spectrum BA, but also in disease detection and therapy monitoring (Banerjee, 2020). The multiplexing capability and applicability for analysing complex samples such as air, water, culture medium, bodily fluids, and food have significant worth for the functionality of MS-based techniques in biothreat scenarios.

The challenges of bringing MS technology into on-site settings are twofold: the technical barriers and the high cost. There are several versions of MS, each designed for particular applications. Over the last two decades, two noteworthy techniques - ambient ionization (Wang et al., 2023) and miniature MS (Rampler et al., 2021) brought significant advancements in addressing the abovementioned challenges and creation viable solution for point-of-care (POC) MS analysis. While ambient ionization methods offer the advantage of direct sampling from the natural environment, eliminating the need for pre-treatment procedures, the miniature MS systems were designed to be compact enough to be used on-site, and simple enough to deliver user-friendly analytical reports understandable to the end users without complex analytical training. By overcoming the technical barriers and cost issues, this integrated approach lays the foundation for more accessible and practical POC MS analysis, enabling quicker and more informed decision-making in on-site settings (Zhou, Zhang & Ouyang, 2022).

One example of a successful practical application of these achievements is portable Membrane Inlet Mass Spectrometric (MIMS) technology presented and tested in 2021 at the U.S. Army Dugway Proving Ground, military testing, and research centre for various types of military equipment, including chemical, biological, radiological, and explosive (CBRE) defence systems. Presented MIMS technology provides a comprehensive strategy for the application of MS technology for Chemical Warfare Agents (CWAs) detection wherein the mass spectrometric aspect allows specificity in fragmentation and precise molecular peak detection, enabling the determination of the chemical structure of any relevant toxic agents. The application of a portable MIMS system displays an effective and consistent performance for qualitative and quantitative detection in both indoor and outdoor scenarios. It can detect and quantify low-mass permeable compounds with parts-per trillion limits of detection and within seconds after the initial exposure time, which represents a significant improvement compared to Fourier Transform Infrared (FTIR) spectroscopy and Lightweight Chemical Detectors (LCD) systems, detection technology currently used by the U.S. government (Virgen et al., 2021).

Mass spectrometry imaging (MSI) is another important innovative method using MS technology to identify BA by visualizing the spatial distribution of molecules, as biomarkers, metabolites, or proteins by their molecular masses. Prevalent ionization technologies in MSI are Desorption Electrospray Ionization (DESI) imaging, Matrix-Assisted Laser Desorption/Ionization (MALDI) imaging, and Secondary Ion Mass Spectrometry (SIMS) imaging (Watrous & Dorrestein, 2011). The principle of these methods is that after acquiring a mass spectrum from a specific location, the sample is repositioned to reach another area, and this process is repeated until the entire sample has been scanned. By choosing a peak in the resultant spectra that corresponds to the compound of interest, the MS data is coupled to record its distribution through the sample, pixel by pixel. Each dataset consists of an array of images

as any peak within the individual spectra can be converted into a spatial map. Although MSI generally represents a qualitative approach, the produced signal is proportional to the substance's relative concentration, thus quantification is also possible.

As single MS struggles with notable limitations in analysing complex microbial cultures, food, and environmental samples, and detecting rare or novel-emerging pathogens a tandem mass spectrometry (MS/MS) was developed to overcome those shortcomings. MS/MS is a two-step procedure for analysing a sample either by using two or more connected mass spectrometers, or a single mass spectrometer by several analysers settled one after another. MS/MS is particularly valuable for analysing complex mixtures and consists of two stages of MS. In the initial stage of MS/MS, a predefined array of m/z ions is sequestered from the rest of the ions coming from the ion source and fragmented by a chemical reaction. In the second stage, mass spectra are generated specifically for these resulting fragments (Büyükköroğlu et al., 2018). Peptide fragments can be analysed for *de novo* peptide sequencing, peptide mass fingerprinting (PMF) (He et al., 2010), or amino acid sequencing of the peptide, succeeded by protein database examination of the resulting fragments (Feist & Hummon, 2015). The identification of the unknown protein can be accomplished by matching the resulting and theoretical peptide masses, using search engines for database search, like Proteome Discoverer, Mascot, X!Tandem (Jayathirtha et al., 2021), ProFound, as well as open-source software tools like MSTracer (Zeng & Ma, 2021) and OpenPepXL (Netz et al., 2020). Moreover, MS/MS is capable of generating sequence information and therefore can significantly improve the specificity for BA detection based on the recognition of unique peptides. For instance, a combined bottom-up and top-down proteomics approach is used, employing a high-resolution/high mass accuracy LTQ-Orbitrap instrument to identify specific markers of *Bacillus anthracis* spores, efficient in discriminating subtle similarities with *Bacillus cereus* biovar anthracis strains CI, CA, and *Bacillus thuringiensis* 9727 (Chenau et al., 2014).

