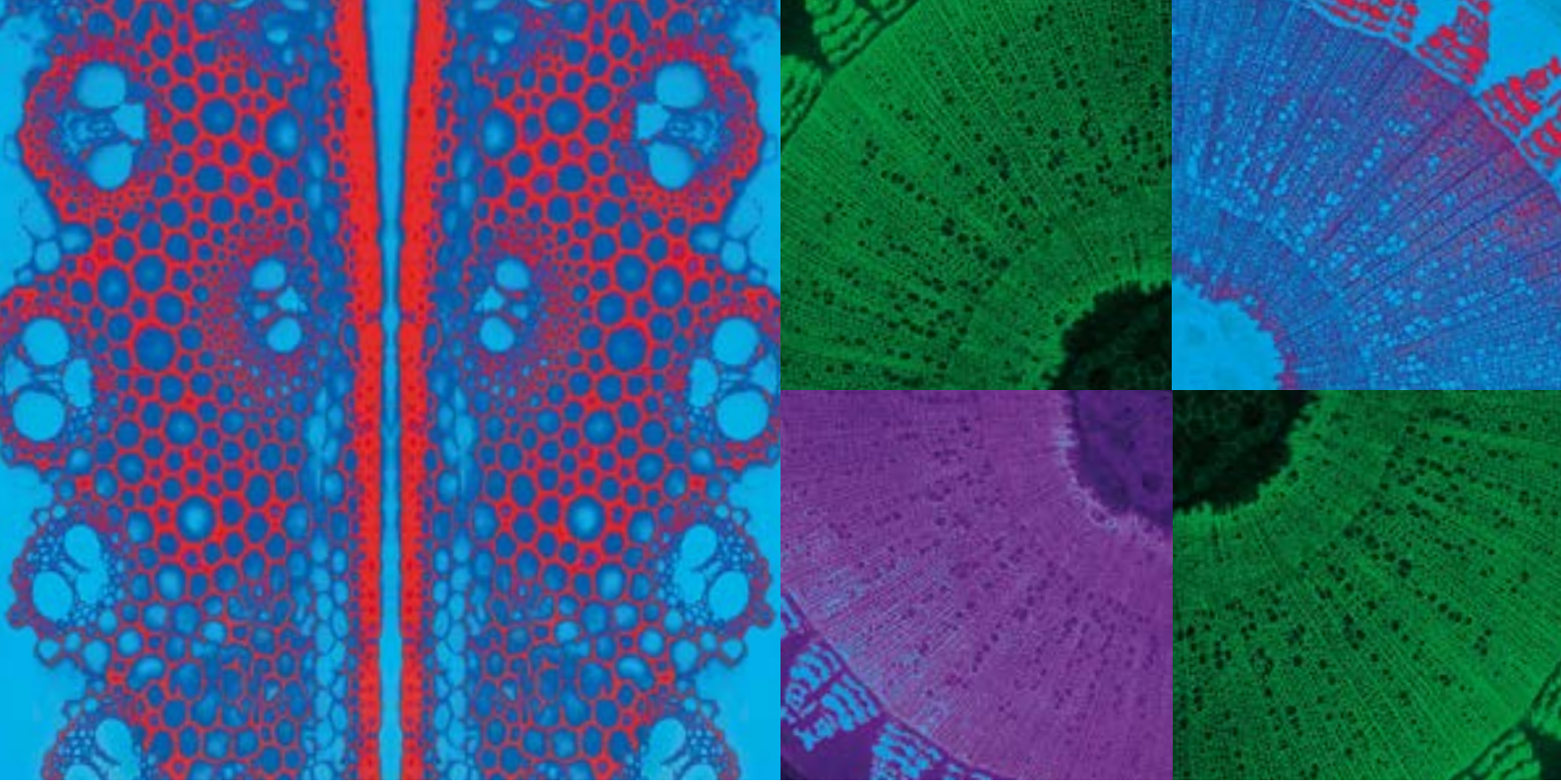

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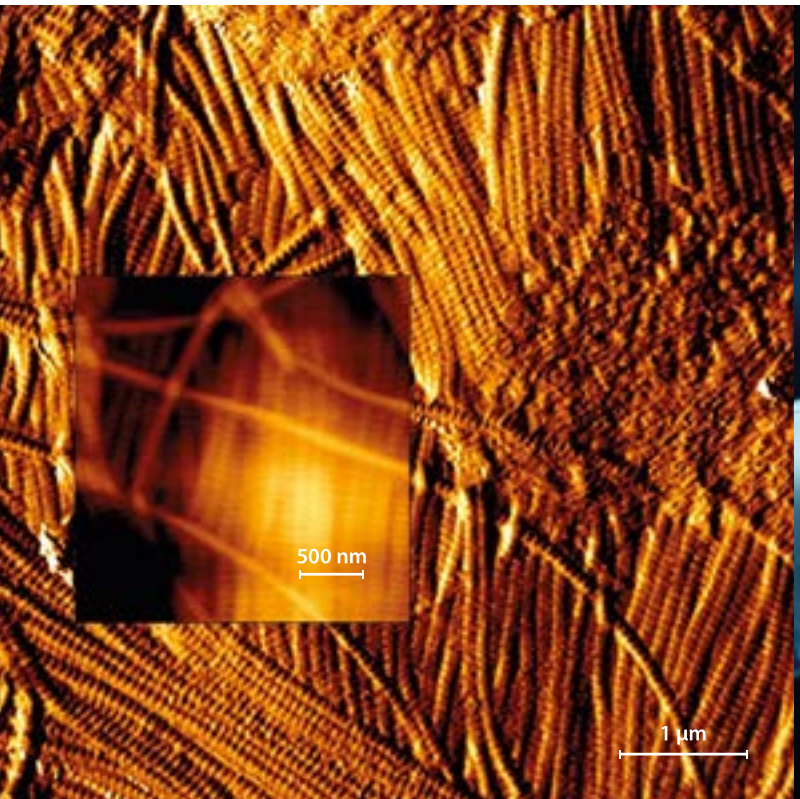

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In this promotional issue, we highlight selected publications to showcase a variety of studies and research approaches within the journal's scope. We present five articles that stand out in different ways. They were chosen by the editors (Editor's Choice) and readers as particularly interesting, as they are among the most frequently accessed and preferred (Most Read).

We wish you a pleasant reading experience. We also hope this selection inspires you to further explore the scientific scope of *BioTechnologia*.

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Cutting-edge AI tools revolutionizing scientific research in life sciences

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Abstract

Artificial intelligence (AI) is becoming a transformative force in the life sciences, pushing the boundaries of possibility. Imagine AI automating time-consuming tasks, uncovering hidden patterns in vast datasets, designing proteins in minutes instead of years, and even predicting disease outbreaks before they occur. This review explores the latest AI tools revolutionizing scientific fields, including research and data analysis, healthcare, and tools supporting scientific writing. Beyond data processing, AI is reshaping how scientists draft and share their findings, enhancing processes ranging from literature reviews to citation management. However, with great power comes great responsibility. Are we prepared for this leap? This review delves into the forefront of AI in the life sciences, where innovation meets responsibility.

Key words: deep learning, machine learning, AI-driven discovery, predictive modeling, artificial intelligence, 2024 Nobel prizes.

Introduction

Artificial intelligence (AI) is revolutionizing scientific research, particularly in the field of life sciences. AI advances science by enabling the analysis of data and addressing challenges that were previously beyond the scope of traditional research methods. Tools such as **AlphaFold**, which has revolutionized structural biology and contributed to a Nobel Prize-winning breakthrough, and **BioBERT**, a natural language processing model for biomedical text analysis, are prime examples of how AI supports scientific research. AI tools assist in analyzing large datasets, automating repetitive tasks, predicting, optimization and modeling complex processes, thereby accelerating scientific discovery. Figure 1 illustrates the application possibilities of artificial intelligence in the fields of healthcare, clinical trials, biosimulation, omics research, personalized medicine, early disease detection, vaccine and drug discovery, bioimaging, robotic laboratory equipment, and streamlining scientific paper writing.

Below, an overview of the latest AI tools and their potential applications in life sciences is presented, along with tools that aid scientists in writing scientific publications.

AI in proteomics

AI plays a transformative role in proteomics by revolutionizing the prediction of protein structures. Tools like **AlphaFold**, developed by DeepMind, predict protein structures with unprecedented accuracy based solely on amino acid sequences. This breakthrough has significantly accelerated research on protein functions and their roles in diseases, facilitating drug development and disease research. The significance of AI in this field was underscored in 2024 when the Nobel Prize in Chemistry was awarded to three pioneers for their development of AI-based methods for predicting protein structures (Callaway, 2024). Demis Hassabis and John Jumper of Google DeepMind were recognized for their AI programs that accurately predict the 3D shapes of proteins,

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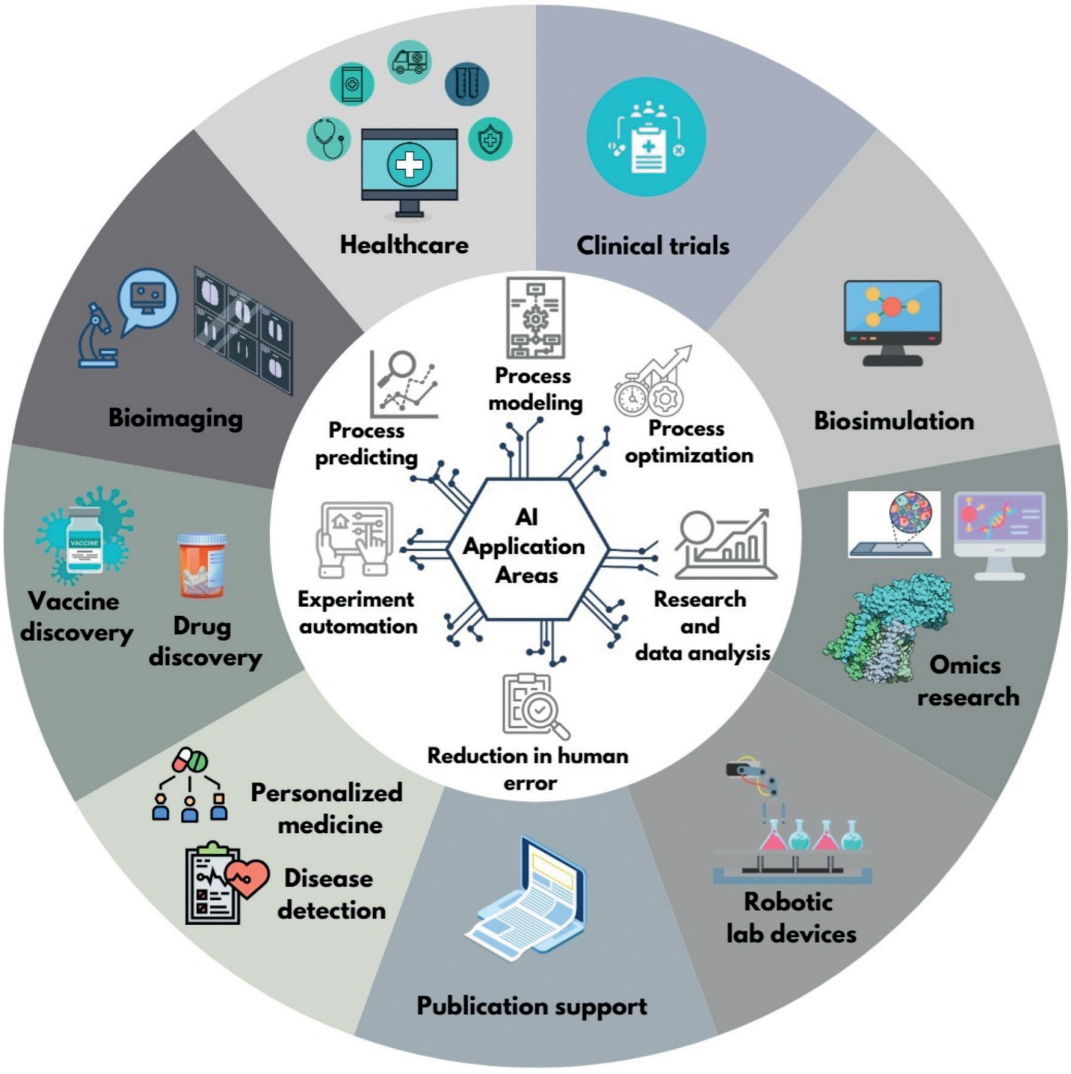

Figure 1. Applications of AI in scientific research

while David Baker from the University of Washington was honored for his work in designing novel proteins. These advancements are essential for understanding biomolecular functions and for developing drugs and vaccines (Protein designer and structure solvers, 2024; Royal Swedish Academy of Sciences, 2024).

Previously, imaging techniques such as X-ray crystallography and cryo-electron microscopy were the primary methods for determining protein structures. However, these methods were time-consuming, expensive, and often challenging to apply to all proteins. In 2020, the introduction of **AlphaFold 2** revolutionized protein structure prediction. By leveraging vast databases of protein structures and amino acid sequences, it achieved performance comparable to imaging methods and delivered over 200 million predictions (Service, 2020). In 2021, DeepMind launched Isomorphic Labs to leverage its AI tools for drug design, inspiring pharmaceutical companies worldwide to adopt similar approaches to combat cancer, infectious diseases, hypertension, and obesity (Protein designer and structure solvers, 2024). Within just 3 years, AlphaFold 2 enabled 1.8 million researchers to map approximately six million different protein structures. In a groundbreaking development, DeepMind recently released **AlphaFold 3**, which provides results in minutes instead of years of laboratory work (Abramson et al., 2024). Unlike earlier versions, which focused on modeling amino acid strands folding into 3D protein shapes, AlphaFold 3 also predicts how folded proteins bind and interact with other molecules, including DNA, RNA, and other proteins. AlphaFold 3 was released shortly after **RoseTTAFold All-Atom**

(Krishna et al., 2024), a similar AI tool from the University of Washington led by David Baker. To promote widespread adoption, DeepMind introduced AlphaFold Server, a free online platform allowing users to generate AlphaFold 3 models of proteins interacting with nearly any biomolecule (AlphaFold Server, 2024).

Understanding interactions between proteins and other biological molecules is essential for drug design and the study of cellular processes. **AlphaFold**'s breakthroughs in protein structure prediction are critical for understanding protein functions, with profound implications for drug development, biotechnology, and disease research. These advancements highlight the transformative role of AI in advancing proteomics and biomedical research. **AlphaFold** has ushered in a new era in science and medicine, revolutionizing our understanding of protein interactions and enabling groundbreaking advancements in drug development and biotechnology.

AI in genomic research

In biological research, especially in genomics, proteomics, metabolomics, and transcriptomics, vast quantities of data are generated. AI has become a cornerstone in clinical laboratory genomics, assisting in tasks such as identifying variants in DNA sequencing data, predicting the effects of DNA variants on protein structure and function, linking phenotype ontologies to genetic variants for faster diagnosis, correlating genomic data with tumor staging and treatment, utilizing natural language processing (NLP) to identify critical medical literature, and using interactive chatbots for genetic testing qualification and education (Aradhya et al., 2023). AI addresses challenges in genomic data analysis arising from high-throughput sequencing (Boulesteix and Wright, 2022). It facilitates GWAS and PheWAS to identify genotype-phenotype associations, improves pharmacogenomics, supports clinical decisions, predicts risks, identifies causal SNPs, enhances EHR-based phenotyping, and designs CRISPR guide RNA (Lin and Ngiam, 2023). Traditional analysis methods often fall short, prompting the adoption of AI, which enhances both the speed and accuracy of genomic data analysis. AI tools, including computer vision (CV), machine learning (ML), neural networks, and NLP, have become indispensable for addressing challenges in genomic data analysis (Guo et al., 2023). They assist in the analysis, interpretation, modeling, and processing of large-scale genomic data. These tools are utilized in genetic and genomic research across various diseases (Libbrecht and Noble, 2015; Yan, et al., 2016; Dias and Torkamani, 2019; Xu et al., 2019; Alimadadi et al., 2020; De Marvao et al., 2020), biomarker discovery studies (Seashore-Ludlow et al., 2015; Mamatjan et al., 2017), annotating genomic sequence elements (Libbrecht and Noble, 2015), predicting gene functions, understanding regulatory networks, and identifying disease-associated variants.

Recent studies increasingly focus on integrating machine learning techniques to process and analyze high-dimensional genomic data. AI is transforming biomedical genomics by improving data analysis, enhancing disease prediction, and advancing personalized medicine. The integration of AI techniques, such as machine learning and deep learning, allows for efficient processing of complex genomic data, leading to significant progress in biomarker discovery and genetic engineering. AI tools also automate labor-intensive processes, improve diagnostic precision, and facilitate the interpretation of complex genomic data, ultimately revolutionizing genomic research and its clinical applications. A comprehensive review in 2023 summarized 82 high-quality AI-driven biomedical genomic studies, emphasizing AI's contributions to disease diagnosis, prediction, and treatment (Guo et al., 2023). The review highlighted various AI techniques, including **ML**, deep neural networks (**DNN**), transfer learning (**TL**), **CV**, graph representation learning (**GRL**), and **NLP**. In this manuscript, we briefly discuss each of the AI techniques mentioned above:

- **ML** is widely used for analyzing large genomic datasets and identifying patterns and relationships within the data. ML encompasses techniques such as linear regression, logistic regression, decision trees, random forests (RF), support vector machines (SVMs), and neural networks (Guo et al., 2023). These methods are indispensable in medicine, particularly for diagnosing SARS-CoV-2 infections, neurological conditions (Deo, 2015; Rajkomar et al., 2019; Sidey-Gibbons and Sidey-Gibbons, 2019; Helmy et al., 2022), identifying genes linked to sepsis-induced ARDS, pinpointing chronic pain locations, uncovering genetic risk factors for various conditions, and diagnosing cancers such as leukemia, breast and lung cancer, and endometrial carcinoma (Guo et al., 2023).

- Convolutional neural networks **(CNNs)** are a prominent type of **DNN**. They have demonstrated significant effectiveness in identifying cancers by analyzing gene expression profiles. In one study, researchers analyzed 6136 samples from 11 cancer types, integrating gene expression profiles with protein–protein interaction (PPI) networks to create 2D images (Chuang et al., 2021). These images were used to develop a CNN that achieved high accuracy in distinguishing normal samples from tumors and identifying specific cancer types. CNNs are not only valuable for diagnosing and predicting cancer outcomes but also for identifying various cancer biomarkers. In the field of cancer research, DNN-based applications have been applied for detection purposes (Zhang et al., 2022). By utilizing medical images and omics data, CNN techniques were used to detect metastasis indicators, cancer cell types, and molecular subtypes using medical images and omics data, providing critical information essential for therapeutic management (Chuang et al., 2021).
- **Transfer learning** is a sophisticated AI approach in genomics particularly useful in cross-population studies (Zhao et al., 2022). It enhances predictive performance for disease risk (Jónsson et al., 2019), predicts patient data trends, and provides new insights into clinical findings from genotypic information (Dong and Boyle, 2019). Transfer learning also improves functional variant prediction accuracy, enhances enhancer-promoter interaction predictions, imputes missing RNA-sequencing data, and predicts rare diseases using gene expression datasets (Guo et al., 2023). In healthcare, transfer learning boosts diagnostic accuracy for cancer detection, mutation identification, cancer type detection from circulating tumor cells, and studying circular RNAs in nonobstructive azoospermia (Guo et al., 2023).
- Another AI tool widely used in genomic analysis is **recurrent neural networks (RNNs)**. RNNs are particularly valuable for analyzing biomedical data collected over time. While CNNs excel in image analysis, they do not account for temporal dimensions. RNNs, designed for sequential data, are ideal for analyzing time-series data such as patient health records. By incorporating temporal aspects, RNNs enable the understanding of data progression and changes over time, which is crucial for accurate predictions and informed decisions in genomics (Guo et al., 2023). For instance, RNNs significantly enhanced genomic analysis for predicting chronic diseases, as demonstrated in a Type 2 diabetes study using UK Biobank data by Srinivasu et al. (2022).
- **CV** uses mathematical methods to derive three-dimensional shapes and appearances of objects from images. Popular architectures such as **AlexNet**, **VGGNet**, **GoogLeNet**, and **ResNet** have broad applications in biomedical genomic research, including linking imaging phenotypes to tumor genetic profiles (Bodalal et al., 2019).
- **GRL** is used to transform complex genomic data into simplified, low-dimensional vectors, facilitating easier analysis and interpretation. In genomics, GRL is particularly valuable for converting sequencing data into graph structures based on gene associations or expression similarities (Guo et al., 2023). Applications of GRL techniques include diagnosing diseases such as multiple sclerosis using graph attention networks on single-cell RNA sequencing data, classifying cancers with graph convolutional networks that integrate gene expression and protein–protein interaction data, and identifying cancer subtypes and intracluster heterogeneity using dimensionality reduction techniques. GRL also aids in identifying new candidate disease genes, leveraging gene–disease associations for clinical investigation, performing link prediction tasks, integrating heterogeneous associations, constructing networks with various gene nodes, and predicting gene–disease associations and disease classifications (Guo et al., 2023).
- In genomic research, **NLP** assists by converting textual data, such as Electronic Health Records (EHRs), into computable features for Genome-Wide Association Studies (GWAS) and Phenome-Wide Association Studies (PheWAS). It also extracts detailed phenotypic characteristics for diagnosing genetic disorders (Guo et al., 2023). **BioBERT**, a revolutionary NLP tool, has significantly advanced biomedical research (Lee et al., 2020). Pretrained on vast amounts of biomedical literature, BioBERT enables researchers to understand and interpret complex biomedical information, making it invaluable for biomedical text mining, drug discovery, disease research, and biotechnology (Lee et al., 2020). The rapid expansion of biomedical literature underscores the need for tools like BioBERT

to handle the influx of information effectively. Its open-access model (Lee et al., 2020), which includes pre-trained weights and fine-tuning source code, allows researchers worldwide to leverage this technology without incurring significant costs.

The volume of data on AI applications in gene and genome analysis continues to grow. Recently, Hu et al. (2024) in *Nature Methods* highlighted the effectiveness of large language models (LLMs) such as **GPT-4**, **GPT-3.5**, **Gemini Pro**, **Mixtral Instruct**, and **Llama2 70b** in gene function analysis. These models demonstrated greater specificity and broader gene coverage compared to traditional methods, illustrating their potential to automate functional genomics research. LLMs like GPT-4 can facilitate the understanding of gene functions and interactions.

AI has proven instrumental in identifying complex structural variants (cxSVs) in whole-genome sequencing data. Researchers at Stanford Medicine have developed **ARC-SV**, an AI-based method leveraging machine learning to improve the precision of detecting and reconstructing cxSVs from standard datasets. This technique not only uncovers rare genetic variations but also connects them to neural genes and regions of rapid human-specific evolution. Furthermore, it links cxSVs to differences in gene expression and chromatin accessibility across various brain regions, advancing the genetic understanding of major psychiatric disorders (Zhou et al., 2024).

The advent of artificial intelligence has driven transformative breakthroughs in genetic medicine. Genomic medicine—the application of genomic information in clinical treatments—holds immense potential for personalized and targeted medical interventions. The integration of AI algorithms into genomic data processing has yielded remarkable successes. These algorithms excel at decoding complex genetic patterns, predicting disease probabilities, and enhancing precision medicine. A study by Hassan et al. (2022) in *Nature Medicine* demonstrated that machine learning algorithms can accurately predict patient responses to cancer immunotherapy based on genetic profiles. This finding underscores AI's transformative impact on healthcare, particularly in advancing genomic medicine, providing insights into disease causation, and enabling personalized treatment plans. By leveraging advanced algorithms and large datasets, AI is poised to revolutionize healthcare, tailoring medical treatments to individual patients.

The pursuit of fast, affordable, and precise DNA sequencing remains a critical goal in advancing personalized medicine. Conventional bioinformatics methods often struggle to efficiently process and interpret the vast volumes of generated data. In contrast, deep neural networks (DNNs) excel at recognizing complex patterns, predicting phenotypes, and classifying genomic variants. Recent advancements in deep learning have demonstrated its effectiveness in various biomedical applications, especially in Next-Generation Sequencing (NGS). These methods employ advanced neural networks to process extensive genomic data, enhancing sequencing accuracy and efficiency. By automating data analysis and uncovering patterns within complex datasets, deep learning significantly advances our understanding of genetic variations and their health implications (Özgür and Orman, 2023). Integrating machine learning algorithms into NGS workflows has the potential to reveal hidden insights, accelerate discoveries, and drive breakthroughs in genomics.

AI can enhance our understanding of how single amino acid changes in proteins impact their function. The deep learning model **AlphaMissense**, building on the protein structure prediction tool **AlphaFold2**, utilizes vast biological sequence data and predicted structural contexts to assess the pathogenicity of gene variants. This capability is essential for understanding the implications of genetic variations in disease contexts (Cheng et al., 2023). AlphaMissense predictions play a pivotal role in elucidating how genetic variants affect protein function, aiding in the identification of pathogenic missense mutations and previously undetected disease-causing genes. This advancement improves diagnostic accuracy for rare genetic disorders and fosters the development of advanced tools for predicting protein variant effects based on structural models.

In 2024, researchers introduced another therapeutic application of AI: designing and validating engineered cis-regulatory elements (**CREs**) using AI models. These CREs were tailored for targeted gene expression in specific cell types (Gosai et al., 2024). AI-engineered synthetic CREs demonstrated the potential to target gene therapies to particular cell populations. Deep neural network modeling was used to predict CRE activity across different cell types, while a high-throughput method called **Massively Parallel Reporter Assays (MPRAs)** enabled the rapid empirical

testing of thousands of designed CREs. Results showed that synthetic CREs were more effective for targeted gene expression than natural sequences derived from the human genome, highlighting their utility in therapeutic and biotechnological applications. By leveraging AI in the design of synthetic CREs, scientists can create programmable regulatory elements with precise targeting capabilities, enhancing the efficacy and specificity of gene therapies (Gosai et al., 2024).

PEACOCK is another example of an AI tool used to analyze large datasets typical in genomic research, through machine learning techniques. This tool efficiently analyzed vast amounts of genomic data to identify potential regulatory links that may not be easily discernible through traditional methods. This model could predict cell type-specific enhancer-gene regulatory links and was trained using various cell lines and a selected set of experimental data validated and published in scientific literature. The efficiency and scalability of the PEACOCK were proved by its ability to score a vast number of enhancer–gene pairs across the entire genome (~17 million pairs). The ability to score enhancer–gene pairs quantitatively allows researchers to incorporate these scores into broader statistical analyses of disease-associated variants. The scores generated by the PEACOCK model have significant implications for disease research, particularly in understanding the role of enhancers in gene regulation. The quantitative scores allow researchers to prioritize enhancer–gene pairs that are most likely to be involved in disease processes. By focusing on high-scoring pairs, researchers can concentrate on the most promising enhancer–gene pairs in diseases like cancer, can gain insights into the molecular mechanisms underlying diseases and hopefully lead to new therapeutic targets. Moreover, the cell type-specific nature of the scores can help understand how gene regulation may differ across various tissues, which is especially important in diseases where certain genes may be activated or silenced. These scores can be used in the statistical research approach of genomic disease-associated variants identified in a GWAS (Genome-Wide Association Studies, 2024). GWAS surveys the genomes of people, looking for genomic variants that occur more frequently in those with a specific disease compared to those without the disease. Once such genomic variants are identified, they are used to search for nearby variants that contribute directly to the disease. By linking these variants to specific enhancer–gene interactions, researchers can uncover how genetic variations contribute to disease risk and progression, potentially leading to new therapeutic targets. Understanding which enhancers are active or inactive in specific cell types can help us understand how disruption in enhancer function contributes to various diseases and genetic disorders. Scores designed by the PEACOCK model can serve as a useful tool for disease research by enabling targeted investigations, enhancing understanding of disease mechanisms, and linking genetic variants to regulatory relationships. This approach can contribute to the development of new treatments for disease prevention. The **PEREGRINE database** (**www.peregrineproj.org**), a publicly available resource was developed as part of the PEACOCK study. These resources allow researchers to access curated data on enhancer–gene links, facilitating further research and validation of findings in genomic studies (Mills et al., 2023).

AI has proven invaluable in biomarker analysis, particularly during the COVID-19 pandemic. By analyzing omics and clinical datasets, AI and ML algorithms have enabled effective patient stratification and management. These tools have identified critical biomarkers indicating COVID-19 severity and survival, assisting clinicians in prioritizing treatments for patients. Additionally, AI-driven analyses have uncovered gene networks associated with disease severity, underscoring the importance of clinical biomarkers in predicting disease progression (Bello et al., 2023).

AI in metabolomics

Metabolomics has diverse applications, including analyzing metabolic products of the human gut microbiome, identifying biomarkers for disease diagnosis, prognosis, and monitoring, supporting cancer research by uncovering biomarkers and metabolic pathways, aiding studies on neurodegenerative diseases such as Alzheimer's and Parkinson's, investigating xenobiotic exposures, discovering new drug candidates, and studying drug metabolism, toxicology, efficacy, and potential side effects. Additionally, metabolomics integrates with other omics domains to provide a comprehensive view of biological systems (Chi et al., 2024). However, metabolomics generates vast datasets comprising hundreds to thousands of metabolites. Incorporating AI into metabolomics provides deeper insights into metabolic networks, advancing

diagnosis, prognosis, and personalized treatment approaches for various diseases. ML techniques, including **decision trees, deep learning (DL), neural networks (NN), random forests (RF), and support vector machines (SVM)**, are employed to classify, regress, or cluster complex metabolomic data (Galal et al., 2022).