Tandem MS methods can be applied across various domains of biological research, encompassing both fundamental and clinical investigations. These methods are helpful in qualitative and quantitative analysis of proteins and peptides, along with their post-translational modifications (PTMs) and interactions between proteins (PPIs) in a wide range of biological samples allowing the transition from targeted to untargeted proteomic approaches (Neagu et al., 2022). The greatest advantage of tandem MS is its very high specificity, resulting from significantly reduced chemical noise, and generally superior signal-to-noise ratio, compared to single MS.

Generally, there are two MS/MS approaches for the verification of BA: Targeted and Shotgun. Their procedures differ, and the choice between them depends on the experimental goals and the nature of the sample being analysed (Neagu et al., 2022).

The targeted approach uses to screen for the BA from a pre-defined list of pathogens using selected unique marker peptides. This approach is also known as selected reaction monitoring (SRM) or multiple reactions monitoring (MRM). A particular group of precursor ions (also known as parent ions) is selected and isolated during the initial phase of the process. In a second phase, a specific set of fragment ions (referred to as product ions) generated from these precursor ions are monitored. It is employed when one possesses prior knowledge of the expected compounds present in a sample, and the objective is to quantify or identify them with high sensitivity and specificity. This approach is commonly used for quantitative analysis of known BA in complex mixtures.

The shotgun approach intends to generate sequence information for as many peptides as possible and to identify even the unknown or advanced BA. In this approach, also known as data-dependent acquisition (DDA), a broad spectrum of precursor ions is selected from the sample during the initial phase of the process. These precursor ions are usually generated from the entire sample mixture, generating

a complex mixture of fragment ions. Subsequently, the most intense or abundant ions from the mixture are selected for fragmentation in the second stage. This approach is commonly used for identifying a large number of peptides in proteomics experiments, as it provides comprehensive coverage of the peptide content in the sample.

Nucleic Acid-Based Methods

Nucleic acid (deoxyribonucleic acid, DNA and ribonucleic acid, RNA) detection technology refers to the various methods and techniques used to identify and analyse nucleic acids, which are the genetic material found in all living organisms. Nucleic acid detection boasts high sensitivity, specificity, short window periods, and is vital for diagnosing infections, diseases, but also for the detection of pathogens like bacteria, viruses, or toxins - materials with the capacity to be used in biological warfare (Shen et al., 2020).

The process entails several steps that are performed in isolated molecular laboratory zones, connected by designated transfer windows to prevent contamination:

a) Sample preparation – extraction and purification of target nucleic acid is from the cellular components that might interfere with the subsequent steps.
b) Amplification – the process of making multiple copies of the target nucleic acid sequence. The most common method of amplification is PCR which involves repeated cycles of heating and cooling to replicate the target DNA segment. PCR stands as a widely favoured method for detecting pathogens, serves as the gold standard in molecular diagnosis. PCR is very powerful, specific, and sensitive, enabling the exponential amplification of specific DNA or RNA sequences, even when only a few copies are present. Traditionally, PCR is conducted with a small volume of sample (no more than 100 µL), and the reaction is run at temperatures cycled among around 60 °C, 72 °C, and 95 °C. However, conventional PCR machines are complex, sizable, and costly, limiting their use to well-equipped and specialized laboratories and making them unsuitable for point-of-care (POC) detection, particularly in resource-limited settings. Other isothermal amplification methods like LAMP (Loop-Mediated Isothermal Amplification) and NASBA (Nucleic Acid Sequence-Based Amplification) can amplify nucleic acids at a constant temperature without the need for thermal cycling.
c) Detection – third step in which amplified nucleic acid can be detected using various methods including fluorescence, colorimetric, and electrochemical assays. Real-time PCR, for example, monitors the amplification process in real-time by measuring the increase in fluorescence signal.

In the wake of the global COVID-19 outbreak across the world, the intensified demand for nucleic acid detection placed a great pressure on various nations and institutions. To address this issue, companies and scientific research institutions have designed a whole process automated systems for nucleic acid testing. Moreover, in response to the demand for on-site rapid detection, integrated rapid detection devices were also developed.

Automatic Nucleic Acid Detection Systems

According to the established workflow, the automated detection systems can be divided into four modules, each capable of functioning autonomously. These modules are: automated dispensing (cup division), automated nucleic acid extraction, automatic amplification system preparation, and auto-

matic amplification analysis modules. Between modules, materials are transported through a handling device, and each module is coordinated by the master control terminal through the communication bus. To prevent contamination and biosafety risk, each module implements a high-efficiency particulate air filter (HEPA) independently to maintain negative pressure.

The entire procedure, starting from sample preparation to data uploading can be automated, as done in available systems like NeuMoDxTM 288 (Qiagen), Cobas® 6800/8800 (Roche), AutoMolec 3000/1600 (Autobio Diagnostics), and High-protection-level nucleic acid automatic detection system (Cohere). These platforms are entirely automated testing solutions, representing the concept of "input sample, receive result". They can accommodate laboratories with different levels of testing demands, be it low, medium, or high throughput, enabling the simultaneous identification of multiple pathogens (Yuan et al., 2022).

Miniaturized PCR Devices

The emergence of microfluidics, coupled with micro electro-mechanical systems (MEMS) technology, supported the miniaturization of PCR into a compact chip-based process. These innovations brought the potential for ultra-fast speed, reduced expenses, minimal sample usage, portability, high capacity, and automation enabling PCR-based diagnostics to extend their reach to POC applications. For instance, in India, a chip based TrueNAT device using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was introduced for POC detection of *Mycobacterium tuberculosis* and diagnosis of pulmonary tuberculosis (PTB) and extra pulmonary tuberculosis (EPTB) (Mangayarkarasi et al., 2019). Their results demonstrated that the sensitivity/specificity of TrueNAT device were 93.1 / 72.5% for the diagnosis of PTB and 96.77 / 76.4% for EPTB, respectively. Additionally, the device has the ability to identify rifampicin resistance, it displays a portability, extended shelf life, cost-effectiveness, and the capacity to operate within a temperature range of 2 °C to 40 °C, which makes it suitable for integration into various detection environments (Mangayarkarasi et al., 2019).

A group of authors from Mexico compared the sensitivity, reproducibility, and convenience of MiniPCR from Amplyus, with a conventional and commercially available PCR thermocycler for the detection and amplification of Ebola and Zika virus samples. MiniPCR can run 8 amplifications in parallel, has dimensions of 20×5×15 cm, and a weight of 0.7 kg. The unit requires 120 V (AC) and 3.5 A to operate, it has its own blueGel electrophoresis unit powered by 120 AC volts, and photo-documentation can be done using a smartphone camera. Results showed that the performance of both thermocyclers is comparable, but that miniPCR's portability, user-friendly design, and reproducibility make MiniPCR a credible for POC nucleic acid detection and amplification (Liu et al., 2014).

Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-Based Technology

Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-based technologies have emerged as powerful tools for the detection of BA, displaying their innovative and multidisciplinary potential in the realm of biodetection. This revolutionary approach employs the precision of the CRISPR-Cas (CRISPR-associated proteins) system, originally recognized for its genome-editing capabilities, to detect specific biological entities with remarkable accuracy and sensitivity.

The CRISPR-Cas system uses RNA to target a specific DNA sequence, which is then cleaved by the Cas enzyme. This cleavage event can be detected using various methods, such as fluorescence, colorimetry, potentiometry, and lateral flow assay (Kumaran et al., 2023). The versatility of CRISPR-based biodetection extends from DNA to RNA and even proteins, enabling the identification of a wide range of BA, including viruses, bacteria, and specific biomarkers. This adaptability is crucial for combating emerging infectious diseases and monitoring environmental contaminants. Techniques such as lateral flow assays and isothermal amplification have been integrated with CRISPR-based systems to create user-friendly, point-of-care testing devices. Furthermore, the development of smartphone applications and portable devices which can interpret CRISPR-based biodetection made the process more accessible to non-experts. Ma and his team (2022) developed a cutting-edge CRISPR-Cas12a-based visual biosensor, integrated with smartphone technology, designed for the highly sensitive and specific detection of SARS-CoV-2. Basically, this biosensor is activated by the genetic material of SARS-CoV-2, causing the CRISPR-Cas12a system to break down a specific single-stranded DNA that links two gold nanoparticles, inducing the disaggregation of nanoparticles and thus producing colour changes that can be easily distinguished by the naked eye or through a smartphone with a Color Picker App. The proposed biosensor was successfully applied to detect SARS-CoV-2 gene in synthetic vectors, transcribed RNA and SARS-CoV-2 pseudo viruses. This biosensor achieved a remarkable "single copy resolution" with a detection limit of 1 copy per microliter for pseudo viruses, no false-positive results and it provided 100% agreement with qPCR results. The entire process, from sample collection to obtaining results, takes approximately 90 minutes (Ma et al., 2022).