Recently, ML techniques have been applied to metabolomics data from various diseases, offering significant insights into metabolic profiles. In cancer research, AI is used to identify metabolic signatures, develop predictive models for cancer detection, prognosis, and recurrence, and analyze metabolomic data to uncover potential biomarkers and metabolic pathways. These approaches have been applied across multiple cancer types, including ovarian, breast, endometrial, hepatocellular carcinoma, gastric cancer, lung, squamous cell carcinoma, non-Hodgkin's lymphoma, renal cell carcinoma, and osteosarcoma (Galal et al., 2022; Chen et al., 2024). In noncancer conditions, AI analyzes metabolomic data to identify biomarkers, disease signatures, and predictive models for various diseases, such as COVID-19, type 2 diabetes, nonalcoholic fatty liver disease (NAFLD), acute myocardial ischemia (AMI), chronic kidney disease (CKD), celiac disease, multiple sclerosis (MS), major depressive disorder, schizophrenia, and autism spectrum disorders. Additionally, AI applications extend to areas such as determining gestational age (Galal et al., 2022).

AI and cancer diagnosis and prediction

AI tools, including ML and deep learning (DL), are revolutionizing cancer diagnosis by enhancing accuracy, improving efficiency, and offering noninvasive techniques. By analyzing medical data such as genomic sequences, imaging, and electronic health records, AI aids in identifying early-stage cancer biomarkers, improving recovery rates, and reducing mortality. These technologies are becoming integral to oncology and preventive healthcare. Integrating AI with genomic studies helps identify cancer-related genes, supporting precision medicine tailored to patients' genetic profiles.

In addition to AI, technologies like big data analytics, cloud computing, and the Internet of Things (IoT) play vital roles in early cancer detection. Big data enables the analysis of large, complex datasets to uncover early cancer indicators, while cloud computing provides secure and efficient platforms for managing vast medical data. Wearable sensors collect biomarker data, offering real-time updates on potential cancer developments.

Advanced AI tools are improving the accuracy of cancer diagnosis through methods such as mammography and CT scans, often outperforming traditional techniques in detecting cancers like breast and lung cancer. AI has shown significant promise in enhancing breast cancer screening by identifying additional cases, improving positive predictive value, and reducing unnecessary recalls (Ng et al., 2023). For instance, the AI tool **Mia**, developed by Imperial College London and Kheiron Medical Technologies, was found to detect 13% more early-stage breast cancers (Transforming Cancer Diagnostics, 2024). Mia detected more cancers and led to more women being recalled, according to a release from Imperial College London. Mia was named one of the biggest seven medical breakthroughs in 2023 by ABC News (7 of the biggest medical breakthroughs in 2023, 2024). In a recent evaluation by Britain's National Health Service, Mia analyzed mammograms from over 10,000 women. Mia accurately identified patients who had cancer, including 11 cases that were initially missed by human doctors. Mia's impact underscores AI's potential to enhance diagnostic accuracy in breast cancer screening (NHS AI Test, 2024). AI platforms such as **CHIEF** (Wang et al., 2024), **Sybil** (Aro et al., 2024), **IBM Watson for Oncology** (Jie et al., 2021), and **Tempus** (2024) are further transforming cancer diagnosis and prediction by facilitating early detection, personalized treatment plans, and improved patient outcomes. These platforms significantly reduce diagnosis time while meticulously analyzing imaging and genomic data (Arefin, 2024). An AI model achieved 96% accuracy in diagnosing invasive lobular carcinoma (ILC) using genetic mutations as ground truth (Pareja et al., 2024). In prostate cancer, AI systems have matched the diagnostic capabilities of experienced physicians, enabling early and accurate detection (Shucai and Heyuan, 2024). AI is also revolutionizing colorectal cancer (CRC) diagnosis and treatment, with advancements in classification, detection, digital pathology, endoscopic data processing, high-precision medical image analysis, personalized treatment, and robot-assisted surgery. These developments have substantially improved diagnostic accuracy for CRC. Machine learning prediction models enable faster and more accurate early-stage diagnoses, increase treatment success rates, and

reduce colorectal cancer mortality. Furthermore, AI optimizes the allocation and utilization of medical resources, enhancing healthcare efficiency (Sun et al., 2024).

AI in biomedical image analysis and digital pathology

Recently, a team of scientists has created **BiomedParse**, an AI model designed for medical image analysis. BiomedParse can handle nine imaging modalities, enhancing the prediction of systemic diseases by unifying segmentation, detection, and recognition tasks. The model introduces new capabilities, such as segmenting objects through textual descriptions, significantly improving accuracy and expanding applications. To develop BiomedParse, researchers created a dataset of over 6 million triples, including images, segmentation masks, and textual descriptions, using natural language labels from existing datasets. By integrating object recognition, detection, and segmentation, **BiomedParse** outperforms existing tools like **MedSAM** (Ma et al., 2024) and **SAM** (2024), particularly in handling irregularly shaped objects. Its ability to segment and label all objects in an image simultaneously makes it a comprehensive tool for biomedical image analysis, paving the way for efficient and accurate discoveries (Zhao et al., 2024).

Another groundbreaking AI tool is **Prov-GigaPath**, designed to address the unique computational challenges of digital pathology. Gigapixel slides, comprising tens of thousands of image tiles, require advanced models to process their immense size effectively. Prov-GigaPath is an open-access, whole-slide pathology foundation model pretrained on over one billion 256 × 256 pathology image tiles from more than 170,000 whole slides. By incorporating pathology reports into vision-language pretraining, Prov-GigaPath integrates real-world data and supports comprehensive slide modeling. Prov-GigaPath stands out as an open-weight model, excelling in various digital pathology tasks and paving the way for advancements in biomedical discoveries (GigaPath, 2024; Xu et al., 2024).

AI in automated cell tracking and microscopy

Microscopic image analysis is a cornerstone of biological research. AI, particularly deep learning algorithms, has revolutionized this field by enabling faster and more accurate identification of cells and tissue structures. AI is revolutionizing the field of microscopy by enhancing the detection and classification of cells through advanced algorithms. Recently, **DeepTrack 2.0** was introduced, a user-friendly software that simplifies the creation, training, and validation of deep-learning models for digital microscopy. DeepTrack 2.0 supports applications such as particle tracking and cell classification, making deep-learning-enhanced video microscopy more accessible to a broader audience (Midtvedt et al., 2021). Additionally, AI algorithms enable real-time microscopy image analysis, facilitating immediate decision-making during image acquisition. Researchers utilized artificial intelligence for real-time cell detection and classification in automated microscopy, employing a high-dimensional feature extractor and machine learning, particularly random forests, to enhance execution performance while maintaining accuracy in biological image analysis (Balluet et al., 2022). AI has also automated cellular segmentation in microscopy images (Eisenstein et al., 2023), improving the accuracy of cell detection and enabling the extraction of quantifiable cellular features. These advancements are critical for understanding cellular organization in various pathologies (Durkee et al., 2021). A paper from 2020 presents a neural architecture search (NAS) method developed to optimize network designs for cell segmentation, achieving better performance compared to traditional methods (Zhu and Meijering, 2020).

Automated cell tracking has become an invaluable tool in biological research. Advances in optical microscopy and machine learning, especially deep neural networks, have driven the need for improved tracking algorithms. The **Cell Tracking Challenge (CTC, http://celltrackingchallenge.net)** was created to promote the development and evaluation of these algorithms. Since its launch in 2013, the CTC has offered a free repository of annotated microscopy videos and standardized evaluation metrics. AI techniques, including deep learning models like **U-Net**, **HRNet, R-CNN**, and **recurrent neural networks**, have significantly advanced cell tracking, improving accuracy and enabling more insightful research (Maška et al., 2023).

AI and spatial omics

Spatial omics is a transformative field focused on measuring and mapping biomolecules such as RNA, DNA, and proteins directly within their native tissue environments. This innovative approach enables researchers

to observe the spatial organization and interactions of cells, offering unprecedented insights into cellular functions and disease mechanisms. A critical step in spatial omics image analysis is cell segmentation, which facilitates the accurate identification and isolation of individual cells within tissue samples. By preserving spatial context, segmentation allows precise molecular analysis and a deeper understanding of cellular processes. This combination enables highly precise localization and quantification of biomolecules within tissues, allowing for a nuanced view of cellular behavior and interactions. The integration of spatial omics with advanced AI technologies is transforming the landscape of biological research, offering novel ways to explore the complexities of how cells interact and the impact these interactions have on health and disease. This synergy between spatial omics and AI not only enhances our understanding but also opens up new possibilities for diagnostics and therapeutic strategies. Scientists at the Children's Hospital of Philadelphia (CHOP) have recently announced the creation of an AI tool called **CelloType.** CelloType is open-source software available in a public repository for noncommercial use (CelloType, 2024). This model was designed to more accurately identify and classify cells in high-content tissue images (Pang et al., 2024). Another similar AI tool is **NVIDIA VISTA-2D**. This model advances cell segmentation and morphology analysis by leveraging NVIDIA's deep learning technology. It is designed to handle large-scale tasks and improve the accuracy of cell detection and segmentation (NVIDIA DEVELOPER, 2024). Two companies, NanoString Technologies and NVIDIA have joined forces to advance spatial biology research. **NanoString's CosMx Spatial Molecular Imager** (**SMI**) platform integrates NVIDIA's GPU technology, enabling the rapid processing and analysis of large datasets. This collaboration allows for the imaging of the entire transcriptome within cells and tissues, providing deeper insights into cellular functions and disease mechanisms (NVIDIA, 2024a).

AI has also facilitated advancements in 3D cell detection and segmentation. Researchers trained an object detector called **CellFinder** for automated 3D cell detection in large-scale images, including neuronal somata in whole-brain images of mice (Tyson et al., 2021; BrainGlobe, 2024). In 2024, the **CellSAM** tool was introduced, excelling in segmenting images of mammalian cells, yeast, and bacteria (Israel et al., 2024) across various imaging modalities. CellSAM builds upon CellFinder by retraining it to prompt the **Segment Anything Model (SAM)** for segmentation. This innovative approach enables a single AI model to accurately segment images of mammalian cells in tissues and cell culture, as well as yeast and bacteria, collected across various imaging modalities (CellSAM, 2024).

AI and electron microscopy (EM)

Recent advancements in EM have been significantly enhanced by ML-based analysis. Research by Xu et al. (2021) demonstrates the integration of ML techniques in improving focused ion beam-scanning electron microscopy (FIB-SEM). These advancements include enhanced data acquisition, improved signal detection, and faster scanning, enabling the imaging of cellular samples at nanometer resolution. ML-based analysis efficiently processes and interprets complex imaging data, providing detailed visualizations of cellular structures.

Similarly, Heinrich et al. (2021) have made strides in data acquisition and ML-based analysis in microscopy, improving the ability to map and analyze molecular interactions within cells. These developments bring researchers closer to a comprehensive understanding of cellular physiology. Collectively, these studies highlight the use of AI and ML in overcoming traditional microscopy limitations and advancing the field of cell biology (Swedlow and Collinson, 2021).

AI and infectious diseases

AI has made significant advancements in infectious diseases, playing a crucial role in developing diagnostic and therapeutic methods, forecasting outbreaks, optimizing treatment plans, and analyzing diagnostic images. It is instrumental in discovering anti-infective drugs and vaccines and combating the rise of antimicrobial resistance.

AI-powered clinical decision support systems forecast disease outbreaks, assist with precise diagnoses, enhance treatment plans, and track epidemiological trends by analyzing extensive datasets. Furthermore, AI improves the analysis of diagnostic images and disease identification, enabling quicker and more accurate results. By examining large datasets, AI systems boost diagnostic precision and treatment strategies, predict disease outbreaks, and monitor epidemiological patterns

(Aslan, n.d.). Advances in AI applications for infectious diseases hold promise for more effective intervention strategies and improved public health protection (Aslan, n.d.; Singh, 2024).

For instance, AI was used to predict COVID-19 hospitalization and mortality. While these techniques show promise, further validation is required to address related challenges (Shakibfar et al., 2023a). Another study highlighted AI's potential in creating a Disease Risk Score for predicting COVID-19 hospitalization and mortality using health registry data. This approach demonstrates AI's ability to identify high-risk individuals, though it faces issues with generalizability and external validation (Shakibfar et al., 2023b).

Hien et al. (2024) utilized real-world clinical data to develop predictive models for severe COVID-19 outcomes, demonstrating the significant role of machine learning (ML) in the early identification and management of high-risk patients. Similarly, Banoei and colleagues employed ML techniques to forecast mortality among hospitalized COVID-19 patients. Their analysis of relationships between various risk factors identified critical indicators such as low oxygen levels and chronic kidney disease. This study provides a foundational understanding of these risk interactions, aiding in the prioritization of treatment approaches (Banoei et al., 2023).

Expanding beyond COVID-19, Gao et al. (2023) analyzed risk factors and predictive models for pulmonary tuberculosis, introducing **TBINet**, a deep-learning model leveraging CT images to identify pulmonary tuberculosis (PTB) infectivity. Validated by high AUC values and gradient-weighted class activation mapping (Grad-CAM) technology, this approach offers a swift and reliable diagnostic method, showcasing the utility of AI in medical imaging analysis for tuberculosis diagnosis and control. Their study demonstrated the feasibility of using CT images to rapidly and cost-effectively identify PTB infectivity through deep-learning methods.

AI has also been effectively employed in diagnosing Hansen's disease (HD). It excels in rapid case detection, personalized treatment planning, mental health counseling, case classification, ensuring compliance with multidrug therapies, tracking geographical treatment distribution, and identifying adverse drug reactions and antimicrobial resistance. Moreover, AI plays a pivotal role in the early detection of nerve damage, which is crucial for preventing disabilities and planning rehabilitation. These capabilities are especially valuable in regions with a shortage of trained healthcare professionals (Deps et al., 2024).

The integration of AI into the management of infectious diseases holds immense potential to revolutionize diagnosis, treatment, and understanding of disease mechanisms. AI has proven to be highly effective in predicting, detecting, and controlling the spread of infectious diseases, as particularly highlighted during the COVID-19 pandemic. This technology plays a critical role in preventing future health crises by predicting outbreaks, identifying high-risk areas, and aiding vaccine development. Additionally, AI's ability to track and trace infected individuals, identify potential hotspots, limit the spread of infections, and monitor patient symptoms empowers healthcare professionals to deliver more effective treatments (Siddig et al., 2023; Hsu et al., 2024).

AI and vaccine design

AI technology has significantly enhanced the vaccine development process, providing innovative solutions to accelerate and optimize vaccine design. Researchers at Baidu Research developed an AI tool called **Linear Design**, which designed the optimal mRNA sequence for the SARS-CoV-2 spike protein in just 11 min. This breakthrough achieved a 128-fold increase in the COVID-19 vaccine's antibody response and significantly improved vaccine stability (Dolgin, 2023; Zhang et al., 2023).

AI is also revolutionizing bacterial vaccine development. AI tools have identified 22 putative antigens for *Helicobacter pylori*, characterized T-cell epitopes for *Mycobacterium*, and validated the membrane protein FilF as a potential vaccine candidate for *Acinetobacter baumannii*. Additionally, AI is aiding vaccine development for *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Streptococcus pneumoniae* (Gorki and Medhi, 2024). The potential of AI extends to parasitic infections, such as gastrointestinal nematodes (GINs), where AI techniques are being applied to develop effective anti-GIN vaccines. By increasing precision, accelerating design processes, and expanding our understanding of disease mechanisms, AI is transforming vaccine production. Despite these advancements, rigorous laboratory testing and regulatory approval remain essential to ensure vaccine safety and efficacy.

AI models support scientific research

Starting in July 2024, scientists at Los Alamos National Laboratory in New Mexico began evaluating the **multimodal LLM model GPT-4o** (2024) in real-world laboratory settings. This groundbreaking project aims to assess GPT-4o's ability to assist both expert and novice scientists with complex biological tasks through its visual and voice capabilities. Tasks include transformation (introducing foreign genetic material into a host organism), cell culture (maintaining and propagating cells *in vitro*), and cell separation (e.g., through centrifugation). By integrating AI into standard laboratory workflows, the project seeks to drive innovation in biosciences while identifying potential risks associated with AI-assisted research (OpenAI and Los Alamos National Laboratory, 2024; Pannu et al., 2024). The potential of AI-assisted biological research to enhance human health and well-being is clear, yet significant unpredictabilities and risks remain. Researchers have emphasized the need for swift government action to establish comprehensive testing and mitigation strategies, leveraging decades of expertise to address large-scale biological research risks (Pannu et al., 2024). In 2023, Microsoft conducted evaluations of **GPT-4**, revealing its ability to provide detailed guidance for using the Rosetta protein design tool. This tool successfully created an antibody capable of binding to the SARS-CoV-2 spike protein. GPT-4 also demonstrated capabilities in automating biological experiments by converting experimental protocols into code for liquid-handling robots, significantly expediting laboratory workflows (Microsoft Research AI4Science, 2023). Further advancements were demonstrated by researchers at Carnegie Mellon University, who developed **Coscientist**, a system powered by GPT-4 that could design, plan, and execute complex experiments, including chemical syntheses. This system was capable of searching through documents, writing code, and operating robotic lab devices (Boiko et al., 2023). Recently, researchers from Stanford University and the Chan Zuckerberg Biohub introduced a **Virtual Lab.** This system comprises a group of large language model agents powered by GPT-4o that managed to design effective SARS-CoV-2 nanobodies (a type of antibody) with minimal human intervention (Swanson et al., 2024).

AI in drug repurposing and discovery

AI presents an exciting future for rapid drug repurposing and discovery. By analyzing vast biological and chemical datasets, AI uncovers hidden connections between existing drugs, disease targets, and potential new treatments (Singh, 2024). AI's capability to examine large datasets of drugs and disease targets is a potent tool for discovering new therapeutic uses for existing medications (Singh, 2024). By analyzing extensive biological and chemical datasets, AI can reveal hidden links between existing drugs, disease targets, and new therapeutic uses.

For example, Baricitinib, initially developed for rheumatoid arthritis, has shown potential as a COVID-19 treatment due to its anti-inflammatory and antiviral properties. AI analysis of Baricitinib's interactions helped researchers predict and manage potential side effects, enabling safer clinical trials (Cantini et al., 2020; Stebbing et al., 2020; Saber-Ayad et al., 2021; Richardson et al., 2022). Similarly, Lopinavir/Ritonavir, an HIV medication, was investigated for its ability to inhibit a crucial SARS-CoV-2 enzyme (protease). However, AI analysis identified potential adverse effects on liver function, highlighting the drug's limitations. This insight emphasizes the need to develop new, targeted inhibitors specifically designed to combat SARS-CoV-2, potentially overcoming the constraints of repurposed drugs (Parvathaneni and Gupta, 2020; Singh, 2024).

By analyzing chemical structures and predicting their interactions with disease targets, AI can uncover potential candidates across various therapeutic fields. For instance, Alendronate, commonly used to treat osteoporosis, exemplifies the efficacy of AI in drug repurposing. AI identified its ability to inhibit a key enzyme essential for the proliferation of certain cancer cells, suggesting its potential as a cancer treatment and broadening the spectrum of candidate drugs beyond traditional considerations (Saul and Einav, 2020; Alachram et al., 2021; Usha et al., 2021; Singh, 2024).

AI has become an invaluable tool, leveraging extensive datasets to forecast and refine drug characteristics, resulting in safer and more efficient repurposed medications. Current efforts include using AI to optimize dosing schedules of antiretroviral drugs for HIV, enhancing patient adherence and minimizing side effects (Xing et al., 2020; Serghini et al., 2023).

In the pharmaceutical industry, AI addresses significant challenges by detecting biological activity in preclinical screenings, optimizing pharmacokinetic properties for better formulations, predicting early toxicity to reduce attrition rates, and proactively screening for genetic mutations in biological targets to prolong thera-

peutic effectiveness (Serghini et al., 2023). Furthermore, integrating AI with patent data analysis offers a robust approach to identifying and repurposing existing drugs for new uses. This accelerates the development of cost-effective, accessible treatments and enhances the healthcare system's preparedness for emerging diseases.

By examining expired patents, AI can identify drugs no longer under patent protection, making them available for repurposing. This approach is particularly beneficial for antiviral treatments targeting less common viruses, where developing new drugs may not be economically feasible. It accelerates drug repurposing and ensures more efficient use of resources in addressing pathogenic threats. For instance, AI analysis identified Teicoplanin, an antibiotic no longer under patent protection, as having potential antiviral properties against the Zika virus (Dalal and Biswas, 2024; Ishaq et al., 2024).

The integration of computational and experimental methods is critical for discovering and developing molecules to combat deadly diseases. Computational approaches include active site prediction, homology modeling, ligand preparation, molecular dynamics simulation, molecular docking, pharmacophore modeling, target identification, and virtual screening. These AI-enabled capabilities highlight the potential of computer-aided drug design (CADD) to streamline and enhance the drug discovery process (Dalal and Biswas, 2024).

AI also plays a pivotal role in designing clinical trials for repurposed drugs. It can optimize dosages, identify outcome measures tailored to the drug and targeted virus, select highly relevant patient groups most likely to benefit, and determine treatment durations. This approach reduces costs, resource needs, and time to market while enabling researchers to achieve more conclusive trial results (Chopra et al., 2023).

The application of AI in drug development represents a significant breakthrough, enabling more efficient and effective discovery of medications, particularly for chronic diseases. By streamlining the drug discovery process, reducing costs, and accelerating the timeline for bringing new treatments to market, AI has the potential to revolutionize the pharmaceutical industry and improve healthcare outcomes.

AI and biosimulation

Biosimulation, which involves simulating biological systems and processes using mathematical models, leverages AI algorithms for pattern recognition in clinical trials and the analysis of connections between drugs, patients, demographics, and trial parameters. These models empower researchers to address questions related to optimal dosing, medication interactions, and population-level efficacy.

For instance, **VeriSIM Life's BIOiSIM** platform utilizes AI and ML to simulate the effects of chemicals on individual organs and entire bodily systems. This allows researchers to investigate optimal dosages, drug interactions, and overall effectiveness across populations while accelerating drug development and reducing reliance on extensive animal testing (BIOiSIM, 2024).

Other leaders in AI-driven biosimulation include **Certara**, which specializes in pharmacokinetic–pharmacodynamic (PK/PD) simulation and toxicokinetic (TK) modeling (Phoenix WinNonlin™ Software, 2024). Their **SimcypPBPK** platform is widely used to describe drug behavior in various body tissues, predict drug toxicity, and optimize dosing regimens (Simcyp™ PBPK Simulator, 2024).

Simulations Plus develops various models to predict drug toxicity and interactions, aiding research processes. Their **GastroPlus** software is an advanced simulation tool that models different types of drug absorption and pharmacokinetics in both humans and animals. It covers intravenous, oral, oral cavity, ocular, inhalation, dermal, subcutaneous, and intramuscular administration routes (Innovative science-based software, 2024). **Schrodinger** offers tools for molecular modeling and chemical engineering simulations, aiding in the discovery of new drugs (Opening New Worlds for Molecular Discovery, 2024). **Genedata** utilizes AI for data management and simulation in drug development processes, resulting in more efficient and cost-effective drug development (Digitalizing Biopharma R&D, 2024).

AI in clinical trials

AI is revolutionizing clinical trials by speeding up data analysis, optimizing study designs, and streamlining patient recruitment, thereby boosting efficiency and cutting costs (Hutson, 2024). AI is employed in clinical studies to perform tasks such as data analysis, protocol preparation, and patient recruitment. It can help lower clinical trial drop-out rates, analyze videos to ensure medication adherence and answer patient questions

through chatbots like **ChatDoctor** (Li et al., 2023). In a 2024 article published in *Nature* (Hutson, 2024), various AI platforms in clinical trials were discussed, such as **HINT**, which predicts trial success; **SPOT**, which analyzes trial timing; **SEETrials**, which extracts safety and efficacy information; **CliniDigest**, which summarizes clinical trial records; **Trial Pathfinder**, which assesses participation criteria; and **Criteria2Query**, which converts eligibility criteria into database queries. Additional platforms include **DQueST** for helping patients search for trials, **TrialGPT** for matching patients with trials, **Unlearn** for creating digital twins for control groups, **PLIP** for managing and organizing trial data, **AutoCriteria** for extracting eligibility criteria, **ChatTrial** for answering trial-related questions, and **SDQ** from **Saama**, which assists with data cleaning and milestone prediction.

Implementing AI in clinical trials faces challenges such as potential bias, difficulties in reproducing results, data privacy and security risks, over-reliance on AI, and the complexity of algorithms leading to a lack of transparency. Despite these challenges, the FDA recognizes the growing role of artificial intelligence and machine learning (AI/ML) throughout the drug development cycle and their potential to accelerate the process.

For instance, AI/ML methods can assist in clinical trials by selecting patients based on baseline characteristics such as demographic data, clinical information, vital signs, laboratory results, medical imaging, and genetic data to predict clinical outcomes following investigational treatments. These predictive models can identify patients with worse prognoses or those most likely to benefit from a treatment, ultimately aiding in demonstrating a drug's effectiveness.

On November 8, 2022, the FDA issued an Emergency Use Authorization (EUA) for anakinra (Kineret) to treat COVID-19 in hospitalized adults with pneumonia requiring supplemental oxygen (low- or high-flow) who were at risk of progressing to severe respiratory failure (SRF) and likely to have elevated levels of plasma soluble urokinase plasminogen activator receptor (suPAR). Elevated suPAR levels are indicative of increased inflammation or immune response (Winnicki et al., 2019). Anakinra is the first interleukin-1 inhibitor authorized for COVID-19 treatment.

Notably, the FDA developed an in silico scoring rule as an alternative method to identify suitable patients for anakinra treatment in the absence of a commercially available suPAR assay in the United States. This scoring rule utilized AI/ML to analyze clinical characteristics and laboratory tests to predict patients likely to have elevated suPAR levels, a key criterion for anakinra treatment. This marked the FDA's first use of AI/ML to identify an appropriate patient population for drug therapy (Liu et al., 2024).

AI in early disease diagnosis

Hospitals and medical research institutions are increasingly developing AI tools for disease detection. These tools study and analyze symptoms, medical histories, and diagnostic processes to identify whether a patient is at risk for or experiencing the early stages of a disease. Early intervention and therapy facilitated by detection algorithms can slow disease progression or alleviate symptoms. Contract Research Organizations (CROs) may use these algorithms to identify and enroll patients at earlier stages of disease development, particularly during the prodromal phase. **IQVIA** has developed a data-driven illness detection program that evaluates a patient's symptoms and characteristics, provides treatment recommendations–including clinical trials–and makes expert referrals (Leveraging Real World Data to Measure Disease Severity, 2024). Other companies leading in AI-driven disease detection include **AiCure**, which enhances treatment through real-time patient monitoring and data analysis (Patient Engagement, 2024); **United Imaging,** which offers AI-based solutions to improve diagnostic imaging accuracy (Alabama gets its first uCT® ATLAS, 2024); and **Ibex Medical Analytics,** which provides AI solutions for pathology analysis (Trusted Cancer Diagnostics, 2024).

AI and small molecule drugs

AI tools can analyze extensive datasets of existing drugs and drug candidates to uncover groundbreaking new insights. Artificial intelligence systems can forecast interactions between small molecule drugs and their target proteins, predict potential side effects, identify relationships and patterns within drug datasets, and enable researchers to develop new small molecule drugs with improved pharmacokinetic profiles, minimized side effects, and enhanced efficacy (Kirkpatrick, 2022). Recent advancements underscore how AI is revolutionizing the design and discovery of small molecules, with examples including USP1 inhibitors, KAT6A in-

hibitors, INS018_055, and small-molecule candidates in oncology, immunology, neuroinflammation, neurology, and cardiometabolic diseases (Dealmakers, n.d.).

AI voice assistants

AI-driven voice assistants are increasingly being employed in clinical trials to manage various routine monitoring tasks, such as reminding patients of upcoming appointments and tracking their daily activities. AI voice assistants like Alexa, Google Assistant, and Siri have the potential to transform healthcare by turning speech into a valuable health indicator, enabling the early detection and prediction of potential health conditions. Specific acoustic features in speech have been linked to psychiatric disorders such as depression, PTSD, anxiety, and eating disorders (Low et al., 2020). For example, a vocal biomarker has been associated with hospitalization and mortality rates in patients with heart failure (Maor et al., 2020), and vocal biomarkers have been shown to correlate with depression severity and treatment response (Mundt et al., 2012). By capturing and analyzing subtle voice changes, AI can generate a range of health measurements to provide a more comprehensive picture of overall health. Machine learning technology using speech samples, obtained either in clinical settings or remotely, could eventually serve as a biomarker to improve diagnosis and treatment. Current research primarily focuses on using speech's acoustic features to detect conditions like depression and schizophrenia (Low and Bentley, 2020).

Companies engaged in AI/ML-enabled discovery

Over the years, the interest in applying AI to drug R&D has significantly increased, driven by expectations of faster timelines, reduced costs, and the ability to uncover hidden insights from large datasets. More than 150 AI-focused companies have raised substantial funding, particularly in small-molecule drug discovery, through venture capital financings, initial public offerings (IPOs), and high-value partnerships with large pharmaceutical companies (Table 1). Currently, the first AI-based small-molecule drug candidates have entered clinical trials (Kirkpatrick, 2022).

BenevolentAI, an AI-enabled drug discovery company, utilizes a knowledge graph that integrates publicly available biomedical and chemical data with proprietary datasets, allowing AI tools to generate target hypotheses. It focuses on identifying targets for chronic kidney disease (CKD), idiopathic pulmonary fibrosis (IPF), heart failure and systemic lupus erythematosus (Kirkpatrick, 2022; Chopra et al., 2023). **Verge's** AI platform using a proprietary collection of patient brain transcriptomes, identifies targets for amyotrophic lateral sclerosis (ALS) and other neurodegenerative diseases (Kirkpatrick, 2022). **NVIDIA Corporation** launched Clara Holoscan MGX to enable the creation and deployment of real-time AI applications. Clara offers tools for medical imaging, genomics, and AI model development (NVIDIA, 2024b). **Insitro** combines machine learning with high-throughput biology. It generates biological datasets from cellular disease models, integrating them with human clinical data to identify therapeutic targets. Insitro aims to discover novel targets for ALS and frontotemporal dementia (FTD) (Insitro, 2024; Making Medicines, 2024). **Recursion Pharmaceuticals** focuses on generating data from cellular models employing ML. By using image-based profiling of cellular disease models treated with various potential drug leads, Recursion identifies novel targets and medicines in neuroscience and oncology (Kirkpatrick, 2022). **Exscientia** specializes in AI-driven small-molecule drug design, oncology and immunology (Kirkpatrick, 2022). **Insilico Medicine** focuses on AI-driven preclinical research. Insilico initiated a phase 1 trial of the small-molecule inhibitor ISM001-055 for idiopathic pulmonary fibrosis. Using its AI platform, both the target and drug candidate were identified, reducing the time from target discovery to phase 1 trial initiation to less than 30 months. Partnership with Fosun Pharma led to nomination of ISM004-1057D as a first-in-class small-molecule inhibitor of the enzyme QPCTL regulating the CD47–SIRPα pathway (Kirkpatrick, 2022). **Relay Therapeutics** specializes in identifying drug candidates based on protein dynamics. The company's SHP2 inhibitor, RLY-1971 is currently in phase 1 trials for cancer treatment (Kirkpatrick, 2022). **Takeda Pharmaceuticals** aims to develop therapies and diagnostics for inflammatory bowel disease (IBD) using advanced bioinformatics and machine learning tools (Prometheus Biosciences, 2024). Takeda explored over 60 indications for preclinical and clinical molecules, identifying new treatment options within 18 months. A key advancement includes the clinical development of TAK-733 (REC-4881), a MEK inhibitor

Table 1. AI-driven innovations in biotechnology: companies and their key products

Company (Ref.)	AI application area	AI technology	Partnerships/Collaborations
BenevolentAI (Kirkpatrick, 2022; Chopra et al., 2023)	Drug discovery, CKD and IPF, treatment, heart failure, and lupus research	AI-driven drug discovery	Partnership with AstraZeneca
Verge Genomics (Kirkpatrick, 2022)	ALS, neurodegenerative diseases	AI platform for target identification in neurodegenerative diseases	Partnership with Eli Lilly
NVIDIA Corporation (2024)	Medical imaging, genomics, and AI model development in healthcare and life sciences; treatment, diagnosis, and drug discovery	Clara Holoscan MGX	
Insitro (2024); Making Medicines (2024)	Drug discovery, neurodegenerative diseases, ALS and FTD treatment	Machine learning combined with high-throughput biology	Collaboration with Bristol Myers Squibb
Recursion Pharmaceuticals (Kirkpatrick, 2022)	Drug discovery, oncology, neuroscience	AI-guided drug discovery with image-based profiling of cellular disease models	Collaboration with Genentech, Roche
Exscientia (Kirkpatrick, 2022)	Drug discovery, oncology, immunology	AI-driven small-molecule drug design	Collaboration with Bristol Myers Squibb, Sanofi
Insilico Medicine (Kirkpatrick, 2022)	Drug discovery, immuno-oncology	AI-driven preclinical research, small-molecule drug development; ISM004-1057D (a first-in-class small-molecule inhibitor of the enzyme QPCTL regulating the CD47–SIRP pathway)	Partnership with Fosun Pharma
Relay Therapeutics (Kirkpatrick, 2022)	Cancer, drug discovery	Drug discovery, RLY-1971 (SHP2 inhibitor development for cancer)	Collaboration with Genentech
Takeda Pharmaceuticals (Merck, 2024; Prometheus Biosciences, 2024; Takeda, 2024)	Drug development, oncology, IBD treatment	Bioinformatics and machine learning for medical treatments and drug innovation; TAK-733 (REC-4881), a MEK inhibitor for hereditary cancer syndrome	Partnerships with Prometheus Biosciences, Recursion
ReviveMed's AI (2024)	Cancer immunotherapies	AI platform	Bristol Myers Squibb
Sensyne Health (2024)	Study of myeloproliferative neoplasms development	Machine learning	Bristol Myers Squibb
Medidata (2024a, 2024b)	Clinical trials, drug development	AI-powered solutions for drug research and clinical trials	Adopted across the pharmaceutical, biotech, academic, government sectors, hospitals and clinical research organizations (CROs)
Saama Technologies (2024)	Clinical research, drug discovery	AI platform	
Health (formerly Pillsy 2024)	Healthcare	Mobile app for medication compliance monitoring and patient-reported information	
AliveCor (2024)	Cardiology, healthcare	Wearable EKG device for cardiac rhythm detection	Partnership with Medable

for hereditary cancer syndrome (Takeda, 2024). **ReviveMed's** AI platform focuses on the mechanisms of response and resistance to cancer immunotherapies (ReviveMed, 2024). **Sensyne Health** (2024) leverages ML for studying the development of myeloproliferative neoplasms, a group of rare blood diseases. **Medidata AI** (2024b) offers AI tools designed to accelerate drug development, reduce risks, lower costs, and enhance patient outcomes. By harnessing AI algorithms and ML, Medidata AI assists hospitals and clinical research organizations (CROs) in executing clinical trials. Launch Therapeutics has chosen Medidata AI Intelligent Trials to expedite the preparation of clinical trials. This analytics system uses AI to enhance trial design and execution through real-time performance measurements, predictive models, and forecasting capabilities (Medidata 2024a). **Saama** (2024) offers products to expedite clinical research and commercialization, by streamlining key phases of clinical research and automating labor-intensive steps through AI and big data analysis. **Health** has developed a mobile app aimed at improving medication compliance in real-time research. The app includes features like reminder notifications, dosage tracking, educational content, and patient-reported information collection for healthcare providers (Pillsy, 2024). **AliveCor** (2024) focuses on biometric data collection through its wearable electrocardiogram (EKG) device. Using ML, the device detects irregular cardiac rhythms, such as atrial fibrillation (AF), by analyzing real-time data. AliveCor partnered with Medable to facilitate remote clinical trials in cardiology.

AI and biosafety and biosecurity risks

AI developers anticipate that combinations of artificial intelligence techniques, including automation technologies, LLMs, and robotics, will enable experiments—such as the manipulation, design, and synthesis of DNA, drug candidates, or toxins—with minimal human involvement. These advances have the potential to transform biomedical research, but they also pose significant biosafety and biosecurity risks (Urbina et al., 2022).

In response to these growing risks, various governments have implemented measures to address safety concerns associated with advanced AI models. In 2023, the US government secured voluntary commitments from 15 leading AI companies to better manage AI-related risks. Later that year, US President Joe Biden issued an Executive Order to ensure the safe, secure, and trustworthy development and deployment of artificial intelligence. This order mandates that companies must inform the government before launching models primarily trained on biological sequence data and requiring more than 10^23 computing operations (Pannu et al., 2024).

In November 2024, representatives from ten governments participated in the first meeting of the International Network of AI Safety Institutes in San Francisco, California (2024). France is set to host the AI Action Summit in Paris in February 2025 (Elysee, 2024; Pannu et al., 2024). Countries such as Canada, Japan, Singapore, the United Kingdom, and the United States have established government institutes focused on AI safety, creating standards and tools to manage risks. Australia, the European Union (which has set up a safety unit within its AI Office), France, Kenya, and South Korea are the founding members of the International Network of AI Safety Institutes.

In the absence of comprehensive government policies to address urgent risks and mitigation strategies, companies like Anthropic and OpenAI have implemented in-house evaluation protocols. These protocols include automated assessments, red teaming—where humans attempt to elicit harmful capabilities—and controlled trials that compare task performance with and without AI assistance (Pannu et al., 2024). However, these evaluations often focus narrowly on the potential for AI models to aid in the development of bioweapons.

Current evaluations also tend to concentrate on basic laboratory tasks. For instance, OpenAI's tests with Los Alamos researchers assess capabilities that, while critical for beneficial research, could also be used to develop harmful agents, such as crop-destroying pathogens. Additionally, an underexplored concern is the interaction of multiple AI systems. While the US government has highlighted this issue, most companies test only individual models, overlooking the broader risks of combined system behavior (Pannu et al., 2024).

Guidelines on the use of AI tools in research publications

Organizations such as COPE, WAME, and the JAMA Network have established guidelines to address the growing use of AI tools, including ChatGPT and Large

Language Models, in publications (Authorship and AI tools, 2024):

1. AI tools cannot be listed as authors, as they cannot take responsibility for submitted work, manage conflicts of interest, or handle copyright agreements.
2. Authors using artificial intelligence must disclose their use in the Materials and Methods section, specifying how and which tool was used.
3. Authors remain fully responsible for the manuscript's content, including sections produced using AI techniques.
4. Authors must ensure compliance with publication ethics.

Publishers have varying editorial policies regarding the use of AI. Most journals, such as those published by AGU (2024), Elsevier (2024), JAMA (2024), PLOS (2024), PNAS (2024), Sage (2024), Science (2024), Springer Nature (2024), and Taylor & Francis (2024), allow the use of AI tools provided their use is disclosed. According to a study published in December 2024, 78 medical journals have issued guidance on AI use in peer review. Of these, 46 journals explicitly forbid the use of AI, while 32 permit it under the condition that confidentiality is maintained and authorship rights are respected (Li et al., 2024).

AI tools for streamlining scientific paper writing

Presented below are seven subsections highlighting various AI tools designed to assist with text-related tasks (Table 2). These tools are categorized based on their functionalities: literature review, content creation, citation management, proofreading and optimization, formatting, and originality verification. Each subsection illustrates how these tools can enhance the efficiency, accuracy, and quality of scientific work.

AI tools in literature review

AI tools like **SciSpace** (2024), **Microsoft Copilot** (2024), **PDFgear Copilot** (2024) enhance research efficiency by analyzing and summarizing scientific papers, detecting errors, and suggesting corrections, thereby significantly improving the quality and speed of research. **Trinka** (2024) refines grammar, style, and clarity for academic writing, while **ChatPDF** (2024) processes PDF documents to extract key details and create summaries, boosting research efficiency and effectiveness. Another useful tool is **Consensus** (2024) that searches over 200 million scientific articles and provides features like the Consensus Meter, which indicates the general scientific agreement on a topic, helping identify the most relevant and reliable research papers. **Scite**'s (2024) database contains 200 million scholarly sources and over 1.2 billion citations. It evaluates references by showing the context of citations, assisting in assessing the impact and credibility of research. **Research Rabbit** (2024) is citation-based literature mapping tool that facilitates citation mining and organizes collections. Starting with "seed papers," it automatically identifies additional relevant papers, visualizes networks of papers and co-authorships, and

Table 2. AI tools for streamlining scientific paper writing

Purpose	AI Tools
Literature review	SciSpace (2024), Microsoft Copilot (2024), PDFgear Copilot (2024), Trinka (2024), ChatPDF (2024), Consensus (2024), Scite (2024), Research Rabbit (2024), Semantic Scholar (2024), Elicit (2024), Clarivate (2024)
Content creation	BioloGPT (2024), GPT-4 (2024), DeepAI Text Generator (2024)
Citation management	Trinka (2024), Semantic Scholar (2024), Scite (2024), Mendeley (2024), Zotero (2024), EndNote (2024)
Proofreading & optimization	Wordvice AI (2024), Grammarly (2024), Hemingway Editor (2024), QuillBot (2024), Underleaf (2024)
Text formatting	Cite This For Me (2024), Zotero (2024), Scribbr (2024), SciSpace (2024), and Underleaf (2024)
Originality verification	iThenticate (2024), Turnitin (2024), Copyscape (2024), Crossref Similarity Check (2024), ZeroGPT (2024), Originality.AI (2024), SciSpace AI Detector (2024), Content at Scale AI Detector (2024), GPT-2 Output Detector (2024)
Retraction verification	WithdrarXiv (2024)

provides updates on new research. **Semantic Scholar** (2024) is a free AI tool for searching over 200 million academic papers. It generates summaries, highlights key and influential elements of papers, displays key citations, and analyzes articles. **Elicit** (2024) summarizes scientific articles from a database of 125 million academic papers, extracts details into organized tables, and synthesizes data for efficient research. **Clarivate** (2024) offers comprehensive solutions for literature reviews through its research database, **The Web of Science**. This subscription-based platform provides reference and citation data from academic journals and conference proceedings.

AI tools useful in content creation

BioloGPT (2024) is a biology-focused AI that ensures accuracy by rigorously citing sources, generating new hypotheses, and addressing research biases with a critical and empirical approach. **GPT-4o** (2024) is OpenAI's most advanced multimodal model, capable of efficiently generating text and excelling in non-English languages. **DeepAI Text Generator** (2024) offers multiple functions, including text generation, sentence completion, and contextually relevant content prediction, transforming input into coherent text.

AI tools useful in citation management

For citation management, the AI tools such as **Trinka** (2024), **Semantic Scholar** (2024), and **Scite** (2024) mentioned in the "AI tools in literature review" section can be particularly useful. In addition to these, there are several other citation management tools like **Mendeley** (2024). This tool offers smart citation suggestions, research discovery capabilities, and personalized paper recommendations based on reading habits. It integrates with Elsevier journals for priority access to newly published papers and includes a social platform for researchers. **Zotero** (2024) is a popular open-source tool that integrates seamlessly with web browsers. It supports annotation, citation, collection, and organization of references, allowing users to create citations and bibliographies in various styles. Zotero also provides smart recommendations while browsing the web. **EndNote** (2024) is a robust tool for managing references and citations, offering features like intelligent citation matching and advanced collaboration tools for projects.

AI tools useful in text proofreading and optimization

Wordvice AI (2024) enhances grammar, spelling, punctuation, and style. It also assists in paraphrasing to avoid plagiarism, translating text, and summarizing documents. **Grammarly** (2024) offers advanced grammar and style suggestions, making it particularly useful for improving the writing quality of scientific papers. **Hemingway Editor** (2024) improves text readability and simplifies language while maintaining clarity. **QuillBot** (2024) utilizes AI to assist users in enhancing, generating, and paraphrasing content. **Underleaf** (2024) specializes in correcting grammar, improving style, and rewording content tailored for academic writing.

AI tools useful in text formatting

For formatting purposes, tools such as **Cite This For Me** (2024), **Zotero** (2024), and **Scribbr** (2024) can be particularly useful. These tools offer citation generators in various formats. Additionally, tools like **SciSpace** (2024) and **Underleaf** (2024) provide further assistance with formatting tasks.

AI tools useful for originality verification

Ensuring originality and avoiding plagiarism is critical when writing a manuscript. It is also essential to verify that AI-generated content adheres to publication guidelines. Depending on the publisher, AI-generated text might be prohibited outright or require explicit labeling by authors during submission. Publishers often employ advanced tools like **iThenticate** (2024) to detect both plagiarism and AI-generated content. Other useful tools for detecting plagiarism and AI-generated text include: **Turnitin** (2024), **Copyscape** (2024), **Crossref Similarity Check** (2024), **ZeroGPT** (2024), **Originality.AI** (2024), **SciSpace AI Detector** (2024), **Content at Scale AI Detector** (2024), **GPT-2 Output Detector** (2024).

AI tool useful for retraction verification

WithdrarXiv (2024) is the first dataset of withdrawn papers from arXiv, released in December 2024. It includes over 14,000 papers and their retraction comments, covering the repository's history up to September 2024. This dataset is valuable for scientists, aiding in maintaining scientific integrity, improving quality control, developing automated verification systems, addressing ethical concerns, and serving as an educational resource for new researchers.

AI's risks and ethical challenges

AI holds the promise of transforming scientific and medical sectors enabling tasks that previously took years to be completed. Recent demonstrations have shown that AI-designed proteins can tackle the century-old issue of developing new treatments for snakebites, which claim around 100,000 lives each year (Callaway, 2025). However, despite these advancements, AI also poses significant risks and demands careful ethical considerations. Ensuring equitable access, fair distribution of AI technologies and their benefits, and high-quality healthcare services for all – irrespective of disability status, ethnicity, gender, geographic location, socioeconomic status, or race – is paramount. Addressing concerns about algorithmic fairness and biases, data privacy, ensuring informed consent for data usage, and maintaining safety and transparency is essential. Additionally, there are legal and social implications, as well as concerns regarding data security and confidentiality, the validity of research findings, and potential incidents of research misconduct, that must be resolved (Bouhouita-Guermech et al., 2023; Resnik and Hosseini, 2024).

To ensure AI benefits everyone fairly, several ethical challenges must be addressed: Mitigating biases in data collection and promoting diversity in research. Safeguarding privacy and confidentiality. Obtaining informed consent for the use of AI technologies. Ensuring human oversight in AI applications. Developing transparent AI systems. Clearly defining the roles and responsibilities of AI developers, health professionals, and institutions. This comprehensive strategy integrates human values into AI advancements, thereby enhancing public health and overall well-being (Health Equity and Ethical Considerations, 2024).

Beyond these ethical considerations, there are also risks, such as the potential introduction of errors by AI. Generative AI, including large language models (LLMs), are prone to hallucinations (Why scientists trust AI too much, 2025), although the exact mechanisms of the problem are not clear. These errors likely stem from a combination of factors such as data compression, ambiguities or mistakes in the AI's training data, or incorrect facts or assumptions in prompts provided by users. To track this issue, the Hallucination Vulnerability Index was created, which sorts hallucinations into six categories and three degrees of severity. Additionally, the Hallucinations Leaderboard platform that tracks, ranks, and evaluates hallucinations in LLMs was launched (Jones, 2025). It was shown that some chatbots confabulate facts in up to 30% of cases, making up information that isn't in the given document. The issue of false scientific references is particularly problematic. Study from 2024 demonstrates that various chatbots made errors in references between 30% and 90% of the time (Chelli et al., 2024). Thus, it is very important to verify generated information and perform external fact-checking.

Furthermore, other limitations of AI technology must also be considered. One notable example is AlphaFold 3, which has enhanced the precision of predicting biomolecular structures, yet still faces challenges with stereochemistry and requires human assistance (Steinkellner et al., 2025).

AI models for antimicrobial resistance diagnosis, discovery, and treatment often rely on imbalanced datasets, leading to potential reliability issues and representing only certain patient populations or specific biological tests. AI models developed using these biased datasets may struggle to generalize effectively beyond their specific training data (Cesaro et al., 2025).

It is crucial to critically evaluate AI's role in research to prevent overdependence on its abilities. By addressing both ethical concerns and potential risks, while acknowledging the limitations of AI, the scientific community can leverage AI's potential while maintaining research integrity.

Conclusions

AI is transforming biomedical research by improving data analysis, modeling biological processes, and detecting diseases, leading to faster scientific discoveries. Scientists emphasize the usefulness of AI in summarizing scientific data from other researchers, accelerating administrative tasks, speeding up the process of writing scientific papers, generating new hypotheses, and conducting faster peer reviews. Despite its promising future, AI presents ethical challenges, including issues related to data quality, result interpretation, and responsible use. Addressing these challenges requires interdisciplinary collaboration and robust regulation. Ensuring the ethical use of AI – such as preventing plagiarism and maintaining content quality – is essential for safeguarding the integrity and progress of scientific research.

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Ethanol extract from *Argyreia acuta* Lour. leaves exhibit analgesic, antipyretic, and anti-inflammatory effects in mouse models

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Abstract

Background: *Argyreia acuta* has traditionally been used for its analgesic, antipyretic, and anti-inflammatory properties; however, scientific validation of these effects remains limited. This study aimed to evaluate the pharmacological potential of ethanol extract from *A. acuta* leaves (AAEE) in murine models of pain, fever, and inflammation.

Materials and methods: The pharmacological properties of *A. acuta* leaf extract were assessed. Analgesic activity was evaluated using a hot plate and tail-flick assays, while antipyretic effects were tested via a yeast-induced pyrexia model. The anti-inflammatory potential was investigated through carrageenan-induced paw edema and by quantifying pro-inflammatory mediators, including TNF-α, IL-1β, IL-6, COX-2, and PGE_2. Histopathological analysis of paw tissues was performed to confirm inflammatory changes.

Results: AAEE exhibited significant, dose-dependent analgesic effects, as indicated by prolonged latency times and increased pain inhibition ($p < 0.05$), with the 200 mg/kg dose showing the greatest efficacy. In the antipyretic model, AAEE at 200 mg/kg reduced rectal temperature to 36.93°C, corresponding to an inhibition rate of 82.61% ($p < 0.05$). The extract significantly reduced paw edema (41.39% inhibition at 200 mg/kg) and markedly lowered levels of TNF-α, IL-1β, IL-6, COX-2, and PGE_2 ($p < 0.05$). The histological analysis supported these findings, revealing decreased edema and inflammatory cell infiltration in treated groups.

Conclusions: These findings provide scientific support for the traditional use of *A. acuta*, demonstrating its significant analgesic, antipyretic, and anti-inflammatory activities. AAEE may represent a promising natural therapeutic agent for treating pain, fever, and inflammation.

Key words: analgesic effects, antipyretic effects, anti-inflammatory effects, ethanol extract, traditional medicinal plants

Introduction

Argyreia acuta Lour., a member of the Convolvulaceae family, is widely distributed in tropical and subtropical regions, including Southeast Asia, India, and China (Zhu et al. 2001). Traditionally, this plant has been used in folk medicine for its purported analgesic, antipyretic, and anti-inflammatory properties (Li et al. 2021). Pain, fever, and inflammation are fundamental physiological responses to tissue injury or infection and are closely associated with various acute and chronic pathological conditions. These symptoms not only diminish the quality of life but also place a significant burden on healthcare systems (Qi et al. 2024). Chronic pain, in particular, can impair mobility and productivity, while unresolved inflammation and prolonged fever are linked to serious conditions such as arthritis, cardiovascular diseases, and immune dysfunction (Cohen et al. 2022).

Several species within the Convolvulaceae family, including *Ipomoea pes-caprae* and *Ipomoea carnea*, have been scientifically validated for their anti-inflammatory,

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analgesic, and antioxidant activities (Galani et al. 2010; Zankar 2024). Despite its traditional use, *A. acuta* remains understudied, with limited empirical evidence supporting its pharmacological efficacy. Phytochemical analyses suggest that this species is rich in bioactive constituents such as flavonoids (kaempferol, rutin, apigenin), phenolic acids (gallic acid, caffeic acid), alkaloids (berberine), terpenoids (limonene), and saponins – compounds known to modulate inflammatory pathways, inhibit prostaglandin synthesis, and attenuate nociceptive responses (Li et al. 2021; Huang et al. 2022; Wijesekara et al. 2024). These biochemical properties indicate that *A. acuta* may possess significant therapeutic potential for managing inflammation-related disorders.

Nevertheless, the lack of detailed pharmacological studies, particularly those focusing on the ethanol extract of *A. acuta* leaves, limits the current scientific understanding of its medicinal potential. With growing interest in plant-based therapeutics due to their perceived safety and efficacy, comprehensive investigations into *A. acuta* are warranted. Such studies could provide foundational data for developing novel, plant-derived therapeutic agents.

The present study aims to evaluate the analgesic, antipyretic, and anti-inflammatory effects of ethanol extract from *A. acuta* leaves using well-established murine models. By elucidating the extract's biological activities, this research seeks to validate the plant's traditional uses and explore its potential as a natural alternative for treating pain, fever, and inflammation.

Materials and methods

Acquisition of plant material and extraction procedures

A. acuta leaves were collected in April 2024 from the Son Tra area, Quang Ngai Province, Vietnam. The harvested plant material was initially inspected to remove damaged or unsuitable samples, followed by thorough washing with distilled water to eliminate surface contaminants. The cleaned leaves were then shade-dried for two consecutive days to avoid direct sunlight exposure, thereby preserving thermolabile and photosensitive phytochemicals. After drying, the leaves were ground into a fine powder using an MRC Laboratory Grinder (MRC Ltd., Israel) and stored in dry, well-ventilated conditions until further processing.

For extraction, 200 g of the powdered leaf material was macerated in 2,000 ml of absolute ethanol. The mixture was subjected to ultrasound-assisted extraction at a frequency range of 40–60 kHz for 30–60 min, with intermittent stirring to enhance the efficiency of bioactive compound release. Following extraction, the mixture was filtered through muslin cloth to remove solid residues, yielding a clear filtrate. The crude extract was then concentrated under reduced pressure at 50°C using a rotary evaporator (Heidolph Instruments GmbH & Co. KG, Germany) to remove residual ethanol. The final ethanol extract of *A. acuta* leaves (AAEE) was obtained with a yield of 28% and stored in amber-colored, airtight containers at 4°C to ensure chemical stability and prevent degradation before further experimental use.

Phytochemical profiling of Argyreia acuta extract

The preliminary phytochemical composition of the ethanol extract of AAEE was assessed through standard qualitative assays based on colorimetric and precipitation reactions with specific reagents. The presence of major phytochemical classes – including tannins, flavonoids, terpenoids, polyphenols, saponins, steroids, alkaloids, and cardiac glycosides – was determined via characteristic color changes or precipitate formation upon interaction with corresponding chemical agents, as described by Tran et al. (2023a).

Quantitative analysis of selected phytochemicals (flavonoids, alkaloids, and tannins) in AAEE was performed using spectrophotometric methods. Flavonoid content was measured via the aluminum chloride colorimetric assay, in which the extract reacts with $AlCl_3$ to form a yellow-to-orange complex, and absorbance was recorded at 415 nm. Alkaloid content was determined by reaction with Mayer's reagent, producing a white precipitate; the resulting solution was measured spectrophotometrically at 280 nm. Tannin content was quantified by reacting the extract with $FeCl_3$, forming a blue-black complex, with absorbance measured at 765 nm (Nhung and Quoc, 2024a). These methods ensured reliable quantification of key bioactive constituents in the extract.

Animal experiments

Healthy male Swiss albino mice (30 ± 2 g) were procured from the Pasteur Institute, Ho Chi Minh City, Vietnam. Upon arrival, the animals underwent a 7-day acclimatization period under standardized laboratory conditions. During this time, mice were housed in

polypropylene cages lined with rice husks, which were regularly treated with a biological deodorizing agent to control odor. Environmental parameters were maintained at a temperature of 24 ± 2°C, relative humidity of 55 ± 5%, and a 12-h light/dark cycle. Mice were provided with a commercial rodent pellet diet and filtered drinking water ad libitum.

All animal handling and experimental protocols strictly adhered to the ethical guidelines set forth by the International Council for Laboratory Animal Science (ICLAS 2012) and complied with the ARRIVE 2.0 guidelines for reporting animal research (Percie du Sert et al. 2020).

Experimental design

Sample sizes were estimated based on prior literature and refined using power analysis to ensure statistical robustness while adhering to the principles of the 3Rs (Replacement, Reduction, and Refinement). Animals were randomly assigned to experimental groups using a computerized randomization tool to minimize allocation bias. Blinding was implemented throughout data collection and analysis to reduce the risk of subjective bias (Rajput et al. 2023).

Analgesic activity

Hot-plate assay

The hot plate assay was utilized to assess central analgesic effects by exposing the animal to a consistently heated surface and recording their latency to respond. Behavioral indicators such as paw licking, lifting, or jumping were observed when mice were placed on a hot plate (VELP®, Italy) maintained at 50 ± 2°C.

A total of 25 mice were fasted for 12 h with free access to water and randomly divided into five groups, each comprising five individuals. The control group received normal saline (10 ml/kg), while the standard group (TRM) was administered tramadol at 5 mg/kg. The remaining groups were treated with AAEE at doses of 100, 150, and 200 mg/kg (AAEE100, AAEE150, AAEE200). Reaction times were recorded at 0, 15, 30, 45, and 60 min before and after treatment.