In addition to its diagnostic applications, CRISPR-based biodetection plays a pivotal role in biosurveillance and biosecurity. Its ability to provide rapid, specific, and sensitive results makes it an indispensable tool for monitoring biothreats, ensuring food safety, and safeguarding public health. CRISPR, also, enables scientists to conduct larger, faster, and more comprehensive experiments to better understand the key characteristics of pathogens. Moreover, CRISPR streamlines the creation of precise animal models for studying novel diseases, expediting the research process.

Numerous experts and organizations advocate for the seamless integration of life science and biotechnological advancements like CRISPR into both national and international initiatives aimed at strengthening pandemic preparedness and response mechanisms (Watters et al., 2021).

Biosensors

Biosensors, integrated analytical tools, combine biological recognition units with transducers to detect and quantify BA through characteristic interactions with target molecules. Biosensor architecture typically encompasses the biological recognition element, transducer, signal processing unit, and readout system.

Enzymes, antibodies, nucleic acids, hormones, organelles, or whole cells can serve as biological recognition elements. Upon contact with the target analyte which might be affinity-based, catalytic, or linked to metabolic processes, these elements generate a response, an analytically measurable signal, translating the biological event into an electrical or optical output. The signal processing unit amplifies, filters, and converts the raw signal into a format suitable for analysis. The readout system finally presents the results to the user (Metkar & Girigoswami, 2019; Singh et al., 2021).

The first major difficulty in the development of biosensors is the efficient capturing of biorecognition signals and their transduction into different forms like electrochemical, electrical, optical, gravimetric,

or acoustic signals. Secondly, improving the performance of the transducers is a significant challenge. This involves boosting sensitivity, reducing response times, ensuring reproducibility, and achieving low detection limits, to the extent of identifying individual molecules. Lastly, there is the goal of downsizing biosensing devices through the utilization of micro- and nano-fabrication technologies. Advancements in nanomaterials significantly contributed to overcoming these challenges and impacted biosensor performance. Nanomaterials such as nanoparticles, nanotubes, and nanowires have unique properties that amplify the biosensor's ability to detect BA. They offer a larger surface area for biomolecule immobilization, enhancing sensitivity and enabling the detection of BA at lower concentrations. Furthermore, nanomaterials can be engineered to provide a favourable microenvironment for immobilized biomolecules, improving their stability and specificity (Naresh & Lee, 2021). In their recent study, Ziółkowski et al. (2020) introduced a novel, completely portable test for the detection of a specific gene fragment called pagA in the *Bacillus anthracis*. This gene fragment is associated with the anthrax toxin translocating protein. The instrument uses graphene oxide nanoflakes to interact with the pagA fragments, causing them to produce a fluorescent signal upon binding. The process uses a fluorometric-paired-emitter-detector-diode and the entire procedure is completed in 2 minutes. What adds to the practicality of this method is that it relies on readily available components like LEDs, PCR tubes, multimeters, and 2.5V batteries. The instrument's sensitivity of 0.625 μM is better than that of spectrofluorophotometers but still lower than conventional PCR product detection methods. The combination of efficiency, cost-effectiveness, and portability of this test makes it an excellent candidate for use in biosafety facilities, environmental, and emergency testing (Ziółkowski et al., 2020).

An inherent advantage of biosensors is their ability to offer real-time monitoring. This is especially critical in applications such as environmental monitoring and medical diagnostics. Enabling quick and precise detection of BA, biosensors have been pivotal in outbreak detection and control. An illustrative example is an acetylcholinesterase (AChE) inhibition biosensor, based on the inhibition of AchE's catalytic activity through interaction with the targeted agent. Once immobilized onto an electrode surface, AchE enzyme triggers the breakdown of acetylthiocholine chloride (ATCl), eventually producing a detectable electroactive byproduct thiocholine (TCh) which serves as an indicator for the presence of pesticides (Zhang et al., 2015).