To prevent potential tissue damage, the maximum response time was capped at 45 s. Throughout the assay, animals were closely monitored for any signs of discomfort or distress and were immediately removed from the apparatus upon responding. All procedures were conducted under strict ethical oversight (Nhung and Quoc 2024b).

The maximum analgesic effect in the hot plate test (AHP) was calculated using the following formula:

$$\text{AHP [\%]} = \frac{\text{Test reaction time} - \text{Control reaction time}}{45 - \text{Control reaction time}} \times 100$$

Tail-flick assay

The tail-flick assay is a widely recognized method for evaluating analgesic effectiveness, wherein pain is induced through thermal stimulation by immersing the distal segment of the animal's tail in heated water. In this study, 25 mice were used, each subjected to a 12-h fasting period with unrestricted access to water. The animals were randomly assigned to five groups, with each group comprising five mice.

The control group received normal saline (10 ml/kg), while the standard treatment group (TRM) was administered tramadol at a dose of 5 mg/kg. The remaining three groups were treated orally with AAEE at doses of 100, 150, and 200 mg/kg (AAEE100, AAEE150, AAEE200). During the experiment, each mouse was gently restrained in a device with its tail extended outward. The distal 1–3 cm segment of the tail was then submerged in hot water maintained at 50–55°C.

Reaction time, defined as the latency (in seconds) from tail immersion to withdrawal, was recorded before and after treatment at intervals of 0, 30, and 60 min. To minimize the risk of tissue damage, a maximum response latency of 15 s was enforced. Mice were carefully observed throughout the procedure, and any animal that responded was immediately removed from the testing setup. All experimental protocols were conducted in full compliance with strict ethical guidelines (Nhung and Quoc 2024c).

The percentage of pain inhibition in the tail-flick test (PPT) was calculated using the following formula:

$$\text{PPT [\%]} = \frac{\text{Test latency} - \text{Control latency}}{15 - \text{Control latency}} \times 100$$

Antipyretic activity

The antipyretic efficacy of AAEE was evaluated using a yeast-induced fever model with a 20% yeast suspension, following a standardized protocol with minor modifications (Nhung and Quoc 2024d). In this study, thirty mice were randomly assigned to six groups, each consisting of five animals.

The control group received sterile saline (10 ml/kg) without fever induction. The yeast group was administered

a yeast solution (10 ml/kg) to induce hyperthermia but did not receive any subsequent treatment. The yeast plus Paracetamol group (Yeast+PCM) included mice treated with the yeast solution (10 ml/kg) followed by oral administration of Paracetamol at a dose of 150 mg/kg. The remaining three groups – Yeast+AAEE100, Yeast+AAEE150, and Yeast+AAEE200 – received oral doses of AAEE at 100, 150, and 200 mg/kg, respectively, following yeast-induced fever (10 ml/kg).

Throughout the experiment, mice were observed for signs of discomfort, including lethargy, piloerection, and abnormal posturing. Any animal exhibiting severe symptoms was immediately euthanized by the humane endpoint criteria outlined in the ethical protocol.

Evaluation of antipyretic activity via rectal temperature measurement

Before the experiment, the test animals were subjected to overnight fasting with unrestricted access to water. Fever was induced by subcutaneous administration of a 20% yeast solution at a dosage of 10 ml/kg body weight. Baseline rectal temperatures were recorded using a digital thermometer (Microlife, Microlife Corporation, Switzerland).

Eighteen hours after yeast injection, animals exhibiting an increase in rectal temperature ranging from 0.3 to 0.5°C were selected for antipyretic evaluation. The rectal temperatures of these animals were subsequently measured at 1, 2, and 3 h following treatment (Nhung and Quoc 2024d).

The percentage of fever reduction (PFR) was calculated using the following formula:

$$PFR\ [\%] = \frac{T_{inital} - T_{post-treatment}}{T_{inital} - T_{baseline}} \times 100$$

Assessment of cyclooxygenase-2 and prostaglandin E_2 levels

Blood samples were collected via the submandibular vein under light isoflurane anesthesia to minimize distress, by refinement principles. The collected blood was centrifuged at 12,000 rpm for 5 min to separate the serum. The concentrations of cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) were quantified using ELISA kits provided by Absolute Biotech Co., Ltd.

Serum samples and standard solutions were added to ELISA plates precoated with specific antibodies against COX-2 and PGE_2. Horseradish peroxidase (HRP) conjugate was then added, and the plates were incubated at 37°C for 1 h. After incubation, the wells were washed three times with wash buffer to remove unbound substances.

Each assay was performed independently and in triplicate to ensure reproducibility. Reagents A and B were subsequently added and incubated at 37°C for an additional 15–30 min. The enzymatic reaction was terminated by the addition of a stop solution. Absorbance was measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader. The concentrations of COX-2 and PGE_2 in serum samples were determined by comparing the optical density values to those from standard calibration curves (Nhung and Quoc 2023a).

Anti-inflammatory activity

The carrageenan-induced paw edema model was employed to assess the anti-inflammatory effects of AAEE. Thirty Swiss mice were randomly divided into six groups, each comprising five animals (n = 5). The control group received an intraperitoneal injection of sterile saline (10 ml/kg) without inflammation induction. The carrageenan group (CGN) was administered a subcutaneous injection of 50 µl of 1% carrageenan solution to induce inflammation, without any subsequent treatment. The carrageenan plus indomethacin group (CGN+IND) received the carrageenan injection (50 µl of 1% solution) followed by an oral dose of indomethacin at 10 mg/kg. The three experimental groups (CGN+AAEE100, CGN+AAEE150, and CGN+AAEE200) received the same carrageenan injection, followed by oral administration of AAEE at doses of 100, 150, and 200 mg/kg, respectively. Throughout the study, mice were carefully monitored for indicators of discomfort, including reduced activity, fur bristling, and abnormal body posture. Animals exhibiting significant distress were promptly euthanized by the humane endpoint guidelines specified in the approved ethical protocol.

Evaluation of anti-inflammatory activity through paw circumference

The circumference of the right hind paw was measured using digital calipers (Fowler, Fowler High Precision, Inc., USA) immediately before the induction of inflammation. This initial measurement served as a baseline for evaluating edema severity and the anti-inflammatory efficacy of the test substances.

Paw edema was quantified by recording paw circumference at specific time points: 0, 1, 2, 3, 4, and 5 h after carrageenan administration. Measurements were taken with high-precision digital calipers to accurately detect changes in paw size due to inflammation. The data obtained provided a comprehensive view of the progression of the inflammatory response and the impact of each treatment (Nhung and Quoc 2024d).

The percentage inhibition of paw edema (PPE), used as an indicator of anti-inflammatory efficacy, was calculated using the following standard formula:

$$\text{PPE [\%]} = \frac{\text{Circumference}_{\text{control}} - \text{Circumference}_{\text{treated}}}{\text{Circum ference}_{\text{treated}} - \text{Circum ference}_{\text{baseline}}} \times 100$$

Assessment of cytokine concentrations

The concentrations of cytokines IL-6, TNF-α, and IL-1β were quantified using enzyme-linked immunosorbent assay (ELISA) combined with immunoassay techniques. In this procedure, specific antibodies targeting IL-6, TNF-α, and IL-1β were immobilized onto the wells of a 96-well plate. The plates were incubated overnight with the samples to allow the antigens to bind to their respective antibodies.

On the following day, biotinylated secondary antibodies were added to each well after incubation with either tissue samples or antigen standards. Subsequently, streptavidin-conjugated enzymes were introduced to facilitate a colorimetric reaction that changed the substrate color from purple to yellow. Absorbance was measured at a wavelength of 450 nm using an ELISA reader to determine the levels of IL-6, TNF-α, and IL-1β. Cytokine concentrations were calculated and expressed as ng/mg (Nhung and Quoc 2023b).

Histopathological analysis

At the end of the experiment, mice were euthanized by gradual exposure to CO_2 in a dedicated chamber until complete loss of consciousness, followed by cervical dislocation to ensure death. The inflamed paw tissues were collected, fixed in 10% formalin, and processed through standard histological procedures, including dehydration, clearing, paraffin embedding, and sectioning (4–5 μm).

The tissue sections were stained with Hematoxylin and Eosin (H&E) and examined microscopically to evaluate neutrophil infiltration, edema, vascular dilation, and tissue damage.

Statistical analysis

Analysis of variance (ANOVA) was employed to assess variations in experimental parameters. Results are expressed as mean ± SD, with statistical significance set at $p < 0.05$. All statistical analyses were performed using Statgraphics Centurion XX.

Results and discussion

Phytochemical analysis and anti-inflammatory potential of Argyreia acuta ethanol extract

Phytochemical analysis of the AAEE confirmed the presence of tannins, flavonoids, terpenoids, polyphenols, saponins, steroids, and alkaloids, while cardiac glycosides were absent. The quantitative assessment revealed polyphenols as the predominant constituents (70.46 ± 1.42 mg GAE/g), followed by flavonoids (41.75 ± 1.16 mg QE/g) and tannins (7.78 ± 0.24 mg TE/g) (Table 1).

The diversity of phytochemicals present in AAEE suggests significant anti-inflammatory potential. The high concentrations of polyphenols and flavonoids are particularly noteworthy, as these compounds are recognized for their antioxidant and anti-inflammatory activities. They inhibit key pro-inflammatory mediators such as nuclear factor kappa B (NF-κB), cyclooxygenase (COX), and lipoxygenase (LOX), thereby reducing the production of cytokines like TNF-α, IL-1β, and IL-6. This suppression mitigates inflammation by decreasing oxidative stress and preventing immune cell activation (Intharuksa et al. 2024).

Tannins contribute by stabilizing cell membranes, reducing capillary permeability, and inhibiting histamine release, thus limiting inflammatory cell infiltration (Molnar et al. 2024). Terpenoids inhibit the synthesis of nitric oxide (NO) and prostaglandins (PGs) by downregulating inducible nitric oxide synthase (iNOS) and COX-2. Saponins enhance macrophage function and reduce inflammatory cell migration (Nhung and Quoc 2025). Steroids found in AAEE suppress inflammatory gene expression and help minimize tissue damage (Ferreira et al. 2024). Additionally, alkaloids exert anti-inflammatory effects by modulating mitogen-activated protein kinases (MAPKs) and NF-κB signaling pathways (Tran and Tran 2021).

Previous studies have identified various phytochemicals in species of the *Argyreia* genus and assessed their anti-inflammatory potential. For instance, research on

Table 1. Phytochemical screening and quantification of ethanol extract from *Argyreia acuta* leaves

Phytoconstituents	Test	Observation	Present in AAEE	Quantification of phytochemicals
Tannins	2 ml AAEE + 2 ml H_2O + 2–3 drops $FeCl_3$ (5%)	Green precipitate	+	7.78 ± 0.24 mg TE/g
Flavonoids	1 ml AAEE + 1 ml $Pb(OAc)_4$ (10%)	Yellow coloration	+	41.75 ± 1.16 mg QE/g
Terpenoids	2 ml AAEE + 2 ml $(CH_3CO)_2O$ + 2–3 drops conc. H_2SO_4	Deep red coloration	+	NT
Polyphenol	2 ml AAEE + 2 ml $FeCl_3$	Bluish-green appearance	+	70.46 ± 1.42 mg GAE/g
Saponins	5 ml AAEE + 5 ml H_2O + heat	Froth appears	+	NT
Steroids	2 ml AAEE + 2 ml $CHCl_3$ + 2 ml H_2SO_4 (conc.)	The reddish-brown ring at the junction	+	NT
Cardiac glycosides	2 ml AAEE + 2 ml $CHCl_3$ + 2 ml CH_3COOH	Violet to blue to green coloration	–	–
Alkaloids	2 ml AAEE + a few drops of Hager's reagent	Yellow precipitate	+	NT

Phytochemicals in AAEE are (+) present, (–) absent, and (NT) not tested

the botanical characteristics of *A. acuta* has provided a foundation for further investigation into its chemical composition and biological activities (Li et al. 2021). Additionally, studies on other medicinal *Argyreia* species have offered valuable insights into their phytochemical profiles (Zankar 2024; Galani et al. 2010). These findings demonstrate that *Argyreia* species contain a diverse array of bioactive compounds, supporting their potential application in anti-inflammatory therapies.

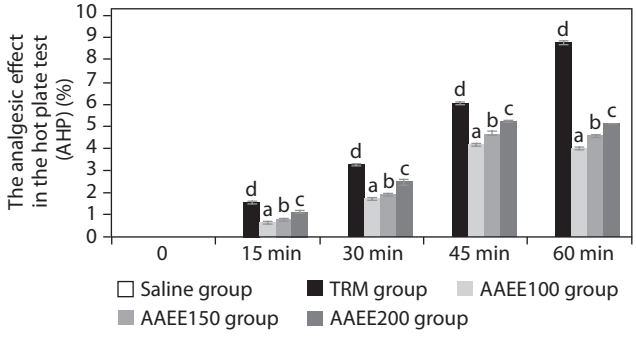

Figure 1. Time-dependent analgesic effects of ethanol extract from *Argyreia acuta* leaves in hot plate test. Results are expressed as mean ± SD, with letters (a, b, c, and d) indicating statistically significant group differences ($p < 0.05$)

Analgesic potential of AAEE

Hot-plate assay

Table 2 and Figure 1 illustrate the analgesic efficacy of AAEE in the hot-plate assay. The extract exhibited dose-dependent analgesic activity, with significantly enhanced paw-licking latency and AHP% at higher doses compared to the saline-treated control group ($p < 0.05$). The saline group showed no significant changes in paw-licking latency or pain inhibition percentage (AHP%) at any time point, confirming the absence of an analgesic effect.

In contrast, the standard treatment group (TRM) displayed a substantial and sustained increase in paw-

Table 2. Effects of ethanol extract from *Argyreia acuta* leaves on reaction time in hot plate test

Time	Saline group	TRM group	AAEE100 group	AAEE150 group	AAEE200 group
0 min	4.52 ± 0.06^a	4.62 ± 0.05^b	4.55 ± 0.07^a	4.53 ± 0.04^a	4.54 ± 0.02^a
15 min	4.45 ± 0.04^a	5.08 ± 0.02^e	4.71 ± 0.06^b	4.77 ± 0.02^c	4.91 ± 0.06^d
30 min	4.21 ± 0.03^a	5.54 ± 0.02^e	4.91 ± 0.03^b	4.99 ± 0.02^c	5.22 ± 0.06^d
45 min	3.99 ± 0.03^a	6.47 ± 0.02^e	5.68 ± 0.05^b	5.91 ± 0.06^c	6.13 ± 0.04^d
60 min	3.81 ± 0.03^a	7.42 ± 0.04^e	5.45 ± 0.04^b	5.68 ± 0.04^c	5.91 ± 0.03^d

Values are expressed as mean ± SD and letters (a, b, c, d, and e) represent the difference between groups ($p < 0.05$)

Table 3. Time-dependent changes in reaction time during tail-flick assay across different treatment groups

Time	Saline group	TRM group	AAEE100 group	AAEE150 group	AAEE200 group
0 min	7.50 ± 0.04[a]	7.51 ± 0.05[a]	7.40 ± 0.03[a]	7.44 ± 0.05[a]	7.50 ± 0.03[a]
30 min	7.00 ± 0.04[a]	12.38 ± 0.03[e]	10.73 ±0.03[b]	11.18 ± 0.03[c]	11.63 ± 0.03[d]
60 min	6.60 ± 0.02[a]	13.52 ± 0.04[e]	9.99 ± 0.02[b]	10.43 ± 0.02[c]	10.88 ± 0.02[d]

Values are expressed as mean ± SD and letters (a, b, c, d, and e) represent the difference between groups ($p < 0.05$)

licking latency, reaching 7.42 ± 0.04 s and an AHP% of 8.77% at 60 min ($p < 0.05$), indicating robust central analgesic activity. Among AAEE-treated groups, both latency and AHP% increased in a dose-dependent manner.

The 100 mg/kg dose produced moderate effects, with latency peaking at 45 minutes (5.68 ± 0.05 s) and AHP% reaching 4.13%, followed by a slight decline at 60 min. The 150 and 200 mg/kg doses induced more pronounced and sustained analgesic effects, with latencies of 5.91 ± 0.06 s and 6.13 ± 0.04 s and corresponding AHP% values of 4.68% and 5.21% at 45 min, respectively. These higher doses continued to show significant analgesic activity at 60 min compared to the saline group ($p < 0.05$).

Tail-flick assay

Table 3 and Figure 2 depict the analgesic effects of AAEE in the tail-flick test, revealing dose-dependent efficacy with significant improvements over the saline control ($p < 0.05$). The saline group showed no significant changes in tail-flick latency or pain percentage threshold (PPT%), confirming the absence of analgesic activity. In contrast, the TRM group demonstrated strong and sustained analgesic effects, reaching a tail-flick latency of 13.52 ± 0.04 s and a PPT% of 82.43% at 60 min ($p < 0.05$).

AAEE-treated groups exhibited dose-dependent increases in tail-flick latency and PPT%. The 100 mg/kg dose peaked at 30 min (10.73 ± 0.03 s, PPT%: 46.60%) but declined by 60 min. The 150 mg dose reached 11.18 ± 0.03 s and 52.25% PPT at 30 min, followed by a slight reduction. The 200 mg/kg dose displayed the highest efficacy, with a latency of 11.63 ± 0.03 s and a PPT% of 57.83% at 30 min, maintaining elevated values at 60 min compared to lower doses ($p < 0.05$). These findings support the analgesic potential of AAEE, particularly at 150 and 200 mg doses.

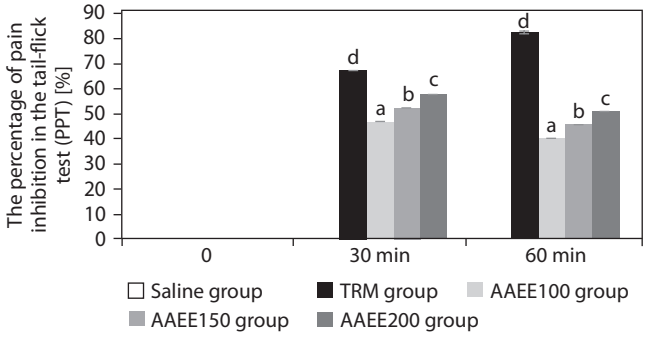

Figure 2. Percentage of pain inhibition in tail-flick assay over time. Results are expressed as mean ± SD, with letters (a, b, c, and d) indicating statistically significant group differences ($p < 0.05$)

The analgesic effects of AAEE are likely mediated through the inhibition of inflammatory enzymes COX-1 and COX-2, thereby reducing prostaglandin synthesis – an essential mediator of pain (Iolascon et al. 2021). In addition, AAEE contains flavonoids and polyphenols, which act as antioxidants, mitigating oxidative stress in the nervous system by scavenging free radicals and reducing pain perception (Nhung and Quoc 2024a). Interaction with central opioid receptors may further inhibit pain signal transmission, similar to the mechanism of centrally acting analgesics (Nhung and Quoc 2024b).

The combination of anti-inflammatory and central analgesic mechanisms enhances AAEE's pain-relieving potential. Notably, the sustained effects observed at 150 mg suggest that the prolonged release or metabolism of active constituents contributes to its efficacy. These results are consistent with previous studies on *A. speciosa* and *A. argentea*, which also demonstrated significant analgesic activity in hot-plate tests via inhibition of inflammatory mediators, antioxidant activity, and opioid receptor interactions (Lalan et al. 2015; Dina et al. 2010). This consistency underscores the therapeutic promise of the *Argyreia* genus as a source of natural analgesics and validates AAEE's potential as an effective analgesic agent.

Antipyretic properties of AAEE

Evaluation of antipyretic activity via rectal temperature measurement

Table 4 and Figure 3 demonstrate that AAEE exhibits significant antipyretic effects in a yeast-induced fever model in mice, with efficacy increasing in a dose-dependent manner ($p < 0.05$). The group administered yeast to induce pyrexia showed a marked rise in rectal temperature, peaking at 39.45°C after 3 h ($p < 0.05$). The reference drug reduced rectal temperature to 36.91°C, corresponding to a fever inhibition rate of 91.06% after 3 h.

AAEE-treated groups exhibited significant reductions in rectal temperature compared to the untreated yeast-induced group. At 100 mg/kg, AAEE lowered the temperature from 39.21 to 37.51°C after 3 h, with inhibition rates of 19.14, 38.28, and 61.48% at 1, 2, and 3 h, respectively. The 150 mg/kg dose reduced the temperature to 37.28°C, with corresponding inhibition rates of 24.04, 43.86, and 72.85%. The 200 mg/kg dose produced the most pronounced antipyretic effect, reducing the temperature to 36.93°C, with inhibition rates of 32.08, 55.30, and 82.61% at the respective time points–closely approximating PCM's efficacy.

Assessment of cyclooxygenase-2 and prostaglandin E_2 levels

Figure 4 illustrates that AAEE significantly reduced COX-2 and PGE_2 levels in yeast-induced fever, supporting its anti-inflammatory and antipyretic properties. The yeast group showed elevated COX-2 and PGE_2 levels (13.55 and 1.75 ng/ml) compared to the control group (7.53 and 0.97 ng/ml). PCM treatment lowered these levels to 8.29 and 1.07 ng/ml, respectively. AAEE reduced COX-2 and PGE_2 in a dose-dependent manner: 100 mg/kg decreased levels to 10.54 and 1.27 ng/ml; 150 mg/kg to 9.79 and 1.27 ng/ml; and 200 mg/kg to 8.67 and 1.12 ng/ml, approaching PCM's effects.

AAEE's antipyretic activity is attributed to the modulation of inflammatory pathways, as evidenced by lowered rectal temperatures and reduced COX-2 and PGE_2 concentrations. By inhibiting the COX-2 pathway, AAEE

Table 4. Changes in rectal temperature over time to assess antipyretic effects of AAEE in yeast-induced fever model

Experimental group	Initial [°C]	Fever [°C]	1 h [°C]	2 h [°C]	3 h [°C]
Control group	36.78 ± 0.03[a]	36.84 ± 0.05[a]	36.82 ± 0.04[a]	36.79 ± 0.03[a]	36.81 ± 0.02[a]
Yeast group	36.51 ± 0.05[a]	39.11 ± 0.04[d]	39.31 ± 0.04[f]	39.41 ± 0.04[f]	39.45 ± 0.04[f]
Yeast+PCM group	36.71 ± 0.01[c]	38.98 ± 0.05[a]	37.89 ± 0.01[a]	37.21 ± 0.04[a]	36.91 ± 0.04[a]
Yeast+AAEE100 group	36.61 ± 0.04[b]	39.21±0.06[c]	38.71±0.05[d]	38.21±0.03[d]	37.51 ± 0.03[d]
Yeast+AAEE150 group	36.61 ± 0.04[b]	39.11 ± 0.05[d]	38.51 ± 0.04[e]	38.01 ± 0.06[e]	37.28 ± 0.05[e]
Yeast+AAEE200 group	36.49 ± 0.04[a]	39.01 ± 0.04[b]	38.19 ± 0.02[c]	37.61 ± 0.05[c]	36.93 ± 0.04[c]

Values are expressed as mean ± SD and letters (a, b, c, d, e, and f) represent the difference between groups ($p < 0.05$)

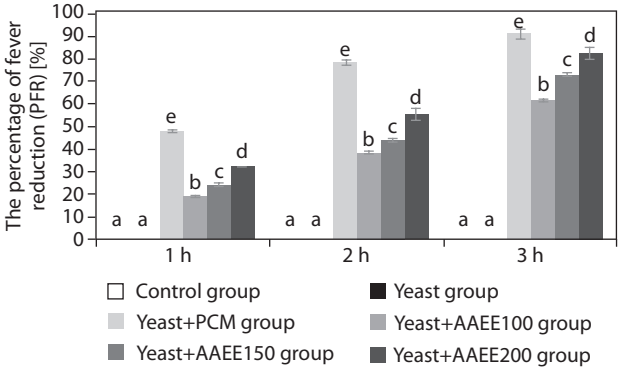

Figure 3. Percentage of fever reduction over time in the antipyretic assay. Results are expressed as mean ± SD, with letters (a, b, c, d, and e) indicating statistically significant group differences ($p < 0.05$)

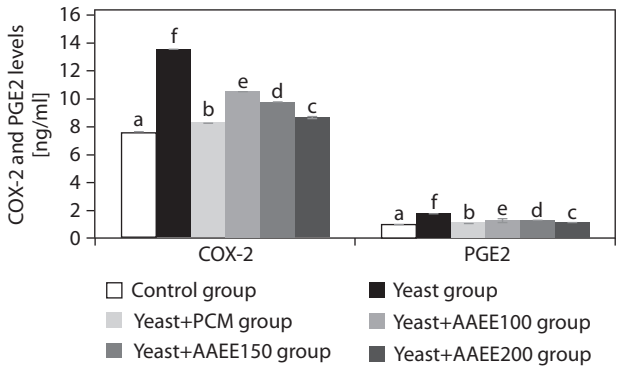

Figure 4. Effects of ethanol extract from *Argyreia acuta* leaves and PCM on COX-2 and PGE_2 levels in antipyretic assay. Results are expressed as mean ± SD, with letters (a, b, c, d, e, and f) indicating statistically significant group differences ($p < 0.05$)

Table 5. Effects of ethanol extract from *Argyreia acuta* on paw edema thickness in carrageenan-induced inflammation in mice

Experimental group	"0" [mm]	1 h [mm]	2 h [mm]	3 h [mm]	4 h [mm]
Control group	22.63 ± 0.19^{a}	22.69 ± 0.19^{a}	22.68 ± 0.13^{a}	22.71 ± 0.24^{a}	22.66 ± 0.19^{a}
CGN group	22.68 ± 0.21^{a}	31.55 ± 0.28^{f}	34.73 ± 0.29^{e}	37.68 ± 0.31^{e}	39.73 ± 0.32^{d}
CGN+IND group	22.65 ± 0.20^{a}	29.44 ± 0.26^{d}	27.04 ± 0.23^{d}	24.75 ± 0.22^{d}	23.19 ± 0.26^{a}
CGN+AAEE100 group	22.65 ± 0.21^{a}	30.87 ± 0.27^{e}	28.08 ± 0.24^{c}	25.40 ± 0.22^{d}	24.77 ± 0.14^{c}
CGN+AAEE150 group	22.63 ± 0.20^{a}	30.34 ± 0.26^{b}	28.08 ± 0.04^{c}	25.32 ± 0.07^{c}	23.92 ± 0.11^{b}
CGN+AAEE200 group	22.64 ± 0.21^{a}	30.11 ± 0.27^{c}	27.19 ± 0.15^{b}	24.83 ± 0.12^{b}	21.53 ± 0.21^{a}

Values are expressed as mean ± SD and letters (a, b, c, d, e, and f) represent the difference between groups ($p < 0.05$)

decreases PGE_2 synthesis, thereby stabilizing hypothalamic temperature regulation (Kulesza et al. 2023). Phytochemicals in AAEE – such as flavonoids, alkaloids, and phenolic compounds – likely act as COX-2 inhibitors or antioxidants, reducing oxidative stress and systemic inflammation (Nguyen et al. 2021). This dual mechanism aligns AAEE's efficacy with that of PCM, positioning it as a promising natural alternative for fever management.

These findings are consistent with previous studies on *A. speciosa* and other medicinal plants such as *Caryota urens* and *Plukenetia volubilis*, which demonstrated antipyretic effects through similar COX–PGE_2 pathway modulation (Nhung and Quoc 2024a, 2024c; Lalan et al. 2015). The consistency across species reinforces *A. acuta*'s therapeutic potential as a natural antipyretic agent and supports its traditional use in ethnomedicine for fever treatment. Further phytochemical and mechanistic studies are warranted to fully elucidate AAEE's medicinal properties.

Anti-inflammatory properties of AAEE

Anti-inflammatory effects in carrageenan-induced paw edema model

Table 5 and Figure 5 illustrate the effects of AAEE on carrageenan-induced paw inflammation in mice, as assessed by changes in paw circumference and percentage paw edema inhibition (PPE%). The control group maintained a stable paw circumference (22.63–22.71 mm) and exhibited no inflammation inhibition (PPE% = 0). In contrast, the carrageenan group (CGN) displayed a significant increase in paw circumference, peaking at 39.73 mm at 4 h, thereby confirming the successful induction of inflammation. Treatment with indomethacin (CGN+IND) resulted in a marked reduction in paw swelling, from 29.44 at 1 h to 23.19 mm at 4 h,

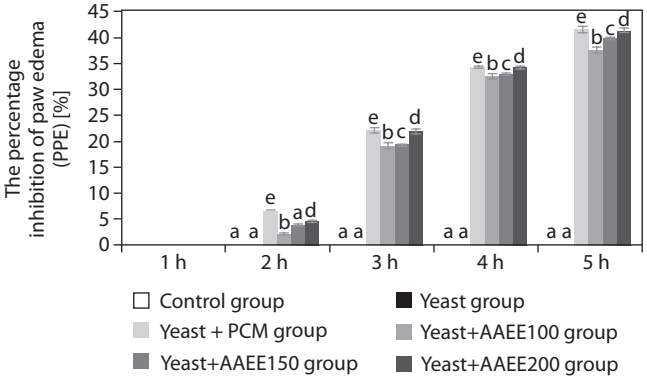

Figure 5. Percentage inhibition of paw edema in carrageenan-induced inflammation in mice following treatment with ethanol extract of *Argyreia acuta*. Results are expressed as mean ± SD, with letters (a, b, c, d, and e) indicating statistically significant group differences ($p < 0.05$)

and a corresponding increase in PPE% from 6.68 to 41.62%, demonstrating strong anti-inflammatory activity.