Biosensor platforms have also integrated microfluidic systems to enhance detection capabilities. Microfluidics enables precise control over sample volumes, reducing detection time and reagent consumption. This technology, when combined with biosensors, enhances portability and it is easy to use. Lab-on-a-chip biosensors are emerging, allowing multiple tests to be conducted simultaneously on a single device (Fu et al., 2021).

Paper-Based Biosensors for Field Testing

Paper-based biosensors offer a sophisticated yet accessible solution for rapid, on-site detection of BA and toxins. These devices employ patterned paper strips that are impregnated with specific biochemical agents, such as antibodies or aptamers that selectively react with target molecules. When a sample is applied to the paper, it interacts with the immobilized agents, leading to visible colour changes or other detectable signals. For warfare and emergency response, paper-based biosensors provide a rapid and cost-effective means of assessing the presence of hazardous substances. They are particularly useful in resource-limited settings where sophisticated laboratory equipment is unavailable. Their simplicity, portability, and ability to provide results without the need for specialized training make them valuable tools for on-the-ground assessment and decision-making (Singh et al., 2018).

Due to their simplicity, cost-effectiveness, and portability, paper-based biosensors have gained substantial attention in recent years. These biosensors are classified into three main categories: dipstick tests, lateral flow assays (LFAs), and microfluidic biosensors on microfluidic paper-based analytical devices (μPADs).

Dipstick, also known as test strips or test cards, involves the usage of specially designed paper strips containing chemical reagents which once immersed into a sample, changes colour based on the presence of specific substances or BA. Dipstick tests are commonly used in various fields, including clinical diagnostics, environmental monitoring, and food safety.

LFAs are a more advanced form of paper-based biosensors that have gained widespread use. LFAs operate on the principle of capillary action, where the sample flows through the paper channels by capillary forces. LFAs consist of a sample pad, conjugate pad, nitrocellulose membrane, and absorbent pad. The sample pad is where the sample is applied, and the conjugate pad contains labelled detection reagents. As the sample flows through the membrane, it interacts with immobilized capture molecules, leading to the formation of a visible test line. LFAs are extensively used for rapid point-of-care diagnostics, such as pregnancy tests and infectious disease detection. For the detection of bioterrorism-relevant agents, lateral flow strips are suitable for the measurement of *Bacillus anthracis*, *Francisella tularensis*, *Brucella sp*., *Yersinia pestis*, staphylococcal enterotoxin B, botulinum toxin, and orthopox viruses. The strips are made for one, five, or eight BA contemporary measurable in one step. Two USA manufacturers - Advnt Biotechnologies x and Alexeter Technologies are active in this area (Pohanka, 2019).

Microfluidic biosensors on μPADs combine the advantages of microfluidics and paper-based devices. These biosensors incorporate intricate paper-based microfluidic structures to control and manipulate small volumes of fluids. μPADs can perform complex assays, including multiplexed analyses and sample pre-concentration. The channels and reservoirs on μPADs can be designed to facilitate sequential fluid flow, enabling multi-step assays. They offer increased sensitivity, reduced sample and reagent consumption, and improved analytical performance compared to traditional paper-based biosensors (Kuswandi & Ensafi, 2020). Recent developments in electrogenerated chemiluminescence paper-based microfluidic analytical devices include the use of 3D-origami devices and devices utilizing self-powered and bipolar electrodes. These advancements offer the potential for rapid and accurate point-of-care testing, enabling early and effective diagnosis and treatment (Chinnadayyala et al., 2019).

Advanced Imaging and Spectroscopic Techniques

Advanced imaging techniques play a pivotal role in the accurate identification and characterization of BA, offering intricate insights into their structural and functional features. Commonly used are fluorescence-based imaging which leverages fluorescent labels to visualize agents' presence, hyperspectral imaging which analyses spectral data for accurate identification, and terahertz (THz) spectroscopy which probes molecular vibrations for non-invasive detection. These cutting-edge approaches offer precise, rapid, and non-destructive means of identifying BA in various scenarios, from research laboratories to security applications.

Fluorescence-based imaging is a powerful method that exploits the inherent fluorescence properties of certain BA. In this procedure, specific molecules are tagged with fluorescent labels, which enable visualization of their presence. After exposure to light of a particular wavelength, these labelled molecules emit light of different wavelengths, which can be detected and analysed. Fluorescence-based im-

aging is broadly used to identify different pathogens, viruses, and biomolecules in complex samples. Recent innovations include the development of high-resolution fluorescence microscopes and novel fluorescent probes that can enhance sensitivity and enable simultaneous identification of multiple agents (Etrych, Janoušková & Chytil, 2019).

Hyperspectral imaging implicates capturing images at numerous narrow and contiguous wavelengths across the electromagnetic spectrum. This results in a hyperspectral cube containing spectral and spatial information for each pixel in the image. In the context of BA detection, hyperspectral imaging can distinguish different agents based on their unique spectral signatures (Manganiello et al., 2021). This technology is particularly useful when dealing with mixed samples, where multiple agents might be present. By analysing the spectral data, algorithms can differentiate between BA, pollutants, and other materials, thus enabling accurate identification.

THz spectroscopy is an emerging imaging technique that utilizes electromagnetic waves in the THz frequency range, falling between microwaves and infrared radiation. It enables the detection of bacterial cells based on their specific spectral characteristics as a result of the interactions between THz radiation and the low-frequency molecular motions of the cellular components of bacteria. The photon energy of THz radiation is comparable with the excitation energy of the rotational transitions in molecules; thus, THz spectroscopy contains information such as molecular vibration and rotation - the fingerprint characteristic of THz spectroscopy owing to which it can be applied to substance detection and recognition (Yang et al., 2016). THz spectroscopy is also characterized by good biosafety. Unlike the X-rays, THz waves have very low photon energy (4 meV at 1 THz) and are non-ionizing, so besides providing valuable information about the composition and structure of BA, they do not cause damage to biological samples. THz waves can penetrate general dielectric materials including plastics, clothing, and ceramics, which makes them suitable for non-invasive screening of individuals and objects and, thanks to which THz spectroscopy has the potential to enhance security screenings at airports, border crossings, and other critical checkpoints (Fu et al., 2022).

Among the important spectroscopy technologies that needed to be mentioned as powerful tools in the detection and identification of BA are also Raman spectroscopy, Surface-Enhanced Raman Spectroscopy (SERS), Tip-Enhanced Raman Spectroscopy (TERS), and Mid-Infrared (Mid-IR) spectroscopy. When combined with biomarkers, these technologies offer unparalleled sensitivity and specificity, making them indispensable in safeguarding against biowarfare threats.

Raman spectroscopy is non-invasive detecting method which operates on the principle of inelastic light scattering, where incident photons interact with molecular vibrations within the sample. This interaction generates a Raman spectrum that provides valuable information about the composition and structure of BA. By analysing specific vibrational modes in the Raman spectra, it is possible to identify and characterize various biomolecules, such as proteins, DNA, and specific markers associated with biological threats (Oshima et al., 2023).

In the study of Pahlow et al. (2023) they introduced a functionalized magnetic bead-based sample preparation method for the purpose of distinguishing positive and negative samples using Raman spectroscopy. They used the ACE2 (Angiotensin Converting Enzyme 2) receptor protein, as a recognition element, allowing the selective capture of SARS-CoV-2. Subtle differences in the Raman spectra for SARS-CoV-2, Influenza A H1N1 virus and negative control were detected using correlation coefficients, specifically the Pearson and Normalized cross correlation coefficients. Thus, this method offers a promising initial step toward the use of conventional Raman spectroscopy for the detection and classification of various viruses by simply changing the recognition element, thereby improving its user-friendliness and reproducibility (Pahlow et al., 2023).

SERS takes this a step further by enhancing the Raman signal through nanostructured surfaces, thereby increasing sensitivity to trace amounts of biomolecules. SERS analysis encompasses two distinctive approaches: label-free and labelled methods. In the label-free SERS technique, bacteria are directly attached to either a solid SERS substrate or an active SERS nanoparticle substrate in a solution. Conversely, the labelled SERS method involves the attachment of a Raman reporter to the surfaces of plasmon resonance silver (Ag) or gold (Au) nanoparticles, followed by a subsequent modification with specific antibodies or aptamers of BA as recognition molecules to generate SERS tags that produce enhanced Raman signals (Zheng et al., 2018).

Recently, a novel SERS-based aptasensor, linking Au nanopopcorn as the substrate and a spike protein DNA aptamer as the receptor, has demonstrated superior sensitivity and specificity in SARS-CoV-2 detection, achieving a detection limit of less than 10 pfu/mL within just 15 minutes (Chen et al., 2021), in contrast to NAAT (Nucleic Acid Amplification Test) or serological tests which require at least 30 minutes. In addition, SERS can detect both live and dead SARS-CoV-2 viruses at any phase of infection, while NAAT and serological tests are most suited for pre-infection and post-infection, respectively.