AAEE exhibited a clear dose-dependent inhibition of inflammation. At 100 mg/kg, paw circumference decreased from 30.87 to 24.77 mm, with a PPE% of 37.65%. A dose of 150 mg/kg further improved the anti-inflammatory response, resulting in a PPE% of 39.93%. The highest dose of 200 mg/kg reduced paw circumference to 23.92 mm and achieved a PPE% of 41.39%, a value closely matching that of the indomethacin-treated group.

Cytokine level modulation by AAEE

Table 6 presents the concentrations of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 following carrageenan-induced inflammation. The control group exhibited the lowest levels of these cytokines, with TNF-α at 142.16 pg/ml, IL-1β at 276.24 pg/ml, and IL-6 at 27.11 pg/ml, consistent with the absence of inflammation. In contrast, the carrageenan group showed

Table 6. Effects of ethanol extract from *Argyreia acuta* on pro-inflammatory cytokine levels in carrageenan-induced inflam-mation in mice

Experimental group	TNF-α [pg/ml]	IL-1β [pg/ml]	IL-6 [pg/ml]
Control group	142.16 ± 1.26[a]	276.24 ± 2.06[a]	27.11 ± 0.14[a]
CGN group	255.89 ± 1.73[f]	497.23 ± 4.25[f]	48.80 ± 0.24[f]
CGN+IND group	156.37 ± 1.40[b]	303.86 ± 2.72[b]	29.82 ± 0.16[b]
CGN+AAEE100 group	213.24 ± 1.68[e]	414.36 ± 3.81[e]	40.66 ± 0.23[e]
CGN+AAEE150 group	199.02 ± 1.50[d]	386.74 ± 3.50[d]	37.95 ± 0.21[d]
CGN+AAEE200 group	170.59 ± 1.44[c]	331.49 ± 3.16[c]	32.53 ± 0.18[c]

Values are expressed as mean ± SD and letters (a, b, c, d, e, and f) represent the difference between groups ($p < 0.05$)

a marked elevation in cytokine levels, with TNF-α increasing to 255.89 pg/ml, IL-1β to 497.23 pg/ml, and IL-6 to 48.80 pg/ml, confirming a strong inflammatory response.

Treatment with indomethacin significantly suppressed cytokine levels, with TNF-α reduced to 156.37 pg/ml, IL-1β to 303.86 pg/ml, and IL-6 to 29.82 pg/ml. AAEE demonstrated a similar dose-dependent cytokine suppression. At 100 mg/kg, TNF-α, IL-1β, and IL-6 levels decreased to 213.24, 414.36, and 40.66 pg/ml, respectively. The 150 mg/kg dose further reduced these levels to 199.02, 386.74, and 37.95 pg/ml. The highest dose of 200 mg/kg showed the strongest suppressive effect, with TNF-α at 170.59 pg/ml, IL-1β at 331.49 pg/ml, and IL-6 at 32.53 pg/ml, approaching the values observed in the indomethacin-treated group.

Morphological and histopathological analysis

Figure 6A depicts rat paw morphology across the different treatment groups. The control group (Figure 6A-a) exhibited normal paw structure without signs of swelling, redness, or deformation. The toes were fully extended and well separated, indicating the absence of inflammation. In contrast, the carrageenan group (CGN; Figure 6A-b) showed pronounced swelling, redness, and edema, particularly in the joints and dorsal surface of the paw. Toes appeared contracted and less flexible, confirming the presence of severe inflammation. Indomethacin treatment (CGN+IND; Figure 6A-c) markedly reduced swelling and redness, nearly restoring normal paw morphology and toe separation, indicative of effective anti-inflammatory action.

AAEE at 100 mg/kg (CGN+AAEE100; Figure 6A-d) resulted in mild edema reduction, although some swelling, redness, and toe contraction remained, suggesting a moderate anti-inflammatory effect. The 150 mg/kg dose (CGN+AAEE150; Figure 6A-e) produced a more pronounced reduction in inflammation, with visibly decreased swelling and improved toe extension, indicating enhanced efficacy. The CGN+AAEE200 group (Figure 6A-f) demonstrated the strongest anti-inflammatory response among the AAEE-treated groups, with paw morphology nearly restored. Significant edema reduction and improved toe flexibility were observed, comparable to the effects of indomethacin.

Histological analysis further supported the morphological findings. The control group (Figure 6B-a) exhibited intact tissue architecture, with well-preserved hair follicles and connective tissue, and no signs of inflammation or edema. In contrast, the carrageenan group (CGN; Figure 6B-b) showed severe edema and inflammatory cell infiltration, along with disrupted tissue integrity. Additional features included reduced connective tissue density, swollen and irregular hair follicles, mild epithelial degeneration, dilated blood vessels, and separated muscle fibers with mild degeneration – hallmarks of significant inflammatory damage. Indomethacin treatment (CGN+IND; Figure 6B-c) resulted in notable recovery, with reduced edema and inflammation, although minor structural alterations persisted. AAEE at 100 mg/kg (CGN+AAEE100; Figure 6B-d) produced slight reductions in edema and inflammatory infiltration; however, the presence of loose connective tissue, dilated vessels, and swollen hair follicles suggested incomplete recovery. The 150 mg/kg, AAEE (CGN+AAEE150; Figure 6B-e) resulted in further improvement, with decreased edema, more organized connective tissue, and reduced inflammatory cell infiltration. The highest AAEE dose (CGN+AAEE200; Figure 6B-f) exhibited the most significant histological

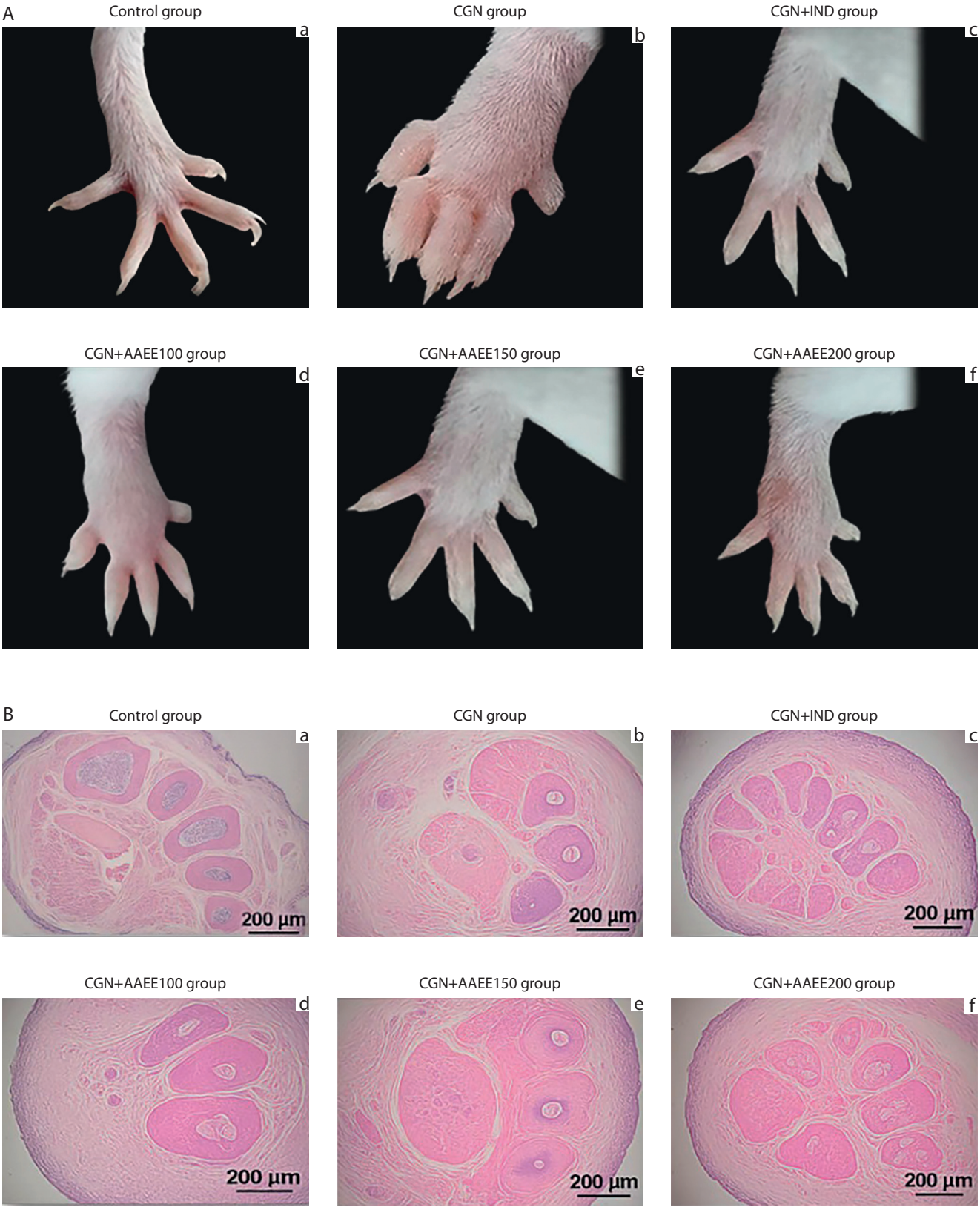

Figure 6. Effects of ethanol extract from *Argyreia acuta* on carrageenan-induced paw edema in mice. **A**) Representative macroscopic images of mouse paws from different experimental groups. **B**) Histological analysis of paw tissue sections stained with hematoxylin and eosin (H&E), magnification ×200

recovery, with minimal inflammation, restored hair follicle structure, and well-preserved tissue structure.

AAEE demonstrated significant anti-inflammatory effects, as evidenced by reduced paw edema, increased inflammation inhibition, suppression of pro-inflammatory cytokines, and improved tissue morphology. The data indicate a clear dose-dependent anti-inflammatory response, with higher doses (150–200 mg/kg) approaching the efficacy of indomethacin (IND), a widely used standard anti-inflammatory agent. CGN-induced inflammation occurs in two phases: the early phase (0–2 h), primarily mediated by histamine and serotonin, and the late phase (3–4 h), driven by prostaglandins and pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Berrueta et al. 2023). AAEE significantly reduced paw circumference and increased PPE%, suggesting effective modulation of both phases of inflammation. The early-phase inhibition may result from mast cell stabilization or suppression of histamine release, while the late-phase activity likely involves interference with the arachidonic acid cascade, potentially through COX inhibition and suppression of prostaglandin synthesis (Tran et al. 2023b).

In addition, AAEE significantly reduced TNF-α, IL-1β, and IL-6 levels, indicating its ability to interfere with NF-κB signaling and downregulate the production of inflammatory mediators, similar to the known mechanism of indomethacin (Pal et al. 2023). Histopathological analysis confirmed these findings by demonstrating that AAEE mitigated tissue damage, reduced inflammatory cell infiltration, and preserved overall tissue structure (Santoso et al. 2024). Higher doses (150–200 mg/kg) effectively reduced edema, vascular congestion, and inflammatory infiltration, restoring connective tissue structure and hair follicle morphology. These results suggest that the anti-inflammatory action of AAEE is mediated through multiple pathways, including the inhibition of prostaglandin and leukotriene synthesis via COX and LOX enzymes, antioxidant effects, and free radical scavenging activity that together help reduce oxidative stress and cytokine overproduction. The observed suppression of TNF-α and IL-1β further supports the notion that AAEE downregulates NF-κB signaling, thereby attenuating the inflammatory response (Zhang et al. 2022).

Numerous studies have validated the anti-inflammatory efficacy of herbal extracts by demonstrating reductions in edema, increases in inhibition percentages, suppression of pro-inflammatory cytokines, and improved histological profiles. For example, the ethanol extract of *Spondias mangifera* fruit significantly reduced joint swelling and cytokine levels (TNF-α and IL-6) in a CFA-induced arthritis model in rats, indicating strong anti-arthritic and anti-inflammatory properties (Khalid et al. 2021). Histopathological evaluation confirmed its efficacy through reductions in synovial inflammation and cartilage degradation, along with preservation of joint space integrity. Similarly, a standardized methanolic extract of *Muntingia calabura* leaves significantly reduced paw edema in a carrageenan-induced inflammation model in rats, further confirming its potent anti-inflammatory activity (Jisha et al. 2019).

Furthermore, the ethanol extract of *Mahonia bealei* significantly inhibited pro-inflammatory cytokines TNF-α and IL-6 in vitro, underscoring its potential for inflammation management (Hu et al. 2016). Collectively, these findings support the conclusion that various herbal extracts exert anti-inflammatory effects by reducing edema, enhancing inflammation inhibition, downregulating pro-inflammatory cytokines, and improving histopathological features. These outcomes are consistent with the present study on AAEE, further reinforcing its potential as a natural anti-inflammatory agent.

Conclusions

AAEE exhibits potent anti-inflammatory, analgesic, and antipyretic activities, which can be attributed to its rich phytochemical composition – including polyphenols, flavonoids, tannins, terpenoids, saponins, steroids, and alkaloids. AAEE effectively reduced carrageenan-induced paw edema and significantly suppressed pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), achieving results comparable to those of indomethacin. It also provided dose-dependent analgesia in both hot plate and tail-flick tests and effectively lowered yeast-induced fever. Histopathological analysis further confirmed AAEE's ability to minimize tissue damage and inflammatory cell infiltration. Taken together, these findings highlight *A. acuta* as a promising natural therapeutic candidate for the management of inflammation, pain, and fever.

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Author contributions

Initiation of the idea for the article, mentoring, supervision, and reviewing were done by T.T.P.N. and L.P.T.Q. Literature search and writing–original draft preparation was done by T.T.P.N., L.P.T.Q., and D.T.K.T. Critical editing and figure preparations were done by T.T.P.N. and L.P.T.Q. All the authors have read the manuscript and agree to its submission.

Conflict of interest

The authors declare that they have no conflict of interest.

Competing interests

The authors declare that they have no competing interests.

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Delivery and adjuvant: liposomes for SARS-CoV-2 vaccines

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Abstract

The global COVID-19 pandemic has highlighted the critical role of vaccines in controlling infectious diseases, with liposome-based formulations emerging as a pivotal advancement in vaccine technology. Liposomes are spherical vesicles composed of lipid bilayers that serve as drug delivery systems and versatile adjuvants, enhancing vaccine efficacy through improved antigen stability, targeted delivery, and immunogenicity. This review explores the potential of liposomes as adjuvants in both mRNA and protein subunit SARS-CoV-2 vaccines, detailing their composition and dual impact on innate and adaptive immune responses. Notably, liposome-based mRNA vaccines, such as those developed by Pfizer and Moderna, have demonstrated high efficacy by utilizing lipid nanoparticles to encapsulate mRNA and stimulate antigen-presenting cells, thereby inducing robust immune responses. Despite their advantages, challenges remain, including the optimization of lipid compositions and the mitigation of adverse immune effects. This review also examines the broad applications of liposomes in nanomedicine – from cancer therapy to antifungal treatments – and their potential for future vaccine development. By bridging the gap between engineering and immunology, the study of liposomes underscores their transformative potential in addressing current and emerging global health challenges.

Key words: liposome, drug delivery, vaccine, adjuvant

Introduction

Since the emergence of the SARS-CoV-2 virus, which caused the global COVID-19 pandemic in late 2019, numerous vaccines have been developed to combat the spread of this infectious agent worldwide. According to Viana et al. (2022), approximately 10 billion doses of COVID-19 vaccines had been distributed globally as of March 2022. Vaccination influences the body's innate and adaptive immune responses in specific ways, inducing protection through the formation of immunological memory (Vetter et al. 2018).

To date, COVID-19 vaccines can be classified into four categories based on their platforms: whole virus vaccines, protein-based vaccines, viral vector vaccines, and nucleic acid vaccines (Ndwandwe and Wiysonge 2021). Among whole virus vaccines, inactivated or live attenuated types are the most common in the industry. Inactivated vaccines can be produced using several inactivation methods, including gamma rays, ultraviolet light, and formaldehyde treatment (Khoshnood et al. 2022). Because these vaccines contain the entire attenuated pathogen, they typically induce a stronger immune response and more effective lymphocyte stimulation than other vaccine types.

However, nucleic acid vaccines, such as DNA vaccines, also show great promise due to several advantages, including ease of large-scale production, the ability to stimulate both cellular and humoral immunity, and low production costs (Silveira et al. 2021). Although promising, the immunogenicity of DNA vaccines is generally

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lower than that of other platforms when administered *in vivo*, mainly due to suboptimal cellular uptake and limited plasmid delivery to antigen-presenting cells (APCs) as a result of DNA degradation (Eusébio et al. 2021). To address this limitation, adjuvants are often incorporated to enhance the immunogenicity of DNA-based vaccines (Narayanan et al. 2022).

Despite their limitations, mRNA vaccines – part of the nucleic acid vaccine category – along with viral vector vaccines, have been approved for COVID-19 (Yang et al. 2023). According to Doan et al. (2022), Virofree, a herbal medicine, has also shown potential in treating SARS-CoV-2 by targeting various viral entry and replication stages in the Delta and Omicron variants. Although these vaccines exhibit high efficacy due to their antigen components, nearly all depend on the inclusion of an adjuvant (Facciolà et al. 2022).

An adjuvant is a substance added to a vaccine to enhance and stimulate the immune response more effectively (Pulendran et al. 2021). Adjuvants work by improving antigen uptake by APCs, thereby activating antigen-specific immune responses and enhancing vaccine immunogenicity and efficacy, particularly in neonates, immunocompromised individuals, and the elderly (Mohan et al. 2013).

Adjuvants play a critical role in vaccine development and are typically classified based on their mechanism of action as either immune potentiators or delivery systems. Immune potentiators stimulate innate immunity via pattern recognition receptors such as Toll-like receptors (TLRs) and NOD-like receptors, or through cytokine signaling, whereas delivery systems aid in transporting antigens into immune cells (Facciolà et al. 2022; Fan et al. 2022; Haensler 2010; Zhao et al. 2023). Although immune potentiators show promise, particularly in tumor immunotherapy, they are often associated with toxicity and systemic side effects due to rapid diffusion into circulation (Abhyankar et al. 2021).

As a result, delivery-based adjuvants – such as aluminum salts, emulsions, liposomes, and polymers – are preferred for their improved safety profiles (Alving et al. 2016; Facciolà et al. 2022; Huang et al. 2024; Zhao et al. 2023). Among licensed adjuvants, aluminum salts and emulsions have proven effective for pathogen-based vaccines but are insufficient for diseases like malaria, tuberculosis, and acquired immunodeficiency syndrome (AIDS), which require robust cellular immune responses. These adjuvants may also lead to systemic reactogenicity, including fever, inflammation, and pain (Fan et al. 2022; Huang et al. 2024; Mohan et al. 2013; Pulendran et al. 2021).

In contrast, liposomes have emerged as a safe and effective adjuvant platform. They offer excellent biocompatibility, structural adaptability, high antigen encapsulation efficiency, and protection from degradation. Liposomes are also suitable for various routes of administration and enhance immune responses through targeted delivery, lysosomal release, and antigen cross-presentation (Nsairat et al. 2022; Tretiakova and Vodovozova 2022). Besides liposomes, other lipid nanoparticle (LNP) systems such as cationic lipids consisting of 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) often combined with phospholipid or polymers to form stable complexes (lipoplexes) and improve encapsulation efficiency, cellular uptake, as well as endosomal escape – critical barriers in nucleic acid therapeutics (Hou et al. 2021). However, these LNPs systems are often limited by higher toxicity, poor stability, lower nucleic acid loading, and manufacturing complexity (Tada et al. 2015). Thus, other lipid adjuvants are needed for a superior balance of safety, efficacy, and scalability as the preferred platform for clinical drug delivery. One of the most common lipid adjuvants can fulfill these criteria: liposomes (Milicic et al. 2012).

Liposomes are spherical, lipid-bilayer vesicular structures that enclose various compartments, as illustrated in Figure 1. Extensive studies have been conducted

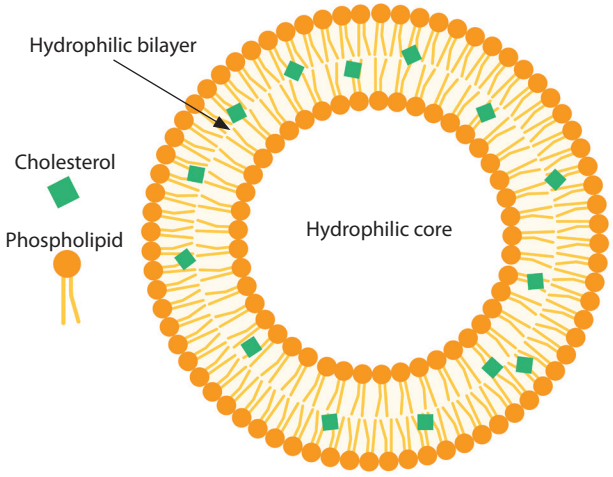

Figure 1. Schematic structures of liposomes. Created in https://BioRender.com

on liposomes as drug delivery systems due to their encapsulation capacity, which helps reduce antigen degradation and preserve a wide range of molecules. Additional advantages of liposomes include their ability to improve drug delivery stability and enhance efficacy by targeting specific cells and tissues (Henriksen-Lacey et al. 2011; Orosco and Espiritu 2024).

Furthermore, several liposomal characteristics – such as nanoparticle size, surface charge, bilayer rigidity, lipid composition, and preparation method – play critical roles in shaping the immune response to the target antigen (Nordly et al. 2009; Nsairat et al. 2022; Yanasarn et al. 2011). In addition to their established function in drug delivery, approximately 15 clinically licensed liposomal products are currently in use (Perrie et al. 2017).

Krasnopolsky and Pylypenko (2022) also reported the use of licensed liposomes and LNPs as adjuvants in vaccines, two of which are the Pfizer-BioNTech and Moderna COVID-19 vaccines. These formulations include LNPs composed of ionizable lipids, polyethylene glycol–lipid (PEG–lipid), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and cholesterol. Specifically, the Moderna formulation includes ionizable lipid, DSPC, cholesterol, and 2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (PEG-2000-DMG).

Therefore, liposomes have been demonstrated to serve as a drug delivery tool and a vaccine adjuvant in the last decade of studies. Herein, the development and potential clinical use of liposomes in vaccines against COVID-19, as well as their specific effect on the immune system, are reviewed.

Liposomes: from drug delivery to adjuvant

Liposomal formulations as drug delivery systems are currently undergoing clinical trials, with several already approved for use. In recent years, research has increasingly focused on the immunogenicity of liposome-based adjuvants in vaccines and on enhancing immune responses through liposome modifications. To understand these applications, it is essential first to examine the sources and composition of liposomes.

Liposome compositions and applications

The term "liposome" is derived from two Greek words: "lipos" means fat, and "soma" means body. Dr. Alec D. Bangham, a British hematologist at the Institute of Cambridge, England, introduced the term in 1961 (Sharma and Agrawal 2021). In the 1970s, G. Gregoriadis proposed that liposomes could be used to deliver drugs to cell membranes, and their development has progressed rapidly since then (Gregoriadis 2016). Liposomes gained prominence due to their stability, encapsulation capability, and high drug-loading efficiency (Noble et al. 2014).

As shown earlier, liposomes are comprised of an area for an aqueous solution inside a hydrophobic membrane, making them capable of capturing drug molecules, including protein, peptides, carbohydrates, and DNA (Alving et al. 2016; Blanken et al. 2020; Boons 2010). Furthermore, their vesicle size, composition, and surface charge can be modified, providing flexibility for diverse medical applications (Zhou et al. 2023). These applications span from vaccines for viral, microbial, and fungal infections to cancer therapy (Huang and Anderson 2002; Orosco and Espiritu 2024).

Three key components contribute to the effectiveness of liposomes in biomedical and nanomedicine applications: (i) phospholipids, (ii) cholesterol, and (iii) PEG (Jiang et al. 2024).

Phospholipids typically consist of a hydrophilic headgroup, hydrophobic fatty acid chains, and a glycerol backbone (De Carvalho and Caramujo 2018). One of the most common sources of phospholipids is bacterial membranes (Kozhikhova et al. 2018). For instance, liposomes prepared from *Deinococcus radiodurans* and loaded into a respiratory syncytial virus vaccine were injected into mice and found to enhance vaccine efficacy (Huang and Anderson 2002). Halophilic bacteria have also been shown to offer strong potential for liposome production due to their ability to achieve high drug-loading efficiency (Baserisalehi 2020).

In addition to bacterial sources, several cationic lipids are well known for their gene delivery capabilities. These include 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and DOTMA (Ponti et al. 2021).

Chen et al. (2017) investigated a drug-in-cyclodextrin-in-liposome (DCL) system for enhancing the delivery of lipophilic antitumor drugs. They encapsulated FITC-labeled hydroxypropyl-β-cyclodextrin (FITC-HP-β-CD) into liposomes composed of soybean-derived phosphatidylcholine (SPC) and modified with transferrin (Tf). The results showed that Tf-modified liposomes

significantly improved stability and cellular uptake compared to PEGylated liposomes. SPC-based liposomes demonstrated the highest tumor cell internalization and lipophilicity, highlighting the potential of optimized Tf-DCL systems for targeted cancer therapy.

Other studies utilized microfluidics to optimize the production of monodispersed, drug-loaded liposomes for breast cancer treatment. Two types of PCs with varying acyl chain lengths were tested to control the release of doxorubicin hydrochloride. Liposomes were produced under optimized conditions (TFR 500 μl/min, FRR 0.1), resulting in six stable formulations (< 200 nm) with high encapsulation efficiency (> 80%) and sustained *in vitro* release. DMPC-based liposomes exhibited slower doxorubicin release than DSPC, and binary formulations showed higher cytotoxicity (IC_{50} ~1 μM) against MCF7, MDA-MB-231, and BT474 breast cancer cell lines, comparable to free doxorubicin. These findings support microfluidics as a robust platform for producing size-controlled liposomal nanomedicines for prolonged chemotherapeutic delivery (Gkionis et al. 2021). PC also showed potential in other drug delivery with thioether phosphatidylcholine (SPC-based) stealth liposomes as promising alternatives to conventional phospholipids for targeted, ROS-responsive drug delivery (Du et al. 2019). Serrano et al. (2015) demonstrated ascorbate PC liposomes for their preventive antioxidant and anti-inflammatory properties on whole human skin irradiated with UVA/UVB while allowing nonstable hydrophilic active ingredients to reach epidermis and dermis, preventing photodamage to the skin.

Another common phospholipid used in liposome formulations is phosphatidylethanolamine (PE), the second most abundant glycerophospholipid in eukaryotic cells. PE has recently gained attention due to its association with Alzheimer's and Parkinson's diseases (Calzada et al. 2016). Fan et al. (2017) investigated the pH-responsiveness mechanism of PE-based liposomes (PSLs), identifying them as key components in pH-sensitive liposomal drug delivery systems for tumor-targeted therapy.

In addition to PE, another study evaluated two radiotracers – ^{18}F-duramycin (PE-targeting) and ^{18}F-Zn-DPA (phosphatidylserine [PS]-targeting) – for their potential in apoptosis imaging, with possible clinical relevance in cancer diagnosis and therapy monitoring (Li et al. 2019). Regarding anticancer activity, other phospholipids such as sphingomyelin (SM) and cardiolipin (CL) have also shown promising therapeutic potential (Ahmadpour et al. 2020; Alrbyawi et al. 2022; Wang et al. 2024; Zhu et al. 2023). Sphingomyelin-based liposomes (SMLs) have notably advanced the development of lipid-based nanocarriers (Lim et al. 2021).

Meanwhile, CL, a signature phospholipid of mitochondrial membranes, is often linked to therapies for cardiac disorders (Gasanoff et al. 2021; Shen et al. 2015). Consequently, phospholipids play a vital role as the main structure of liposomes while also regulating the functional components inside liposomes (Jiang et al. 2024).

Cholesterol in cell membranes helps modulate the fluidity, stability, and permeability, sustaining the lipid bilayer's rigid parts of the cell (Briuglia et al. 2015). The phospholipid structure is usually formed by four fused rings of hydrophobic lipids (Cerqueira et al. 2016). When the phospholipid's hydrophobic tails interact with the nonpolar part of cholesterol and the polar part of cholesterol binds to the phospholipid's hydrophilic headgroups, this results in a controlled release of molecules inside liposomes (Kaddah et al. 2018).

PEG is inherently hydrophilic, which imparts a "stealth" property to the surface of liposomes, helping them evade detection and degradation by the body's immune system (Andra et al. 2022). In addition to prolonging the *in vivo* circulation time of liposomes, PEG as a polymer can enhance liposome stability (Shen et al. 2018). For example, Doxil® was the first PEGylated liposomal formulation to encapsulate doxorubicin for anticancer treatment and has been approved for use against Kaposi's sarcoma, breast cancer, and multiple myeloma (Mohamed et al. 2019). Thus, PEG is considered a critical component in boosting the drug delivery potential of liposomes.