TERS system is based on metallic tip, typically composed of Au or Ag, to concentrate the incident light field at its apex. The tip serves as a nano-scale light source and local field amplifier, significantly enhancing Raman sensitivity by factor of 10^3 - 10^7. It also minimizes the probed volume to the "nano" region immediately below the tip (Chen et al., 2019). TERS has been effectively used to detect various BA, including bacteria, viruses, and toxins. Dou et al. (2020) presented an innovative diagnostic method for the label-free identification and structural analysis of individual viruses which combines nanoscale Raman and infrared spectroscopy techniques. By employing atomic force microscopy-infrared (AFM-IR) spectroscopy, the researchers successfully examined the structural organization of the virions of Herpes Simplex Type 1 viruses and bacteriophage MS2. Additionally, authors showed that TERS could be used for revealing the protein secondary structure and amino acid composition of the virus's surface. The results showed that AFM-IR and TERS offer different yet complementary insights into the structure of complex biological specimens. The authors also noted that this structural data can be used for fast and reliable identification of viruses, as well as for investigating the origin of viral polymorphism and study mechanisms of virion assembly (Dou et al., 2020).

Mid-IR spectroscopy is based on the principle that different molecules have distinct vibrational modes, resulting in unique absorption spectra in the Mid-IR region (4000–400 cm^{-1}). Absorptions in midIR spectroscopy are linked to the fundamental vibrations of chemical bonds within molecules. When a molecule interacts with midIR light, its chemical bonds vibrate more vigorously, causing changes in molecular vibration and rotation. MidIR spectra typically display multiple absorption peaks, which are categorized into four main regions: X–H stretching (4000–2500 cm−1), triple bonds (2500–2000 cm−1), double bonds (2000–1500 cm−1), and the fingerprint region (1500–600 cm−1). The fingerprint region is a complex area with numerous bands that reveal specific molecular structural details, often overlapping. Moreover, valuable quantitative data can be extracted from mid-IR spectra by measuring the absorbance (band height or, more precisely, band area), which is proportional to the number of functional groups, following Lambert–Beer's law (Türker-Kaya & Huck, 2017). By identifying biomarkers that are specific for given BA, researchers can also create a spectral fingerprint that allows its precise identification. The success of Mid-IR in detecting BA depends on the careful selection of biomarkers - specific molecular components or functional groups that are characteristic of the agent of interest. For biowarfare detection, biomarkers could include proteins, nucleic acids, or lipids unique to the target organism. The obtained Mid-IR spectra are then subjected to rigorous data analysis techniques, often involving pattern recognition and machine learning. By comparing the sample's spectral

data to a database of known biomarker signatures, the presence of a specific BA can be confirmed (Farrugia et al., 2023). This targeted approach not only improves sensitivity but also minimizes false positives. Another great advantage of Mid-IR combined with biomarkers is its usefulness for real-time monitoring. This is particularly important in biowarfare scenarios where quick and accurate identification is crucial for response and containment efforts.

Combining two standoff technologies, Laser-Induced Fluorescence (LIF) and Light Detection and Ranging (LIDAR), enables a robust and complementary approach to enhance the accuracy and reliability of BA detection. The identification and classification of microorganisms is based upon fluorescence properties of specific biomolecules within subcellular structures (fluorophores) when exposed to appropriate wavelengths of light. LIF-LiDAR system employs a laser source emitting light of specific excitation wavelengths toward an object, such as bioaerosols. A receiving system collects and amplifies signals, including scattered light and fluorescence, from the target which are visualized using data acquisition platform (Owoicho, Olwal & Quaye, 2021).

The integration of LIF and LIDAR technologies in biosecurity demonstrates a significant improvement in our capacity to detect and respond to biological threats. These methods are not only non-invasive but also offer several advantages over traditional detection methods, including the ability to detect and identify BA from a safe distance, minimizing the risk to personnel and the environment. Additionally, the superiority of remote techniques compared to point detectors results from the possibility to survey large territories, up to tens of kilometres, with a fine spatial resolution of several meters, and without the need to physically approach the target (Kwaśny et al., 2023). Currently, extensive defence research has been undertaken worldwide to develop LIF-LIDAR technology for remote detection of BA attack (Li, Huang & Sun, 2019).