Liposome applications range from general drug delivery to targeted cancer therapy. For instance, to address glioblastoma multiforme (GBM), Shi et al. (2019) developed a dual-functionalized, thermosensitive liposomal system (DOX@P1NS/TNC-FeLP) co-loaded with doxorubicin (DOX) and superparamagnetic iron oxide nanoparticles (SPIONs). The surface was modified with a GBM-targeting peptide (P1NS) and an antibody (TN-C) to achieve targeted delivery, while an alternating magnetic field triggered localized drug release. This system successfully crossed an *in vitro* blood–brain barrier (BBB) model, demonstrated GBM-specific uptake and drug release, and inhibited proliferation of U-87 GBM cells

without affecting healthy brain cells. These results support the potential of DOX@P1NS/TNC-FeLP as a promising platform for BBB-penetrating, targeted GBM therapy.

In another approach, Rodà et al. (2023) employed Raman spectroscopy (RS) to characterize functionalized liposomes designed for targeted drug delivery in neurological disorders such as glioblastoma and Alzheimer's disease. Furthermore, a novel strategy by Chen et al. (2020) involved embedding stiff nanobowls inside the aqueous core of DOX-loaded liposomes (DOX@NbLipo). This modification improved liposome stability against plasma proteins and shear forces during circulation, reducing drug leakage, enhancing tumor targeting, and increasing antitumor efficacy.

Liposomes composed of SPC, cholesterol, and CL and loaded with levofloxacin have demonstrated antibacterial activity against *Mycobacterium tuberculosis* (Gaidukevich et al. 2016). Another study addressed the challenge of achieving high liposomal loading efficiency for antibiotics by evaluating three clinically relevant drugs – vancomycin hydrochloride, teicoplanin, and rifampin – with varying degrees of hydrophilicity (Gonzalez Gomez et al. 2019). Encapsulation techniques were assessed based on encapsulation efficiency, lipid requirements, and mass yield. Hydrophobic antibiotics such as teicoplanin and rifampin showed higher encapsulation efficiencies with specific methods, while the hydrophilic vancomycin exhibited no clear preference. The study also highlighted methodological biases introduced by different quantification approaches, recommending ultrafiltration and methanol bursting as more accurate alternatives. These findings provide valuable insights for optimizing liposomal antibiotic delivery and inform broader nanocarrier design strategies (Ferreira et al. 2021; Gonzalez Gomez and Hosseinidoust 2020).

Liposomes have also proven useful as antifungal drug carriers, particularly against infections caused by *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. Ambati et al. (2019) improved treatment efficacy and reduced toxicity by utilizing amphotericin B-loaded liposomes coated with sDectin-2, a mannan-binding domain that targets fungal cell wall components. These targeted liposomes demonstrated significantly enhanced binding and antifungal activity compared to untargeted formulations, allowing for lower effective doses. This approach shows promise for developing broad-spectrum antifungal liposomal therapies.

Anidulafungin-loaded liposome nanoparticles have also shown antifungal activity against both planktonic and biofilm forms of *Candida albicans* (Vera-González et al. 2020). In a related study, Bezerra et al. (2020) investigated the antifungal potential of farnesol, a bioactive compound, delivered via liposomes against various *Candida* strains. Farnesol-loaded liposomes enhanced antifungal efficacy and more effectively inhibited fungal dimorphism than free farnesol. Moreover, liposomal farnesol synergized with fluconazole, whereas free farnesol in combination with fluconazole exhibited antagonistic effects. These findings highlight the potential of farnesol-loaded liposomes for antifungal drug development, though further research is needed to understand their influence on drug resistance. Besides its applications in the medical field, liposomes have also gained interest in the food and pharmaceutical industry through the development of garlic extract encapsulated within PC and oleic acid (OA) as an antifungal agent in wheat bread, showing its potential as a natural antifungal agent in bakery products (Pinilla et al. 2019).

In addition to liposome applications, Snyder et al. (2016) compared pain control in total knee arthroplasty (TKA) patients who did not receive a femoral nerve block. Participants were administered either an intraoperative injection of bupivacaine liposome suspension (EXPAREL) or a concentrated multidrug cocktail. The study found that patients in the multidrug cocktail group reported significantly higher pain levels on postoperative days 1 and 2 and experienced more adverse events than those in the bupivacaine liposome group. Moreover, the bupivacaine liposome group reported greater satisfaction with both pain control and overall experience.

Similarly, Sporer and Rogers (2016) investigated the use of liposomal bupivacaine for postoperative pain management following primary TKA. Their study demonstrated a reduced need for breakthrough pain medication, improved pain scores at 12 h, and earlier ambulation when compared to a combined femoral nerve block and periarticular bupivacaine injection. These results highlight liposomes as a promising therapeutic strategy in pain management (Ji et al. 2021).

Mennini et al. (2015) further explored this potential by developing both conventional and PEGylated liposomes to enhance the pain-relieving effects of opiorphin. Using a rat tail-flick test, they compared the antinociceptive

effects of these formulations to free opiorphin and morphine solutions (all at 5 mg/kg). Conventional liposomes increased opiorphin's area under the curve (AUC) by 28% compared to the free peptide. PEGylated liposomes yielded even greater improvements, with AUC values 80, 60, and 40% higher than those of free opiorphin, morphine, and conventional liposomes, respectively. Additionally, PEGylated liposomes extended the duration of analgesic effect by over 50%, likely due to improved drug protection and prolonged circulation time. These findings suggest that opiorphin-loaded PEGylated liposomes could serve as a promising alternative to morphine for pain management.

Liposomes have demonstrated tremendous potential in gene therapy due to their advantages, such as biocompatibility, biodegradability, and high encapsulation efficiency (Liu et al., 2020; Tseu and Kamaruzaman 2023; Zylberberg et al. 2017). As a promising strategy for treating neurodegenerative diseases, dual-functionalized liposomes – modified with Tf for BBB targeting and penetratin (Pen) for enhanced cellular penetration – were developed to improve gene delivery. BBB model studies confirmed the ability of these liposomes to cross the barrier and transfect neurons *in vitro*. In *in vivo* studies, PenTf-liposomes accumulated significantly in the brain (12%) without causing cellular or structural damage. These liposomes successfully delivered plasmid DNA encoding β-galactosidase and GFP, resulting in measurable gene expression in mouse brains. These findings underscore the promise of multifunctional liposomes as platforms for gene therapy targeting neurodegenerative diseases (dos Santos Rodrigues et al. 2018).

In a related development, liposome-based systems are emerging as effective delivery platforms for clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene-editing technology, offering improved flexibility and delivery efficiency (Zhen et al. 2017; Zhen and Li 2020).

Chen et al. (2017) introduced liposome-templated hydrogel nanoparticles (LHNPs) as a novel co-delivery system for Cas9 protein and guide RNA. When integrated with minicircle DNA technology, LHNPs outperformed commercial transfection agents such as Lipofectamine 2000 in gene delivery, including to brain tumors. Targeted CRISPR/Cas9 delivery against the *PLK1* gene significantly inhibited tumor growth and improved survival in mice, showing LHNPs' potential as a flexible platform for cancer gene therapy.

In another study, a nano-liposomal carrier system encapsulating therapeutic Cas9 with single-guide RNA (sgRNA) ribonucleoprotein (Cas9-RNP) demonstrated strong potential to enhance gene-editing therapies for human liver diseases (Cho et al. 2019).

Lower cholesterol content in liposomes increases membrane fluidity and enhances drug transport across the stratum corneum (Soni et al. 2016). While plain liposomes are not ideal for transdermal drug delivery due to limited skin penetration, they are effective for cosmetic use as they tend to remain within the upper layers of the skin (Musielak et al. 2024; Soni et al. 2016). Larger liposomes (≥ 1000 nm) are typically confined to the stratum corneum, whereas smaller liposomes (≤ 600 nm) can penetrate deeper (Estanqueiro et al. 2016). Liposomal systems can also enhance drug absorption by adhering to the skin surface, destabilizing or fusing with skin lipids, and disrupting the stratum corneum to facilitate penetration (Ahmadi Ashtiani et al. 2016). These mechanisms enable liposomes to improve the delivery of active ingredients from cosmetic and skincare products into the skin.

Bochicchio et al. (2020) utilized a novel semi-continuous simil-microfluidic technique to produce nanoliposomes loaded with vitamin D_3, K_2, E, and curcumin – compounds known for their antioxidant and skin-healing properties but typically unstable and poorly absorbed. This method yielded stable, negatively charged vesicles (84–145 nm) with high loading and encapsulation efficiency, demonstrating promising potential for cosmetic and dermo-cosmetic applications.

In cosmeceuticals, liposomes serve dual roles: as carriers for active ingredients and as active agents themselves (Ahmadi Ashtiani et al. 2016). In conditions like eczema or dry, damaged skin, empty liposomes can interact with skin lipids, proteins, and carbohydrates, aiding skin repair and restoring the barrier function of the stratum corneum. As delivery systems, liposomes offer numerous advantages: enhanced penetration, solubility, and stability of active ingredients; extended therapeutic effects; protection against environmental degradation; targeted delivery; reduced toxicity; and improved pharmacokinetic and pharmacodynamic control – ultimately making products more cost-effective (Ahmadi Ashtiani et al. 2016; Hameed et al. 2019).

Liposomes have also been used to deliver vitamin D_3 due to their stability and ability to enhance its effectiveness as a skin-protective agent against photoaging (Bi et al. 2019). However, many cosmetic actives used in novel formulations, such as sunscreens and anti-aging treatments, suffer from low penetration, poor solubility, and physicochemical instability (Kim et al. 2018). Lipid carriers like liposomes help address these issues by increasing solubility, improving skin permeation, and enhancing the stability of poorly soluble compounds (Pavlou et al. 2021).

Vovesná et al. (2021) developed stable liposomal formulations containing ceramides and other skin lipids to repair damaged skin barriers – often linked to reduced ceramide levels. Using lipid film hydration and high-pressure homogenization, liposomes were prepared with ceramides 3 and 6, cholesterol, stearic acid, and 10% urea in phosphate-buffered saline. When tested on chemically damaged porcine skin, this formulation significantly improved barrier function, reducing permeation to levels closer to those of intact skin. Notably, nonhomogenized liposomes were more effective than homogenized ones. FTIR analysis supported these results, highlighting the potential of liposomal therapies in skin barrier repair.

In another study, Kim et al. (2015) investigated the moisturizing effects of liposomal serine combined with different cosmeceutical bases. Serine, a key component of the skin's natural moisturizing factors, was hypothesized to enhance hydration when effectively delivered to the stratum corneum. Among the four tested bases, hydrogel showed the best water-holding capacity. Incorporating 1% liposomal serine into the hydrogel significantly improved skin moisturization – 1.62 to 1.77 times more than hydrogel alone, free serine, or blank liposomes. The moisturizing effect was not dependent on serine concentration, supporting liposomal serine as a promising agent for advanced skincare formulations.

Finally, and importantly, liposomes have also been employed as platforms in theranostic applications to encapsulate both therapeutic and diagnostic agents (Xing et al. 2016). Theranostics refers to technologies that integrate therapeutic and diagnostic functions into a single system (Kim and Jeong 2021).

For example, Ren et al. (2019) investigated how the physical and chemical properties of liposomes influence their ability to passively target inflamed joints in rheumatoid arthritis (RA). Using a collagen-induced arthritis mouse model, the researchers evaluated liposomes with varying sizes, surface charges, and PEG modifications. The results indicated that liposomes with a 100 nm diameter, a slightly negative surface charge, and 10% 5 kDa PEG achieved optimal circulation time and selective targeting of RA-affected joints. When loaded with dexamethasone, these optimized liposomes significantly improved antiarthritic effects.

In addition to RA, liposomes have been widely studied for their roles in tumor and cancer-targeted therapies, including drug delivery to gliomas, chemotherapy enhancement, and cancer detection (Alavi and Hamidi 2019; Belfiore et al. 2018; Bozzuto and Molinari 2015; Mojarad-Jabali et al. 2021; Mukherjee et al. 2022; Pérez-Herrero and Fernández-Medarde 2015; Riaz et al. 2018; Wang et al. 2023).

Liposome and LNPs applications in viral infections beyond SARS-CoV-2

Liposomes have also been employed in addressing viral infections and virus-associated diseases beyond SARS-CoV-2. Notably, their use has been explored in the treatment and prevention of HIV, where liposomal formulations enhance drug stability, improve targeted delivery to immune cells, and reduce systemic toxicity (Gao et al. 2018). In a related study, Leaman et al. (2021) developed broadly neutralizing antibodies (bNAbs) targeting the virus's native, membrane-bound envelope glycoprotein (mEnv). Membrane Env liposomes (MELs) are a novel liposome-based platform that displays multivalent, structurally intact mEnv trimers. In this system, purified mEnv spikes are reconstituted onto naked liposomes using a detergent-removal method, resulting in stable nanoparticles (~133 nm) with proper antigen orientation. These MELs preserved native epitopes recognized by bNAbs while excluding non-neutralizing antibody (non-NAb) binding. When used in sequential immunization in rabbits, MELs successfully elicited antibodies capable of neutralizing tier 2 HIV isolates.

Additionally, plasma membrane-derived liposomes have demonstrated robust antiviral activity against herpes simplex virus type 1 (HSV-1), outperforming Chinese hamster ovary (CHO)-derived liposomes (Bhattacharya et al. 2022). Another study reported the development of decoy liposomes functionalized with heparan sulfate octasaccharide (HS-octa) as a broad-spectrum antiviral strategy against HSV, respiratory syncytial virus (RSV), and human parainfluenza virus 3 (hPIV3).

The ability of liposomes to modulate and elicit adjuvant effects through diverse lipid combinations further supports their use in delivering anti-RSV agents (Joshi et al. 2019).

In another application, Croci et al. (2016) developed and evaluated liposome-based formulations of ivermectin – a potent antihelminthic drug shown to inhibit flavivirus helicases *in vitro*, though its clinical use is limited by poor solubility and high cytotoxicity. The engineered liposomes significantly reduced ivermectin's cytotoxicity across multiple cell lines and enhanced its antiviral efficacy against several Dengue virus strains (1, 2, and S221). These results confirmed the antiviral potential of ivermectin and demonstrated the value of liposomes as effective drug carriers that improve pharmacokinetics and therapeutic outcomes. This liposomal approach offers a promising strategy for optimizing ivermectin-based antiviral therapies.

Hongtu et al. (2023) demonstrated a promising approach to rabies vaccination using an mRNA vaccine encoding the rabies virus glycoprotein (RABV-G), encapsulated in LNPs and combined with various nucleic acid-based immune stimulators, including CpG 1018, CpG 2395, and Poly I:C. Among these, the formulation with LNP and CpG 1018 elicited the strongest immune response, inducing high and sustained levels of RABV-G-specific IgG, potent virus-neutralizing antibodies, and robust cell-mediated immunity, including IFN-γ- and TNF-α-producing $CD4^+$ and $CD8^+$ T cells. Notably, this combination provided 100% protection in both pre- and post-exposure models and significantly reduced viral replication. These findings suggest that LNP-formulated RABV-G mRNA with CpG 1018 is a safe, effective, and scalable vaccine candidate that could serve as a next-generation alternative to traditional rabies vaccines. However, the success observed with SARS-CoV-2 vaccines has not been equally replicated in other antiviral activities of certain drugs or vaccines, partly due to the virus's high antigenic variability, dense glycan shield, and the need for prolonged and sequential immunization to guide antibody maturation.

Types of liposomes

Liposomes can be classified in several ways, including by size, lamellarity, and surface charge (González-Rodríguez and Rabasco 2011; Kraft et al. 2014; Liu et al. 2022). Based on size, liposomal nanoformulations typically range from 50 to 500 nm and are well-suited for nanomedicine applications (Lombardo et al. 2019). Smaller liposomes, particularly those under 50–100 nm, can evade immune detection, exhibit prolonged circulation in the bloodstream, and allow for enhanced drug release (Andra et al. 2022; Ren et al. 2019).

Another classification of liposomes is based on lamellarity, which is divided into: unilamellar vesicle (ULV), which contains only one bilayer membrane; oligolamellar vesicle (OLV), which has around 2–5 bilayer membranes; and multilamellar vesicles (MLV) with bilayer membranes resembling onion-like structures with many layers of membranes (Jiang et al. 2024; Pattni et al. 2015).

Lamellarity influences the drug release rate; the more bilayers present, the slower the encapsulated molecules are released (Lombardo et al. 2019). It is also important to note that both the size and lamellarity of liposomes can be controlled through preparation methods and modifications to their surface charge.

DOTAP is an example of a charged, cationic liposome that has been widely studied for its ability to deliver negatively charged macromolecules, such as DNA and RNA (Majzoub et al. 2016). Due to their positive surface charge, cationic liposomes attract negatively charged nucleic acids via electrostatic interactions, facilitating cellular uptake and enhancing drug delivery through easier diffusion across cell membranes (Ochoa-Sánchez et al. 2024). However, despite these advantages, cationic liposomes are often associated with high toxicity and low transfection efficiency (Neves et al. 2016).

In contrast, anionic liposomes are less toxic and typically exhibit shorter circulation times. Although they are less stable and have weaker binding affinity for nucleic acids, their biocompatibility makes them more suitable for transdermal rather than gene delivery applications (González-Rodríguez and Rabasco 2011). For instance, Ibaraki et al. (2019) explored the rigidity of anionic liposomes, such as 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), and demonstrated their potential in dermal drug delivery systems.

Beyond liposome classification, structure, and composition, LNPs are a critical determinant of performance in vaccine delivery systems (Nordly et al. 2009; Song et al. 2012). LNPs are typically composed of ionizable lipids, helper phospholipids, cholesterol, and PEG-lipids, each contributing distinct functional roles (Hou et al. 2021; Schoenmaker et al. 2021). Ionizable lipids aid in endosomal escape and antigen delivery by becoming

positively charged in acidic environments, while cholesterol enhances membrane stability and maintains structural integrity (Cerqueira et al. 2016; Wang et al. 2024). Phospholipids such as DSPC or DOPE influence bilayer fluidity and promote fusion with immune cells (Ponti et al. 2021). PEG-lipids prolong circulation time and prevent aggregation; however, excessive PEGylation can hinder cellular uptake and reduce immunogenicity (Mohamed et al. 2019).

Fine-tuning the composition of these components enables LNPs to balance antigen protection, stability, and delivery efficiency – critical factors for optimizing vaccine performance.

Licensed liposomal drug products

Since liposomes were first recognized as drug delivery systems in the 1970s, hundreds of nanodrug formulations have entered clinical development, with several already licensed for medical use (Gatto et al. 2024; Jiang et al. 2024). This widespread implementation demonstrates the versatility of liposomes as platforms for anticancer therapies, antifungal treatments, and vaccine adjuvants – applications that continue to expand, as summarized in Table 1.

Among these licensed liposome products, anticancer drugs held the first place for having a great deal of quantity, concerning about seven marketed medicines, including the first liposome-approved product by the U.S. Food and Drug Administration (FDA) in 1995: Doxil® for Kaposi's sarcoma, ovarian cancer, and multiple myeloma treatment (Mohamed et al. 2019).

In the antifungal category, liposomal drugs such as Amphotec® and AmBisome® have been approved for clinical use (Jiang et al. 2024). These formulations encapsulate amphotericin B as the active pharmaceutical ingredient (API). Therefore, it suppresses the toxic activity from fungal infection (Nsairat et al. 2022).

The Inflexal® V vaccine was the first licensed liposomal vaccine, formulated with two influenza virus strains (type A and type B) (Asadi and Gholami 2021; Krasnopolsky and Pylypenko 2022). Clinical trials of Inflexal® V demonstrated a strong humoral immune response, with immunogenicity levels several times higher than existing influenza vaccines at the time (Poon and Patel 2020).

Liposomes' high encapsulation capacity also allows them to carry macromolecules such as DNA and RNA, enhancing vaccine efficacy by functioning as adjuvants. During the 2019 COVID-19 pandemic outbreak, researchers leveraged the ability of liposomes to extend the half-life of antigens in the bloodstream, thereby ensuring prolonged exposure to APCs and promoting stronger immune responses (Krasnopolsky and Pylypenko 2022; Mohan et al. 2013).

This was exemplified by the Pfizer/BioNTech and Moderna vaccines, which utilized liposome-based formulations to develop mRNA vaccines against COVID-19. The primary components of these formulations include cationic lipids, PEGylated lipids, phospholipids, cholesterol, and mRNA encoding the viral spike glycoprotein in which the latter were encapsulated inside the liposomes to protect it from enzyme degradation for an efficient antigen protein translation after administered intramuscularly (Figure 2). Upon delivery into human cells, the mRNA is translated into viral spike proteins, triggering both innate and adaptive immune responses that result in localized inflammation (Schoenmaker et al. 2021). Leukocytes, including neutrophils and APCs, are recruited to the site of inflammation, leading to the generation of antibodies that prevent SARS-CoV-2 infection (Gregoriadis 2021).

By contemplating the biodegradability and versatility of liposomal formulations in vaccine fields, it is safe to say that further development of liposomes as another vaccine adjuvant will be looked forward to. Other than being a drug delivery system, liposomes have every potential and significant effect on the human immune system while also achieving synergism with the mRNA-encoded proteins (Hou et al. 2021). Utilizing billions of vaccine doses against SARS-CoV-2 saved countless human lives due to the liposomal formulation and its immunogenicity (Cheng et al. 2021). Moreover, numerous liposomal vaccines are underway, ready to catch up as another licensed drug for medical applications.

Immune system response to liposomes

Ongoing research has led to a growing number of licensed and approved liposome-based drug products each year. Two prominent examples that contributed to combating the COVID-19 pandemic are Spikevax® (Moderna) and Comirnaty® (Pfizer). Further studies are taking place regarding the liposomal formulation in vaccines and its impact on the body. While liposomes offer numerous advantages, they are not without drawbacks. Therefore, understanding their impact on the immune system is essential.

Table 1. A summary of liposomal drug products approved by the U.S. Food and Drug Administration (FDA) as of 2024

Brand name	Year of approval	Liposomes formulation	API	Indication	Route of administration	References
Doxil®	1995	HSPC:CHOL:DSPE-PEG2000	Doxorubicin	Kaposi's sarcoma, ovarian cancer, and myeloma	*i.v.*	Liu et al. 2022; Nsairat et al. 2022; Jiang et al. 2024; Gatto et al. 2024
DaunoXome®	1996	DSPC:CHOL	Daunorubicin	Kaposi's sarcoma	*i.v.*	Liu et al. 2022; Nsairat et al. 2022; Jiang et al. 2024; Gatto et al. 2024
Amphotec®	1996	Amphotericin B:Cholesteryl sulphate	Amphotericin B	Fungal infection	*i.v.*	Jiang et al. 2024
AmBisome®	1997	Amphotericin B: HSPC:DSPG:CHOL	Amphotericin B	Fungal infection	*i.v.*	Liu et al. 2022; Nsairat et al. 2022; Jiang et al. 2024; Gatto et al. 2024
Inflexal® V	1997	Lecithin:Cephalin:Phospholipids	Virus antigen	Influenza	*i.m.*	Jiang et al. 2024
DepoCyt®	1999	DepoFoam™	Cytarabine	Lymphomatus meningitis	Spinal	Liu et al. 2022; Nsairat et al. 2022; Gatto et al. 2024
Myocet®	2000	CHOL:EPG	Doxorubicin	Cancer	*i.v.*	Liu et al. 2022; Nsairat et al. 2022; Jiang et al. 2024
Visudyne®	2000	Verteporphin:DMPC with EPG	Verteporphin	Age-related degeneration	*i.v.*	Liu et al. 2022; Nsairat et al. 2022; Jiang et al. 2024; Gatto et al. 2024
DepoDur®	2004	DepoFoam™	Morphine sulfate	Painkiller	Epidural	Liu et al. 2022; Nsairat et al. 2022; Jiang et al. 2024; Gatto et al. 2024
Mepact®	2004	DOPS:POPC	Mifamurtide	Cancer	*i.v.*	Liu et al. 2022; Nsairat et al. 2022; Jiang et al. 2024; Gatto et al. 2024
Exparel®	2011	DepoFoam™	Bupivacaine	Anesthesia	*i.v.*	Liu et al. 2022; Nsairat et al. 2022; Jiang et al. 2024; Gatto et al. 2024
Marqibo®	2012	SM:CHOL	Vincristine	Leukemia	*i.v.*	Liu et al. 2022; Nsairat et al. 2022; Gatto et al. 2024
Onivyde®	2015	DSPC:CHOL:DSPE	Irinotecan	Pancreatic adenocarcinoma	*i.v.*	Liu et al. 2022; Nsairat et al. 2022; Jiang et al. 2024; Gatto et al. 2024
Vyxeos®	2017	DSPG:DSPC:CHOL	Daunorubicin and cytarabine	Leukemia	*i.v.*	Liu et al. 2022; Nsairat et al. 2022; Jiang et al. 2024; Gatto et al. 2024
Onpattro®	2018	CHOL, DLin-MC3-DMA: DSPC:PEG2000-C-DMG	Patisiran	Hereditary transtyretin-mediated amyloidosis	*i.v.*	Liu et al. 2022; Nsairat et al. 2022; Jiang et al. 2024; Gatto et al. 2024
Comirnaty®	2021	ALC-0315:ALC-0159: CHOL:DSPC	mRNA	COVID-19	*i.m*	Jiang et al. 2024; Gatto et al. 2024
Spikevax®	2022	SM-102:mPEG2000-DMG: CHOL:DSPC	mRNA	COVID-19	*i.m*	Jiang et al. 2024; Gatto et al. 2024

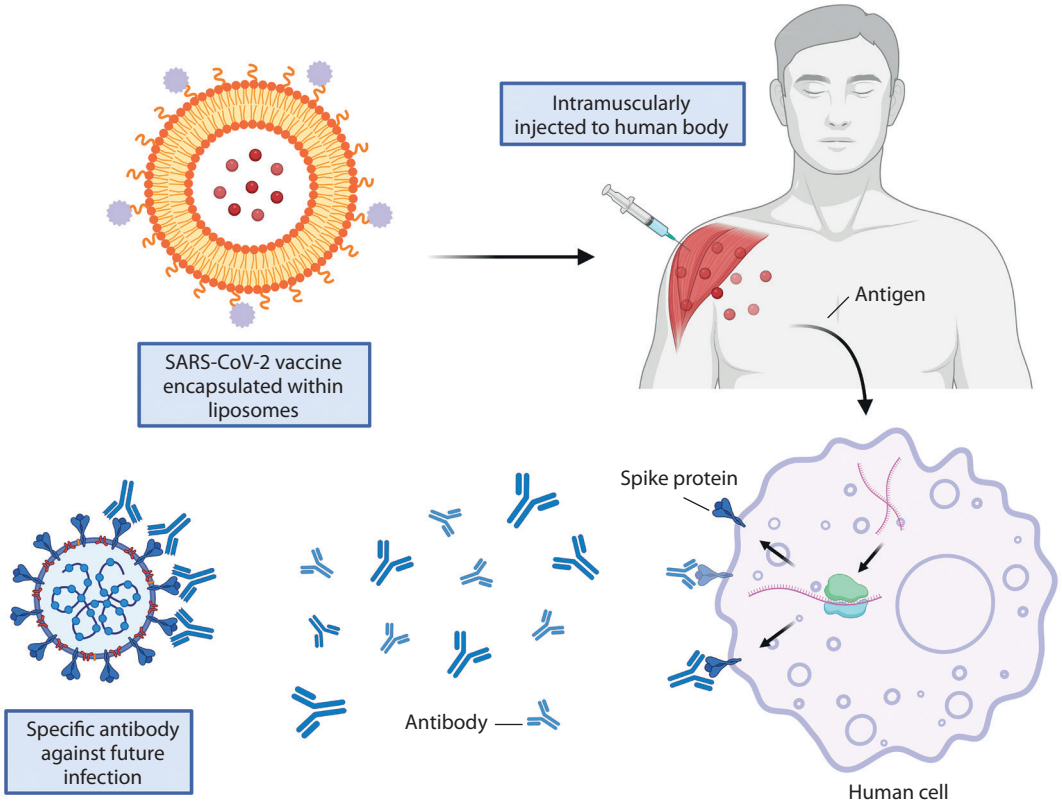

Figure 2. Liposome encapsulation ensures antigen protein translation in SARS-CoV-2 vaccines. Created in https://BioRender.com

Innate immunity and liposomes

When liposomes are introduced into the body, they are treated similarly to other foreign particles – that is, as antigens (Tretiakova and Vodovozova 2022). They are typically recognized and taken up by APCs through phagocytosis or receptor-mediated endocytosis. Innate immunity represents the body's first line of defense and is mediated by macrophages, neutrophils, dendritic cells, eosinophils, and NK cells (Lee et al. 2023).