Artificial Intelligence

The rapid progress in artificial intelligence (AI) and robotics has expanded possibilities for strengthening bioterrorism response strategies. In an ever-evolving bioterrorism landscape, it remains imperative to leverage state-of-the-art technologies to stay ahead of potential attacks and minimize their impact on public health and safety. AI-driven technologies have the potential to revolutionize the field of bioterrorism response by enhancing not only detection, but also prevention, and mitigation strategies.

AI-powered technologies hold considerable promise in bioterrorism response, particularly in the early detection of biological threats. These algorithms adeptly analyse extensive data to unveil patterns and anomalies indicative of the presence of biological risk. For instance, machine learning algorithms can analyse data from environmental sensors, social media sources, and medical records to identify uncommon disease outbreaks or symptoms that may herald a bioterrorism event.

Group of authors (Jo et al., 2017) presented an optical method for fast and label-free screening of *Bacillus anthracis* spores using the combined application of holographic microscopy and deep learning (a state-of-the-art machine learning technique based on deep multilayered neural networks). These authors designed a deep convolutional neural network, HoloConvNet, capable of classification of holographic images of living cells. After training with quantitative phase images of single *Bacillus anthracis*, the network identified new anthrax spores with single-spore sensitivity and subgenus distinctiveness. Its outstanding learning ability enables direct training from raw images by automatically recognizing key biological characteristics encoded in the images (for example, dry mass in the anthrax problem), and presents exceptional accuracy that exceeds previous approaches in all accuracy

measures. Moreover, in addition to *Bacillus anthracis*, this method is applicable to the classification of various single cells without any modification, as the authors demonstrated for the diagnosis of *Listeria monocytogenes*.

Beyond mere detection, AI-driven tools are also poised to contribute to the prevention of bioterrorist acts. AI-powered surveillance systems can monitor high-risk areas such as transportation hubs, public spaces, and critical infrastructure, deploying sophisticated sensors and cameras capable of real-time identification of hazardous BA. Through analysing the accumulated data, AI algorithms can identify potential threats and promptly alert authorities for proactive measures.

In the unfortunate cases of a bioterrorism incident, AI technologies stand primed to mitigate its consequences and expedite response efforts. AI algorithms can model the dispersion dynamics of a BA, thereby aiding authorities in making informed choices pertaining to quarantine protocols, distribution of medical resources, and calibrated public communication. Furthermore, AI-powered robotics can assist in decontamination activities, mitigating risk exposure for first responders and expediting recovery procedures.

An interesting example illustrating the comprehensive role of AI in biological warfare is presented in the work of Su et al. (2021) who examined the potential role of information technologies in preventing and mitigating Biodisaster X. Term "Biodisaster X" is defined as disasters caused by the accidental or intentional misuse of biotechnologies by state or nonstate actors. The primary objective of investigation was to identify: 1) the possible risks Biodisaster X poses to the entire society; 2) solutions employing emerging 6G and AI technologies to help surveillance and manage Biodisaster X risks (6G technologies are the next generation of wireless communication systems following 5G networks) (Su et al., 2021).

Investigation findings showed that Biodisaster X has the potential to destroy economies, lives, and livelihoods, displaying an approaching risk for civilizations on a global scale. To uncover the risks associated with Biodisaster X, authors presented effective AI and 6G-based strategies ranging from natural language processing to deep learning-based image analysis to manage challenges that include early Biodisaster X detection (for example, identification of suspicious behaviours), remote design and development of pharmaceuticals, public health interventions (for example, reactive shelter-at-home mandate enforcement), along with disaster recovery (for example, sentiment analysis of social media posts to clarify the public's feelings and readiness for recovery building) (Su et al., 2021).

The study conclusively demonstrated that for the mitigation and management of Biodisaster X hazards, it may be more economically efficient and operationally feasible to implement technology-based solutions, rather than relying exclusively upon the strained capacity of healthcare practitioners and governmental authorities.

Conclusion

The present study underscores the pivotal role of innovative and multidisciplinary strategies in revolutionizing the detection of BA through the exploitation of contemporary technologies. Current MS-based, nucleic acid-based, and advanced imaging techniques have demonstrated remarkable proficiency in enhancing the sensitivity, specificity, and rapidity of detection processes. Moreover, the integration of AI algorithms empowers real-time data analysis, pattern recognition, and predictive modelling, contributing to the robustness of the detection process. As technology continues to evolve,

the continued exploration, refinement, and integration of these contemporary technologies hold great promise to transform biosensing, biosecurity, and public health surveillance on a global scale.

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