Liposome-based mRNA vaccines for COVID-19 significantly impact the innate immune response through the activation of pattern recognition receptors, such as TLRs, melanoma differentiation-associated gene 5 (*MDA5*), and NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) (Newton and Dixit 2012; Takeuchi and Akira 2010). These receptors are capable of detecting mRNA encapsulated within LNPs, leading to the production of key cytokines – IL-1β, IFN-γ, and IL-6 – which are essential for initiating an antiviral state (Chen et al. 2018; Iwasaki and Medzhitov 2015).

The delivery mechanism of liposomes further influences the innate immune response, as illustrated in Figure 3. Tahtinen et al. (2022) demonstrated that empty LNPs (eLNPs) alone can stimulate cytokine production, functioning as immune potentiators. This indicates that innate immune activation depends not only on the presence of mRNA but also on the lipid composition and vaccine formulation. For example, SM-102 LNPs were found to induce higher IL-1β secretion than other lipid formulations, emphasizing the role of specific lipid components in modulating immune responses.

However, excessive activation of these pathways may lead to systemic inflammation, cytokine storms, and hypersensitivity reactions, including anaphylaxis, which has been associated with PEGylated lipids (Risma et al. 2021). Additionally, the overproduction of type I interferons can suppress mRNA translation, thereby reducing vaccine efficacy (Crow et al. 2019).

Adaptive immunity and liposomes

The adaptive immune response triggered by liposome-based mRNA COVID-19 vaccines involves strong activation of both T cells and B cells (Lee et al. 2023).

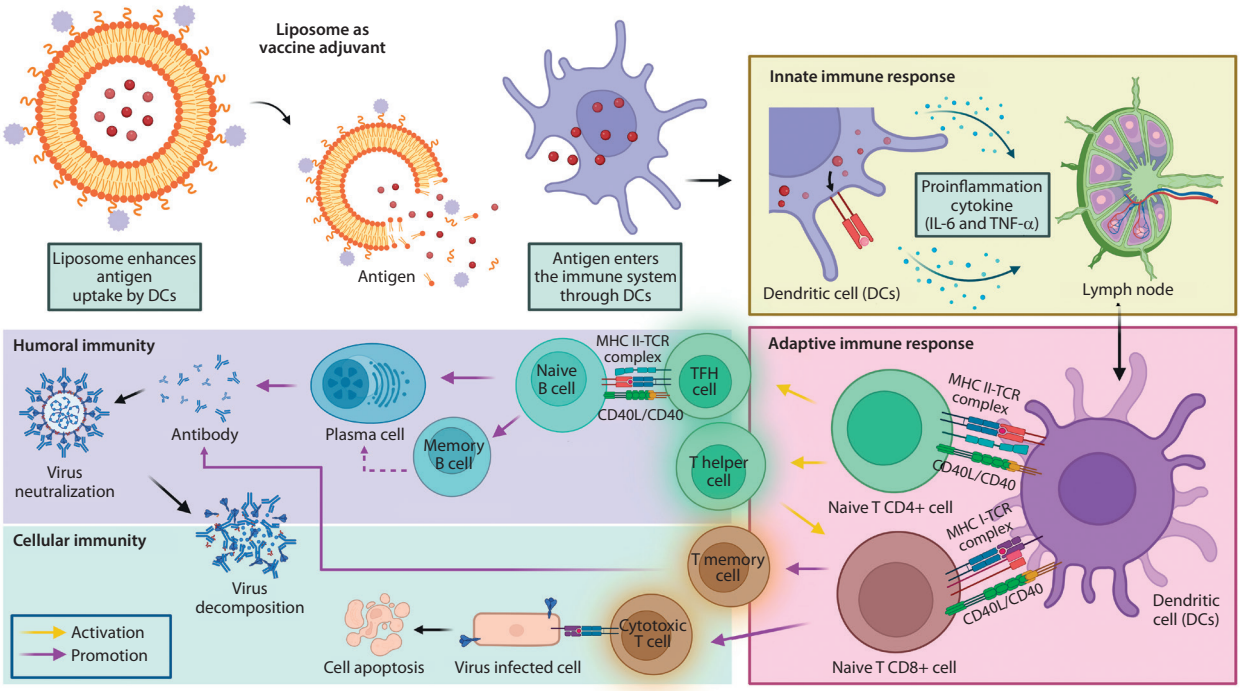

Figure 3. Immune response mechanisms following intramuscular vaccine injection of liposome as an adjuvant. Upon vaccination, antigens are presented on the surface of dendritic cells via major histocompatibility complex (MHC) molecules right after liposomes are ingested, producing inflammatory cytokines, thus called an innate immune response. Liposomes as adjuvants promote lymphocytes within the lymph node, which activate naďve CD4+ and CD8+ T cells. The process initiated an adaptive immune response. CD4+ and CD8+ T cells were the precursors of T follicular helper (TFH) cells, which promote B cell expansion and differentiation, while T helper cells assist in the activation of T memory cells as well as cytotoxic T cells, respectively. Created in https://BioRender.com

These vaccines deliver mRNA encoding specific antigens, which are processed and presented by APCs, thereby promoting $CD8^+$ T cell responses. For example, Keshari et al. (2024) investigated a neoantigen mRNA vaccine in preclinical models and demonstrated its ability to stimulate neoantigen-specific T cell responses.

Liposome-based mRNA vaccines also enhance the differentiation of T follicular helper (TFH) cells, which are critical for B cell activation and antibody production. Among the key cytokines involved in this process, IL-6 plays an essential role in TFH cell differentiation, ensuring a strong humoral immune response (Korn and Hiltensperger 2021; Li et al. 2018).

Furthermore, mRNA vaccines play a role in inducing memory responses that could create long-lasting immunity. The route of administration also plays a role in adaptive immunity since intramuscular injections promote systemic T and B cell activation. Meanwhile, intravenous administration demonstrates enhanced antigen-specific $CD8^+$ T cell responses through RNA-lipoplex (RNA-LPX) vaccines, inducing robust activation of antigen-specific $CD8^+$ T cells, promoting their expansion to comprise up to 60% of the total $CD8^+$ population. These cells also exhibit full effector functions, including the production of IFNγ, TNFα, granzyme B, and expression of degranulation markers such as CD107a/b. Additionally, RNA-LPX vaccination supports forming memory $CD8^+$ T cells capable of rapid recall responses upon antigen re-exposure.

Importantly, the acquisition of effector function depends on type I interferon signaling, as blockade of the IFNAR1 impairs cytotoxic activity despite normal $CD8^+$ T cell expansion (Kranz et al. 2016).

However, the strong immune stimulation can sometimes skew responses, leading to autoimmune reactions or an imbalanced immune profile. Consequently, variability in lipid composition and delivery routes can affect the consistency of neutralizing antibody and T-cell responses. While liposome-based mRNA vaccines have demonstrated remarkable efficacy, these challenges highlight the need for continued optimization to minimize

adverse effects and enhance therapeutic outcomes (Lee et al. 2023).

Applications of liposomes as adjuvant for SARS-CoV-2 vaccines

The rapid development of COVID-19 vaccines during 2020–2021 was propelled by advancements in mRNA technology over the past two decades. Unlike traditional vaccines that deliver inactivated or attenuated viruses, mRNA-based vaccines use messenger RNA to instruct human cells to produce the spike protein of SARS-CoV-2, thereby triggering an immune response (Krasnopolsky and Pylypenko 2022). Liposomes played a critical role in this innovation by protecting the fragile mRNA and facilitating its delivery into cells. This approach – exemplified by the Pfizer-BioNTech and Moderna vaccines – set a new standard in biotechnology by achieving high efficacy while minimizing risks such as oncogenesis (Jackson et al. 2020; Krasnopolsky and Pylypenko 2022).

Liposomes composed of ionizable lipids, cholesterol, and other stabilizing molecules are pivotal for ensuring mRNA vaccine stability, transport, and efficacy. Advances in liposome engineering – including novel mixing methods and lipid compositions – enhance vaccine performance by improving antigen presentation and immune activation. For example, the EG-COVID candidate in South Korea has explored lyophilized lipid systems (LSs) to improve storage and distribution conditions (Hong et al. 2021). Another example includes Pfizer-BioNTech and Moderna vaccines, which utilize liposomes as the mRNA vaccine's adjuvant, licensed and used worldwide, which has been shown in Table 1. In addition to supporting mRNA vaccines, liposomes also enhance the performance of protein subunit vaccines. Numerous liposomal subunit SARS-CoV-2 vaccines are currently under clinical investigation. For example, a ferritin-based vaccine – which self-assembles the SARS-CoV-2 WA-1 spike glycoprotein with a unilamellar liposomal adjuvant containing monophosphoryl lipid A and saponin QS-21 (ALFQ) – elicited robust antibody responses in a Phase 1, first-in-human trial (Ober Shepherd et al. 2024).

Another example is EuCorVac-19 (ECV-19), which uses adjuvanted liposomes combined with the compact receptor binding domain (RBD) of the SARS-CoV-2 spike protein (Mabrouk et al. 2021). Following the recently reported interim Phase 2 trial results, the latest research concluded that the antibody durability of ECV-19 was induced. However, a further Phase 3 trial of ECV-19 is necessary to assess a vast and larger study of the observed antibody responses (Lovell et al. 2024). The development of several SARS-CoV-2 subunit vaccines in a mouse infection model was also being conducted. The first vaccine has a dual TLR ligand liposome adjuvant. It has been proven to protect mice from infection by eliciting local antispike IgA upon challenge (Abhyankar et al. 2021). According to Christensen et al. (2022) that the intranasal boost vaccine (a cationic liposomal adjuvant formulated with Spike Hexa-Pro trimer) was effective against SARS-CoV-2 infection due to the high-magnitude serum-neutralizing antibody responses in the upper respiratory tract of a Syrian hamster model. In addition to this, Ho et al. (2022) formulated a nasal spray vaccine using CpG oligodeoxynucleotides (ODNs) and squalene nanoparticles (PELC) as adjuvants in mice. This approach enhanced APC activation in cervical lymph nodes and significantly improved SARS-CoV-2-specific IgG and IgA antibody production, supporting both systemic and mucosal immunity.

These developments demonstrate the adaptability, versatility, and efficacy of liposome-based and nanoparticle-based delivery systems in modern vaccine design and highlight their critical role in ongoing and future immunization strategies.

The success of liposomal mRNA vaccines during the COVID-19 pandemic has marked a revolutionary advancement in pharmaceutical science. These vaccines achieved efficacy rates of up to 95% and significantly reduced the incidence of severe disease among vaccinated individuals. This innovation has sparked global discussions on the future of biotechnological applications, including cancer immunotherapy and treatments for other infectious diseases. By leveraging the synergistic power of vaccines, nanoparticles, and liposomes, researchers are laying the foundation for next-generation therapeutics. The SARS-CoV-2 pandemic has thus become a defining milestone in the evolution of vaccine technology.

Opportunities and challenges in liposomal applications

Liposomes are among the most extensively studied nanocarrier systems in drug delivery, owing to their

exceptional physicochemical and biological properties (Yan and Huang 2007). One of their most significant advantages is their high biocompatibility, which is attributed to the use of natural or synthetic phospholipids that closely resemble the lipids found in biological membranes (Daraee et al. 2016; Perrie 2008). This structural similarity reduces immunogenicity and cytotoxicity, ensuring that liposomes are biodegradable and safely metabolized within the body. These characteristics make them particularly well-suited for repeated or long-term administration (Haensler 2010; Zamani et al. 2018).

Another crucial advantage is their ability to encapsulate a wide variety of therapeutic agents, including both hydrophilic and hydrophobic molecules (Dimov et al. 2017; Lamichhane et al. 2018; Lombardo et al. 2019; Zununi Vahed et al. 2017). Hydrophilic drugs are retained in the aqueous core of the vesicle, while hydrophobic compounds are incorporated into the lipid bilayer. This dual-loading capacity broadens the range of therapeutic applications, making liposomes especially useful for combination therapy, where multiple drugs with differing solubility profiles must be co-delivered (Riaz et al. 2018; Tyagi et al. 2017; Xing et al. 2016).

In addition, liposomes can be engineered to provide controlled and sustained drug release. By altering the lipid composition, bilayer rigidity, and surface characteristics, researchers can fine-tune the release profile of encapsulated drugs to maintain therapeutic concentrations over extended periods (Gatto et al. 2024; Gregoriadis 2016; Pattni et al. 2015). Such controlled release helps reduce dosing frequency and enhances patient compliance, which is particularly beneficial in chronic disease management (Gonzalez Gomez and Hosseinidoust 2020; Poon and Patel 2020).

Liposomes also significantly improve drug bioavailability, especially for poorly soluble or labile drugs, by shielding them from enzymatic degradation, chemical instability, or rapid clearance from circulation (Alrbyawi et al. 2022; Gonzalez Gomez et al. 2019; Huang and Anderson 2002). Furthermore, their ability to cross biological barriers – such as the BBB – enhances systemic absorption and therapeutic outcomes (dos Santos Rodrigues et al. 2018; Shi et al. 2019).

Another critical advantage of liposomal delivery is the reduction of systemic side effects. Through passive targeting mechanisms – such as the enhanced permeability and retention (EPR) effect in tumors or inflamed tissues – and active targeting via ligand modification, liposomes can preferentially accumulate at the site of disease (Belfiore et al. 2018; Boons 2010; Liu et al. 2020; Noble et al. 2014; Riaz et al. 2018; Wang et al. 2023). This targeted delivery minimizes drug exposure to healthy tissues, thereby reducing off-target toxicity, a common limitation of conventional drug formulations. This property is particularly valuable in oncology and other therapeutic areas requiring high-potency treatments.

Collectively, these advantages highlight the significant potential of liposomes to improve the therapeutic index of drugs, enhance treatment efficacy, and provide safer alternatives in clinical practice (Kim and Jeong 2021; Nsairat et al. 2022).

Despite their promise, liposomes face several challenges that may limit their broader clinical application. One major limitation is stability – liposomes are susceptible to drug leakage, aggregation, and degradation during storage (Gregoriadis 2016; Pasarin et al. 2023). On the manufacturing front, liposome production involves complex, multistep processes that are difficult to scale up while maintaining batch-to-batch consistency in size, encapsulation efficiency, and product quality (Bochicchio et al. 2020; Perrie et al. 2017).

Another concern is immunogenicity, particularly with repeated dosing or cationic liposomal formulations, which can provoke unwanted immune responses or accelerate clearance by the mononuclear phagocyte system (Ochoa-Sánchez et al. 2024; Ponti et al. 2021; Tada et al. 2015). Although PEGylation is widely used to extend circulation time, it can induce the formation of anti-PEG antibodies, contributing to the accelerated blood clearance phenomenon (Mohamed et al. 2019; Nosova et al. 2019; Ren et al. 2019; Shen et al. 2018).

Addressing these issues is essential to fully realizing the clinical utility of liposomal drug delivery systems.

Future directions and perspectives

The pivotal role of liposomes in the development of SARS-CoV-2 vaccines has underscored their broader potential in medicine (Daraee et al. 2016; Perrie 2008). Future research should focus on optimizing lipid composition, improving liposomal stability, minimizing adverse immune reactions, and advancing large-scale manufacturing techniques. Developing multifunctional liposomes capable of co-delivering both antigens and

immunostimulants may further enhance vaccine efficacy and broaden their immunological reach.

In addition, expanding the application of liposomes beyond intramuscular mRNA vaccines to intranasal or oral delivery systems could revolutionize mucosal immunity and improve vaccine accessibility, particularly in low-resource settings. Liposomes also hold immense promise in gene therapy, cancer immunotherapy, and the delivery of CRISPR/Cas9 systems, where precise, targeted delivery is essential to enhance specificity and minimize off-target effects (Zhen et al. 2017; Zhen and Li 2020).

Despite their versatility, key challenges remain, including liposomal stability, immunogenicity, and manufacturing complexity (Gregoriadis 2016; Pasarin et al. 2023). Future efforts should prioritize the design of biodegradable, immune-tolerant liposomal systems that maintain efficacy while reducing toxicity.

Ultimately, sustained interdisciplinary collaboration across immunology, materials science, and nanotechnology will be critical to fully unlocking the potential of liposomes in preventive and therapeutic medicine.

Conclusions

Liposomes exhibit remarkable versatility and potential in biomedical applications, particularly as adjuvants in vaccine development. Their unique structure and composition make them effective delivery systems that enhance vaccine stability, immunogenicity, and targeted delivery. The success of mRNA vaccines, such as those developed by Pfizer and Moderna for COVID-19, has demonstrated the critical role of liposomal formulations in protecting mRNA, facilitating cellular delivery, and activating robust innate and adaptive immune responses.

Despite these advantages, challenges remain – including the need to optimize lipid compositions, minimize adverse effects, and ensure consistent immune responses across populations. As research advances, liposomes continue to demonstrate strong potential not only as drug delivery systems but also as innovative platforms for enhancing vaccine efficacy and addressing a wide range of infectious diseases and medical conditions.

Author contributions

Supervision, review concept, and design: I.P.N. Collection and/or assembly data: A.K., and I.P.N. Data analysis and interpretation: A.K., I.P.N., and T.H.C. Writing the article: A.K., and I.P.N. Critical revision of the article: I.P.N., and T.H.C. Review & Editing, English Proof-read: A.N.S., T., and T.H.C. Final approval of the article: I.P.N. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Gene expression, purification, and functional characterization of recombinant conotoxin μ-TIIIA and TIIIAlaMut in *Escherichia coli* with clinical evaluation of antiwrinkle efficacy

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Abstract

Background: Conotoxins are small peptides known for their potent and selective activity on ion channels, offering potential applications in both medicine and cosmetology. This study aimed to design and validate recombinant conotoxin TIIIA and its mutant TIIIAlaMut, assess their biological activity on the voltage-gated Na^+ (Nav) channel Nav1.4, and evaluate the antiwrinkle efficacy of a topical cream containing the recombinant peptide in a group of volunteers.

Materials and methods: Fusion genes encoding *TRX::TIIIA* and *TRX::TIIIAlaMut* were cloned into the pDM vector and expressed in *Escherichia coli* S4B cells. The proteins were purified using Ni-NTA chromatography, cleaved with CNBr under optimized acidic conditions, and analyzed. Biological activity was assessed using two-electrode voltage-clamp electrophysiology in *Xenopus laevis* oocytes expressing the human Nav1.4 channel. Additionally, a conotoxin-containing cream was applied to 55 human volunteers in an application study assessing its antiaging effects.

Results: Both recombinant genes were successfully expressed, purified, and activated. Electrophysiological measurements demonstrated their ability to inhibit Nav1.4 channel activity, including the version extracted directly from the cream. In the human study, 47% of participants reported a visible reduction in wrinkles. Additional benefits included evening of skin tone, reduced erythema, and balanced sebum production in oily skin types.

Conclusion: This study describes the design, bacterial expression, and functional analysis of recombinant conotoxins TIIIA and TIIIAlaMut. Their bioactivity was confirmed on human Nav1.4 channels. The recombinant toxins, including the form extracted from the cream, showed effects comparable to a synthetic standard. Application tests demonstrated the conotoxin's potential in cosmeceuticals, particularly in reducing periocular wrinkles and improving skin texture and tone.

Key words: conotoxins, recombinant protein production, *Escherichia coli* expression system, patch-clamp method, antiaging activity

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Introduction

Conotoxins are natural compounds found in the venom of cone snails from the *Conidae* family, which use them to immobilize and paralyze their prey (Terlau and Olivera 2004). These snails typically inhabit coral reefs and are predominantly found in tropical and subtropical waters, including the South China Sea, the Australian coast, and the Pacific Ocean. There are approximately 700 species of cone snails, all of which are venomous. Based on their dietary preferences, they can be categorized as worm hunters, mollusk hunters, or fish hunters (Olivera 1997; Duda et al. 2001).

Conotoxins are specialized peptides designed to target ion channels, receptors, and transporters in their prey, ensuring rapid immobilization. Their primary mode of action involves modulating or blocking ion channels, such as voltage-gated Na^+ (Nav), K^+ (Kv), and Ca^{2+} (Cav) channels, as well as ligand-gated ion channels like nicotinic acetylcholine receptors (Lewis et al. 2012). These peptides are structurally diverse and highly selective, having evolved to efficiently capture prey and defend against predators (Bergeron et al. 2013).

Due to their high specificity, conotoxins have become valuable tools for studying ion channels and hold potential therapeutic applications, particularly in targeting specific ion channels and glucose transporters. Ion channels are membrane proteins that regulate the movement of ions across the cell membrane and play crucial roles in both excitable and non-excitable cells, including neurons, muscle cells, renal tubules, and epithelial tissues.

Conotoxins are microproteins, typically under 40 amino acids in length, which facilitate their recombinant expression (Duque et al. 2019). They often form multiple disulfide bonds that stabilize their bioactive conformation, enhancing their potency, selectivity, and resistance to enzymatic degradation. A single cone snail's venom may contain up to 100 different peptides, each serving a distinct function and collectively producing a potent effect on prey. Based on their molecular targets, conotoxins are classified into several types: ω-conotoxins block Cav channels to inhibit neurotransmitter release; α- and ψ-conotoxins block nicotinic acetylcholine receptors, causing neuromuscular blockade; μ- and δ-conotoxins target Nav channels in muscles; κ-conotoxins block Kv channels, increasing neuronal excitability; γ-conotoxins affect cation channels; and σ-conotoxins act as antagonists of serotonin 5HT3 receptors (Mir et al. 2016).

Due to their remarkable specificity, conotoxins are valuable biological tools for distinguishing closely related receptors, making significant contributions to neuroscience research. They have also demonstrated promise in pharmaceutical and cosmetic applications (Becker and Terlau 2008; Del Rio-Sancho et al. 2017; Pope and Deer 2013; Ramirez et al. 2017; Sun et al. 2019). Because many neurological and systemic disorders – such as epilepsy, schizophrenia, Tourette syndrome, Parkinson's disease, and multiple sclerosis – are linked to malfunctioning ion channels, the small size, high potency, and selectivity of conotoxins position them as strong candidates for developing therapeutic and cosmetic solutions (Armishaw and Alewood 2005; Clark et al. 2010; Layer and McIntosh 2006; Miljanich 2004; Netirojjanakul and Miranda 2017).

For further research, we selected conotoxin TIIIA due to its myorelaxant properties, which result from the specific blockade of skeletal muscle Nav1.4 channels. This unique characteristic holds potential for application in cosmetology as part of daily anti-wrinkle therapy.

In this study, we aimed to obtain active recombinant forms of the conotoxin TIIIA and its alanine-substituted mutant TIIIAlaMut using a bacterial expression system. These peptides were subsequently incorporated into the formulation of an antiwrinkle cream intended to counteract skin aging processes at the molecular level.

Materials and methods

DNA manipulation, transformation, and sequencing

DNA restriction, ligation, and gel electrophoresis were performed using standard techniques (Sambrook et al. 1989). All bacterial transformations with plasmid DNA were carried out by electroporation using 1 mm cuvettes (BTX) and a MicroPulser™ electroporator (Bio-Rad, US). Electrocompetent *Escherichia coli* DH5α (New England Biolabs, UK Ltd.) and *E. coli* S4B cells (Mazurkiewicz-Pisarek et al. 2023; WO/2025/057018, this work) were prepared using standard procedures (Sambrook et al. 1989). The gene encoding conotoxin μ-TIIIA was synthesized by GenScript (Rijswijk, Netherlands).

All *E. coli* strains were propagated in LB broth (tryptone 10.0 g/l, yeast extract 5.0 g/l, NaCl 5.0 g/l, pH 7.2–7.5), supplemented with tetracycline (100 μg/ml). Plasmid DNA was isolated using the Plasmid Mini Isolation Kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. All restriction enzymes,

ligase, and DNA ladders were purchased from New England Biolabs (UK Ltd.) and used according to the manufacturer's instructions. A prestained protein molecular weight marker was purchased from GE Healthcare (UK). The correctness of DNA sequences was confirmed by sequencing (Genomed, Poland).

Construction of TRX::TIIIA and TRX::TIIIAlaMut fusion genes

Construction of TRX::TIIIA fusion gene

To obtain a recombinant soluble conotoxin TIIIA protein, a genetic construct encoding the fusion protein TRX::TIIIA was designed. This construct included the gene encoding conotoxin TIIIA and the gene encoding the leader protein thioredoxin (TRX). The TRX protein provides reducing conditions that facilitate the correct folding of proteins containing disulfide bridges. It frequently enables recombinant proteins to be expressed in a soluble form, significantly simplifying the purification process.

The nucleotide sequence of the *TRX* thioredoxin gene was modified using site-directed mutagenesis to replace the amino acid methionine (M) at position 37 with lysine (K). This modification ensured appropriate protein fragments after cleavage with cyanogen bromide (CNBr), which specifically cleaves at methionine residues.

The nucleotide sequence of the *TRX::TIIIA* fusion gene was optimized for bacterial codon usage and ordered from GenScript (Rijswijk, Netherlands). Restriction sites (NdeI, XbaI) were added. To enable protein purification using a Ni-NTA chromatography column, a sequence encoding six histidines (6His) and a short linker consisting of serine-glycine-serine (SGS) was added to the 5′ end of the construct. The *TRX::TIIIA* fusion gene was inserted into the pDM expression vector (Mazurkiewicz-Pisarek et al. 2023; WO/2025/057018), digested with NdeI/XbaI. The nucleotide sequences of the cloned genes were verified.

Construction of TRX::TIIIAlaMut fusion gene

The recombinant fusion gene *TRX::TIIIAlaMut* was generated from the *TRX::TIIIA* gene using site-directed mutagenesis. A specific mutation was introduced to replace the glutamic acid (E) residue at position 15 with alanine (A). The nucleotide sequence of the modified gene was confirmed by sequencing. Schematic diagrams of the genetic constructs and amino acid sequences are presented in Figure 1.

Construction of the E. coli expression strains

Construction of pDM/TRX::TIIIA plasmid

The expression vector pDM (a derivative of plasmid pBR322) was digested with NdeI/XbaI and ligated with a 428 bp NdeI/XbaI insert encoding the hybrid protein TRX::TIIIA. The codon usage of the hybrid gene was optimized for expression in *E. coli*. Transcription initiation in the constructed pDM/TRX+TIIIA plasmid is regulated by the *deoP1P2* promoter and includes a tetracycline resistance marker.

The final plasmid, named pDM/TRX+TIIIA, was used to transform the *E. coli* S4B strain developed in the Science4Beauty LLC laboratory. Figure 2 illustrates the strategy used to construct the pDM/TRX+TIIIA expression vector. A production strain of *E. coli* S4B

Figure 1. Schematic diagrams of (**A**) *TRX::TIIIA* and (**C**) *TRX::TIIIAlaMut* genetic constructs, and amino acid sequences of (**B**) TRX::TIIIA and (**D**) TRX::TIIIAlaMut. The amino acid methionine is marked in red, and the amino acid lysine is marked in yellow. The sequence including 6His and the SGS linker is shown in bold and underlined. The modified amino acid in TRX::TIIIAlaMut is highlighted in green

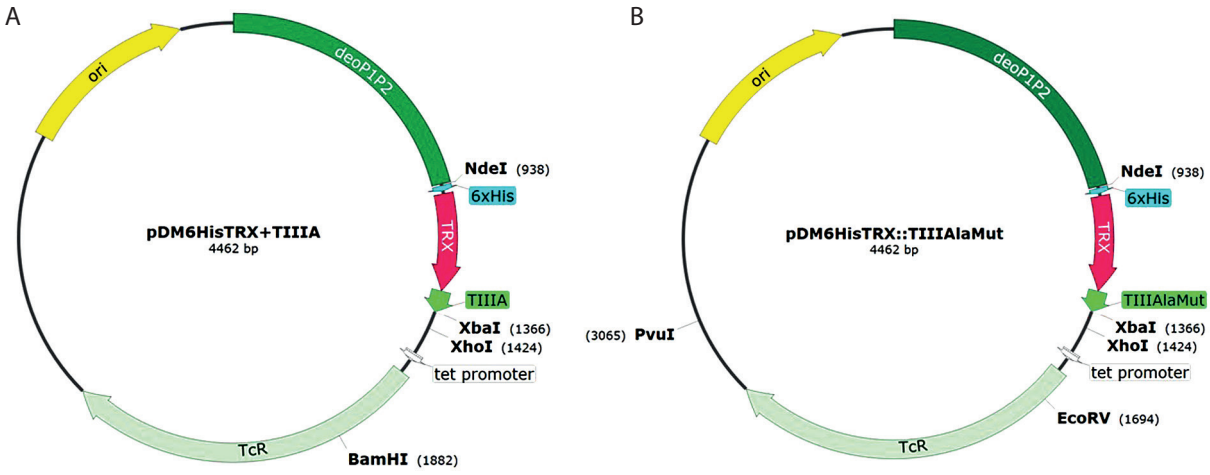

Figure 2. Construction scheme of the expression plasmids pDM/TRX+TIIIA and pDM/TRX+TIIIAlaMut. **A**) pDM/TRX+TIIIA contains the hybrid *TRX:TIIIA* gene under the *deoP1P2* promoter. **B**) pDM/TRX+TIIIAlaMut contains the hybrid *TRX:TIIIAlaMut* gene. Abbreviations: *TRX* – thioredoxin gene, *TIIIA*, *TIIIAlaMut* – conotoxin genes, tet promoter – tetracycline promoter, *TcR* – tetracycline resistance, ori – origin of replication

containing the pDM/TRX+TIIIA construct – where the conotoxin *TIIIA* gene is fused to the thioredoxin leader protein gene, enabling the production of the target protein in a soluble form – was created.

Work was then carried out to optimize the culture conditions for the newly developed genetic construct and bacterial strain to achieve the highest possible expression of the recombinant conotoxin *TIIIA* gene.

Construction of pDM/TRX::TIIIAlaMut plasmid

The recombinant plasmid pDM/TRX::TIIIAlaMut was constructed using the previously prepared *TRX::TIIIAlaMut* fusion gene. The final plasmid retained all regulatory elements of the parent vector, including the *deoP1P2* promoter and the tetracycline resistance marker. The codon usage of the *TRX::TIIIAlaMut* gene was optimized for expression in *E. coli*. The verified construct was introduced into the production strain *E. coli* S4B, developed at Science4Beauty LLC, for recombinant expression of the soluble TRX-fused conotoxin mutant. The construction scheme of the final pDM/TRX::TIIIAlaMut expression vector is shown in Figure 2.

Cell culture and purification of TRX+TIIIA and TRX::TIIIAlaMut fusion proteins

Purification of TRX+TIIIA fusion proteins

The prokaryotic expression vector pDM/TRX+TIIIA was introduced by electroporation into the *E. coli* S4B expression strain. On a laboratory scale, *E. coli* S4B strains were grown in LB broth supplemented with tetracycline (100 μg/ml) at 30°C, 150 rpm, for 18 h, reaching an OD_{600} of 3.2 to 3.5. Cultures were inoculated using stock material stored at –70°C (500 μl stock per 500 ml LB medium). Bacterial stocks were deposited in the strain bank collection at Science4Beauty LLC and prepared by mixing bacterial culture ($OD_{600} \approx 0.8$) with 20% glycerol in a 1:1 ratio.

After 18 h of growth, the cultures were centrifuged (10 min at 8,000 rpm). The biomass from 1 l of culture was resuspended in 50 ml dissolution buffer (50 mM Tris-HCl, pH 7.8; 300 mM NaCl), sonicated (40% amplitude, 15 s on/5 s off, for 75 min, on ice), and centrifuged twice (15 min at 11,500 rpm). The clarified supernatant was applied to a Ni-NTA affinity column pre-equilibrated with calibration buffer.

The column was washed with wash buffer, and the TRX+TIIIA recombinant protein was eluted using elution buffer. The flow rate during sample loading was 1.0 ml/min; washing was performed at 1.5 ml/min; and elution was at 2.0 ml/min. Buffers used for protein purification – calibration buffer: 50 mM phosphate buffer (pH 7.8), 500 mM NaCl, 10 mM imidazole; Wash buffer: 50 mM phosphate buffer (pH 7.8), 500 mM NaCl, 20 mM imidazole; Elution buffer: 50 mM phosphate buffer (pH 7.8), 500 mM NaCl, 150 mM imidazole. Fractions were collected in 5 ml increments. Protein separation was performed on a Bio-Rad DuoFlow system using a chromatography column from the same manufacturer.

After elution, 4 mM GSH (reduced glutathione) and 1 mM GSSG (glutathione disulfide, the oxidized form) were added to the collected fractions to ensure proper formation of disulfide bridges.

Purification of TRX+TIIIAlaMut fusion proteins

The expression plasmid pDM/TRX::TIIIAlaMut was introduced into the *E. coli* S4B strain by electroporation. Cultures were grown in LB medium supplemented with tetracycline (100 μg/ml) at 30°C and 150 rpm for 18 h, reaching an OD_{600} of 3.2–3.5. Inoculation was performed using 500 μl of frozen stock ($OD_{600} \approx 0.8$, stored in 20% glycerol at –70°C) per 500 ml of medium. These strains are maintained at –70°C in the Science4Beauty LLC strain bank. After growth, cells from 1 l of culture were harvested at 8,000 rpm, resuspended in lysis buffer (50 mM Tris-HCl, pH 7.8; 300 mM NaCl), and sonicated under controlled conditions (40% amplitude, 15 s on and 5 s off, for 75 min, on ice). The lysate was centrifuged twice at 11,500 rpm for 15 min, and the resulting supernatant was applied to a Ni^{2+}-affinity column (Bio-Rad DuoFlow System) equilibrated with binding buffer. Purification was performed using standard buffers: binding buffer (50 mM phosphate, 500 mM NaCl, 10 mM imidazole), wash buffer (same buffer with 20 mM imidazole), and elution buffer (with 150 mM imidazole). Elution was carried out at a flow rate of 2.0 ml/min, and fractions were collected every 5 ml. Following purification, 4 mM GSH and 1 mM GSSG were added to the eluted protein to facilitate correct disulfide bond formation.

Cleavage of the TRX::TIIIA and TRX::TIIIAlaMut fusion proteins using CNBr

The purified recombinant fusion proteins were dialyzed for 48 h at 4°C against a buffer containing 50 mM Tris-HCl (pH 7.8) and 10% glycerol, using dialysis tubing with a molecular weight cutoff of 12–14 kDa. The dialysis buffer was replaced after 24 h. CNBr digestion was performed under acidic conditions, as the reagent specifically cleaves at methionine residues. CNBr reacts with the sulfur atom in the methionine side chain, resulting in cleavage of the peptide bond on the carboxyl side. Because methionine is among the least abundant amino acids in proteins, this method allows precise, targeted cleavage of the fusion proteins (Andreev et al. 2010). To separate the recombinant conotoxins from the thioredoxin (TRX) leader protein, cleavage was carried out using a 100:1 molar ratio of CNBr to methionine residues under acidic conditions (Gross and Witkop 1962; Inglis and Edman 1970). The TRX::TIIIA and TRX::TIIIAlaMut fusion proteins each contain two methionine residues, providing the specific cleavage sites required for this reaction. The amount of CNBr used in each reaction was calculated based on the molar mass of the individual fusion proteins. All procedures related to gene construction, expression strain development, and the production of active recombinant peptides are described in the corresponding patent application WO/2025/057018 (Mazurkiewicz-Pisarek et al. 2024).

Electrophysiological testing on Nav1.4 channel genes expressed in Xenopus laevis oocytes

The activity of the recombinant conotoxins was assessed using two-electrode voltage-clamp electrophysiology on Nav1.4 ion channels expressed in *Xenopus laevis* oocytes (Leipold and Olivera 2018; McIntosh et al. 1999). The *Xenopus* oocyte expression system is well-suited for electrophysiological studies of voltage-gated ion channels due to its low background of endogenous channels and the large size of the oocytes (Dascal 1987). The human Nav1.4 (hNav1.4; NM_000334.4, OriGene Technologies, USA) gene was co-expressed with the human β1-subunit (NM_001037.5, GenScript, USA) at a 1:5 molar ratio by microinjecting capped RNA (cRNA) into defolliculated oocytes. Electrophysiological recordings were performed 1–2 days after injection. Oocytes were maintained at 17 °C in Barth's medium (88 mM NaCl, 1 mM KCl, 5 mM HEPES, 2.4 mM $NaHCO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, and 50 μg/ml gentamycin, pH adjusted to 7.4 with NaOH).

Channel kinetics were examined using a two-electrode voltage-clamp setup (OC-725C, Warner Instruments, USA) with a 150 μl recording chamber. Data were filtered at 4 kHz and digitized at 20 kHz using pClamp10 software (Molecular Devices, USA). Microelectrodes filled with 3 M KCl had resistances of 0.5–1 MΩ. The external ND100 recording solution contained 100 mM NaCl, 5 mM HEPES, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$ at pH 7.6 (adjusted with NaOH). All experiments were conducted at room temperature (~21°C). Leak and background conductances, identified by blocking Nav channels with tetrodotoxin (TTX), were subtracted from all current recordings. Voltage-activation relationships were obtained from

peak currents, and conductance (G) was calculated and fit with a Boltzmann function using the equation: $G/G_{max} = [1 + e^{-zf}(V–V_{1/2})/RT]^{-1}$, where G/G_{max} is the normalized conductance, z is the equivalent charge, $V_{1/2}$ is the half-activation voltage, F is Faraday's constant, R is the gas constant, and T is the absolute temperature. Offline data analysis was performed using Clampfit10 (Molecular Devices, USA), Excel (Microsoft Office, USA), and Prism 8 (GraphPad, USA).

Clinical efficacy testing

This application study was conducted between May 2023 and August 2023 and was approved by the Bioethics Committee at the District Medical Chamber in Warsaw in June (protocol code 1444/23 KB). The study was designed to assess the efficacy and user satisfaction of a novel cosmetic cream formulation containing the active ingredient conotoxin TIIIA. The cream was developed specifically for this research, and its complete composition is described in the corresponding patent application WO/2025/052257 (Janczewska et al. 2023). This *in vivo* study focused on evaluating both the performance and the user experience associated with the cream.

Treatment protocol

A total of 55 healthy adult volunteers (37 women and 18 men, aged 25–55 years; average age 40.6) were anticipated for recruitment. All participants provided written informed consent before enrolment. Each volunteer was pre-screened by a licensed physician to confirm eligibility based on the study's inclusion and exclusion criteria.

Inclusion criteria

To qualify for participation in the study, the following inclusion criteria were applied: 1) age 25–55 years, 2) good general health, 3) written consent to participate in the study and acceptance of its procedures.

Exclusion criteria

To ensure the safety and accuracy of the clinical trial involving the cosmetic cream, it was essential to establish exclusion criteria. These criteria helped identify participants at risk of adverse reactions or whose involvement could compromise the reliability of the study results. The exclusion criteria for this study were as follows: 1) taking oral retinoids within the past 6 months, 2) skin and connective tissue diseases (e.g., systemic lupus erythematosus, collagenopathy, cutaneous porphyria), 3) active or frequently recurring Herpes simplex infection (cold sores), 4) use of medications that may affect skin condition (including tetracycline antibiotics, immunosuppressants such as cortisone and its derivatives, and anticoagulants such as dipyridamole and coumarin derivatives) within the past 6 months, 5) immunocompromised conditions (including active HIV infection), 6) pregnancy and breastfeeding, 7) uncontrolled hypertension, 8) unregulated diabetes, 9) vitiligo or disorders of melanin production (e.g., hypermelanosis), 10) tattoos in the treated areas, 11) constant use of anti-inflammatory medications, 12) history of allergic reactions to ingredients of the tested cosmetic formulations, 13) tendency to develop scarring, or having undergone aesthetic medicine or cosmetic surgery procedures within 4 weeks before or during the study.

All clinical assessments, surveys, and imaging procedures were conducted by trained personnel experienced in dermatological evaluation. Skin condition was assessed using high-resolution 3D imaging systems, specifically the VECTRA® H2 (Canfield Scientific, USA) or FOTOMEDICUS (ELFO®, Poland), both of which enable quantitative analysis of wrinkles and skin tone.

Each study participant received two coded products labeled "A" and "B". Participants were instructed to apply product A for 4 weeks, followed by product B for an additional 4 weeks. Product A served as the placebo formulation, while product B differed only in the presence of the active ingredient conotoxin TIIIA; all other excipients and formulation parameters were identical. Between the two application phases, an intermediate clinical assessment was performed, including high-resolution photographic documentation, 3D imaging using the VECTRA® H2 (Canfield Scientific, USA) or FOTOMEDICUS (ELFO®, Poland) system, and a standardized user satisfaction questionnaire.

This within-subject design allowed each participant to serve as their own control, enabling a direct comparison of the placebo and active formulations while minimizing inter-individual variability. Participants remained blinded to the identity of each product throughout the study to reduce bias.

The cream was applied twice daily (morning and evening) to the facial skin. The application period lasted

4 weeks, with a progress questionnaire completed after 2 weeks. After 4 weeks, participants underwent clinical re-assessment and imaging. Primary outcomes included quantitative evaluation of skin texture and tone using 3D imaging and subjective assessments of product performance collected through structured questionnaires.

Methodology – image and data analysis

Image analysis was performed using specialized software capable of quantifying wrinkle depth through color-coded mapping, where red indicates deeper skin depressions and green represents shallower lesions.

Photographs taken before and after product application were analyzed by superimposing corresponding images and examining characteristic facial regions, including forehead wrinkles and periorbital lines.

Survey data were evaluated by the Principal Investigator based on anonymized participant responses to the complete set of questionnaire items.

The results were expressed as the percentage ratio of positive to negative responses, with answers marked as "hard to say" classified as neutral.

Throughout the study period, no adverse dermatological reactions or pharmacologically significant side effects were reported. Application sites remained free from irritation or inflammation, and no participants required medical treatment or withdrawal due to adverse effects.

Results

Designing of the pDM/TRX+TIIIA and pDM/TRX+TIIIAlaMut plasmids

The construction of the conotoxin expression plasmids pDM/TRX+TIIIA and pDM/TRX+TIIIAlaMut, based on the pDM vector, is illustrated in Figure 2. The DNA sequences encoding the *TRX::TIIIA* and *TRX+TIIIAlaMut* fusion genes include a modified thioredoxin fragment that enables the production of the recombinant protein in a soluble form. An *N*-terminal 6×His tag was incorporated to facilitate purification on a Ni-NTA agarose matrix. The presence and correctness of the inserts were confirmed by DNA sequencing.

Protein production and analysis

The newly constructed plasmids pDM/TRX+TIIIA and pDM/TRX+TIIIAlaMut were introduced into *E. coli* S4B electrocompetent cells by electroporation, and intracellular expression was achieved. As expected, the expressed fusion proteins accumulated in the cytoplasm in a soluble form.

Following low-pressure liquid chromatography (LPLC) purification and 48-h dialysis, the TRX+TIIIA and TRX::TIIIAlaMut fusion proteins (1.0 mg/ml each) were subjected to CNBr cleavage. The reactions were carried out under acidic conditions at +4°C and at room temperature in the dark, with continuous stirring. A 100:1 molar excess of CNBr relative to methionine residues was used. For each milligram of fusion protein, 1.375 mg of CNBr was added, corresponding to 2.60 μl of a 5 M CNBr solution in acetonitrile (CH_3CN; density 1.093 g/ml).

The digested samples were analyzed by LC-MS (liquid chromatography-mass spectrometry). Based on these analyses, the optimal digestion conditions for TRX+TIIIA were determined to be 0.1 M HCl for 3 h at room temperature, in the dark, with continuous stirring. SDS-PAGE analysis could not detect the released conotoxins due to their low molecular weight (approximately 2.6 kDa), but the digestion pattern was confirmed by LC-MS. The corresponding SDS-PAGE gel images and LC-MS chromatograms are presented in Figure 3.

Biological activity

Recombinant conotoxins TIIIA, TIIIAlaMut, conotoxin TIIIA extracted from the cream formulation, and the synthetic standard CnIIIC (Alomone Labs, Ltd., Israel) were tested on Nav1.4 channels expressed in *Xenopus laevis* oocytes at concentrations of 1 or 0.1 μM. Rapid and reproducible solution exchange (<300 ms) was achieved using a 150 μl funnel-shaped oocyte chamber combined with a vertical solution flow delivered through a collector positioned next to the oocyte. Voltage-current relationships recorded from the same oocyte before and after administration of the tested compound were compared to assess channel inhibition.

The results of conotoxin activity on *X. laevis* oocytes expressing human Nav1.4 channels, recorded using the two-electrode voltage-clamp technique, are presented in Figure 4. Conotoxin TIIIA (Toxin A) exhibited an IC_{50} value of 9.7 μM, while the alanine-substituted conotoxin TIIIAlaMut (Toxin C) showed an IC_{50} of 3.8 μM. The synthetic standard CnIIIC (Toxin D) demonstrated an IC_{50} value of 33.6 nM. No full dose-response curve was obtained for conotoxin TIIIA extracted from the cream; however, it remained active on Nav1.4 channels at 0.1 μM.

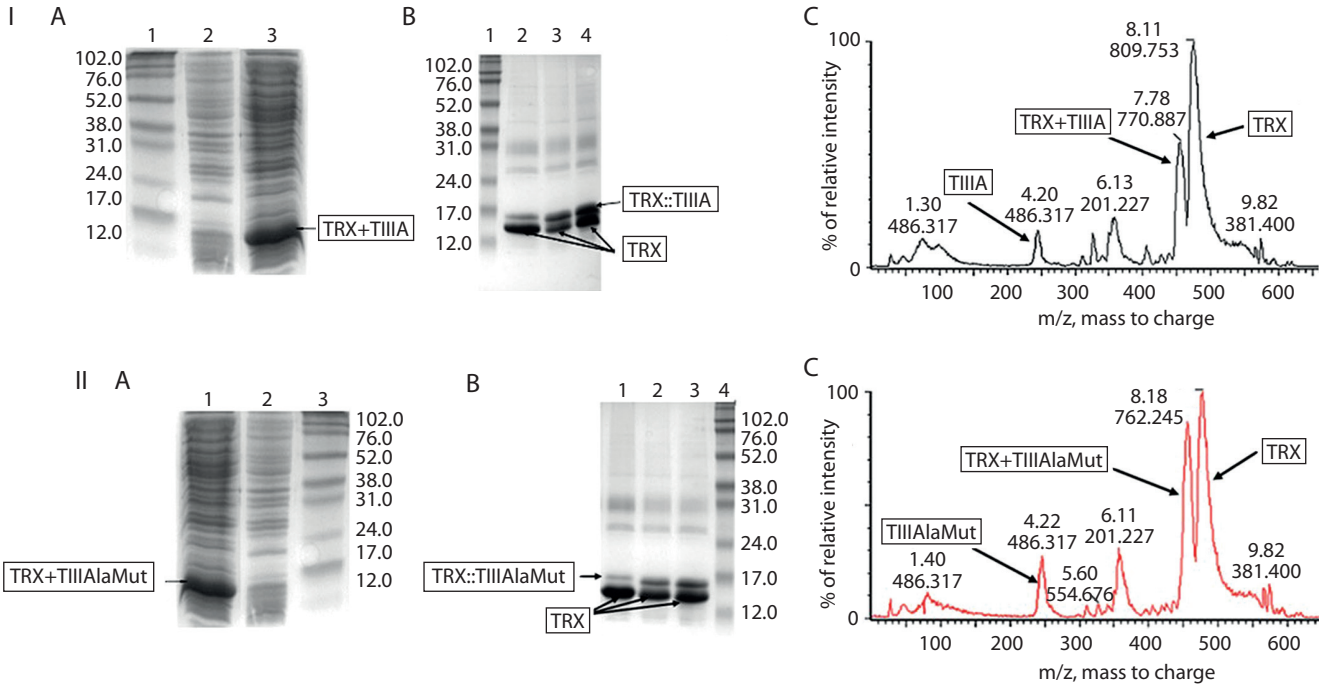

Figure 3. Expression and purification of TIIIA and TIIIAlaMut from *Escherichia coli*. **I**) TIIIA. **A**) SDS-PAGE analysis of TIIIA expression. Lane 1: molecular-weight marker; lane 2: total protein from *E. coli* S4B without insert; lane 3: total protein from *E. coli* S4B/pDM-TIIIA. **B**) SDS-PAGE analysis of CNBr-digested TRX::TIIIA under different conditions. Lane 1: molecular-weight marker; lanes 2–4: digestion in 0.3 M HCl, 0.2 M HCl, and 0.1 M HCl for 3 h, respectively. **C**) LC-MS chromatogram illustrating separation of the TRX::TIIIA fusion protein. **II**) TIIIAlaMut. **A**) SDS-PAGE analysis of TIIIAlaMut expression. Lane 1: molecular-weight marker; lane 2: total protein from *E. coli* S4B without insert; lane 3: total protein from *E. coli* S4B/pDM-TIIIAlaMut. **B**) SDS-PAGE analysis of CNBr-digested TRX::TIIIAlaMut under different conditions. Lane 1: molecular-weight marker; lanes 2–4: digestion in 0.3 M HCl, 0.2 M HCl, and 0.1 M HCl for 3 h, respectively. **C**) LC–MS chromatogram showing separation of the TRX::TIIIAlaMut fusion protein

Efficacy test results

A total of 55 participants completed the full 4-week study period and were included in the final analysis. Results from the post-treatment questionnaires were analyzed descriptively, with data expressed as the percentage of positive responses for each evaluated aspect. Descriptive statistical methods were applied, and results are presented as percentage frequencies of positive responses. Given the observational design of the study, no inferential statistical tests were performed.

Overall satisfaction and user perception

A graphical summary of the patient satisfaction survey, illustrating overall satisfaction and user perception, is presented in Figure 5. The results indicate a high level of user satisfaction: more than two-thirds of participants reported positive impressions, and nearly 80% expressed willingness to continue product use. Additionally, nearly half of the respondents perceived their skin as visibly younger, suggesting a moderate but noticeable aesthetic benefit.

Evaluation of product characteristics

The patient survey results evaluating product characteristics are shown in Figure 6. The cosmetic formulation was very well tolerated in terms of its physical and sensory properties. Over 90% of respondents rated texture, color, and application favorably, demonstrating excellent consumer acceptance.

Perceived improvement in skin condition

The graphical representation of patient satisfaction survey results regarding perceived improvement in skin condition is shown in Figure 7. Most participants reported improvements in hydration, softness, and firmness, confirming the moisturizing and smoothing effects of the tested formulation. Moderate improvements were noted in skin brightness and pore size reduction. Wrinkle reduction

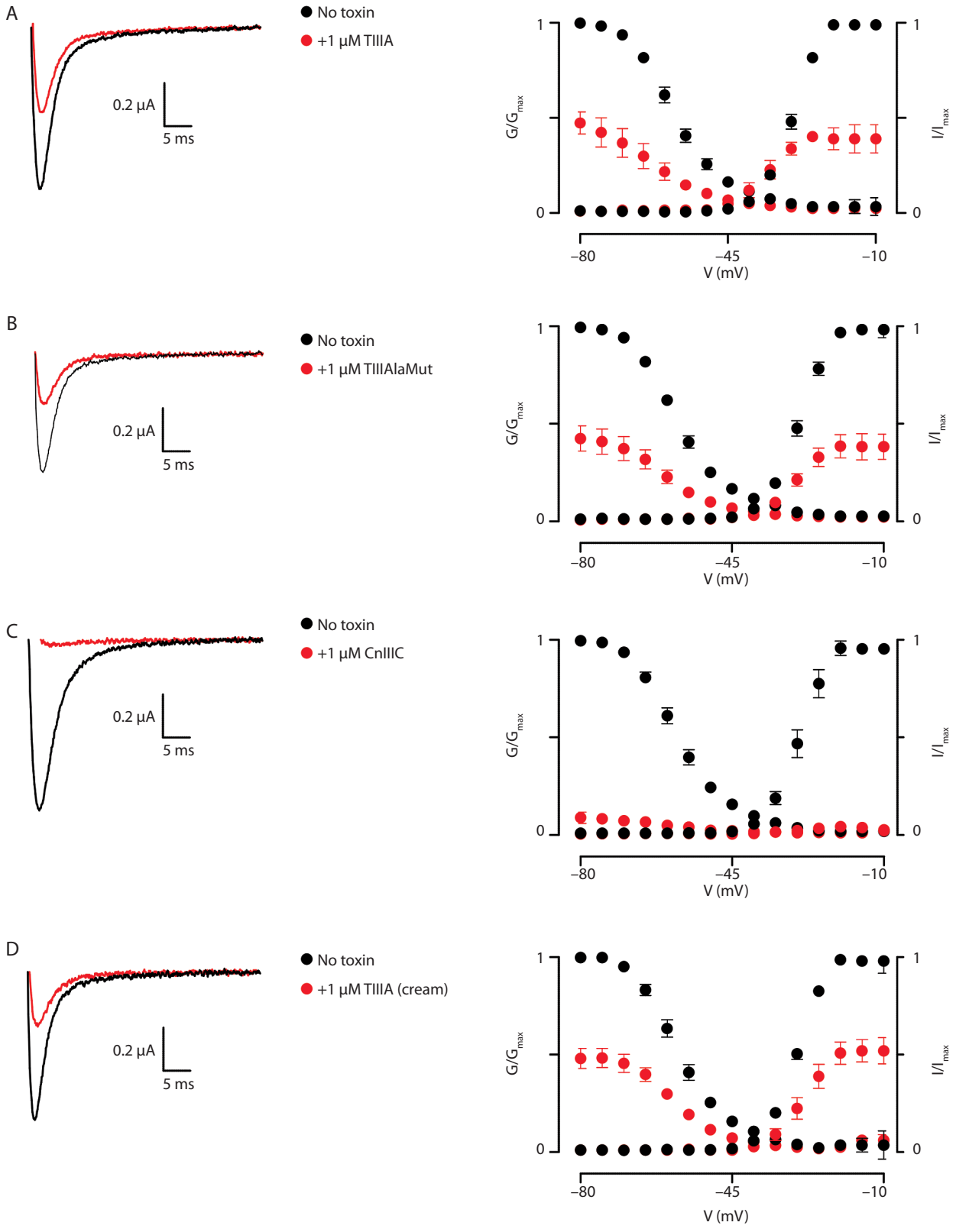

Figure 4. Biological characterization of conotoxin activity using *Xenopus laevis* oocytes expressing hNav1.4for: **A**) conotoxin TIIIA, **B**) conotoxin TIIIAlaMut, **C**) standard CnIIIC, **D**) conotoxin TIIIA extracted from cream. Left: representative hNav1.4 traces without and with toxin, demonstrating inhibitory effects. Right: effects on normalized conductance-voltage (G-V; open circles) and channel availability (I–V; filled circles) relationships. Error bars indicate SEM (n = 6 per condition)

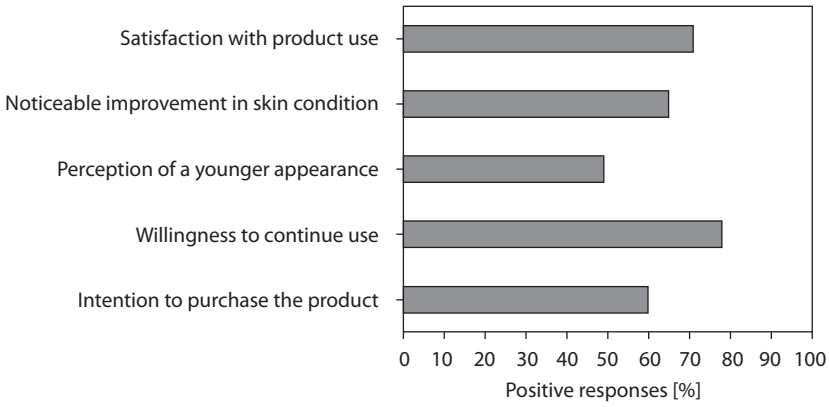

Figure 5. Results of the patient satisfaction survey: overall satisfaction and user perception

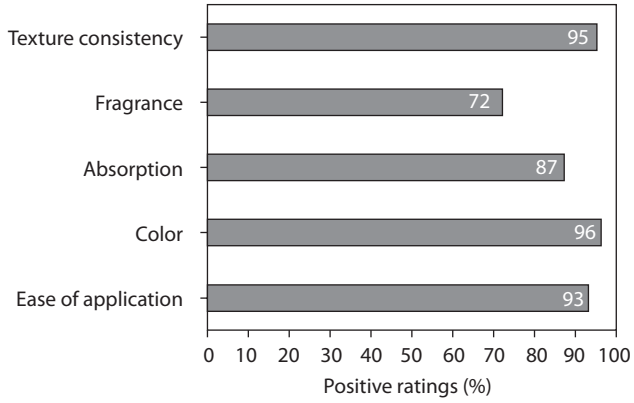

Figure 6. Patient satisfaction survey results for evaluation of product characteristics

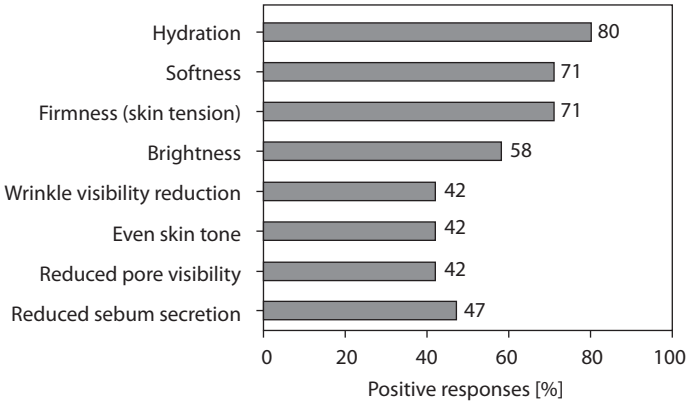

Figure 7. Graphical representation of patient-reported improvements in skin condition

and improvements in skin tone were reported by approximately 40–45% of respondents, suggesting the potential for progressive long-term benefits with continued use.

Perceived changes in wrinkle parameters

The results of the patient satisfaction survey addressing perceived changes in wrinkle parameters are presented in Figure 8. Approximately one-third to two-fifths of participants perceived a visible reduction in wrinkle depth or prominence, which is consistent with the expected activity of the active ingredient, conotoxin TIIIA. These subjective findings complement the objective 3D imaging assessments, together indicating potential anti-aging effects of the formulation.

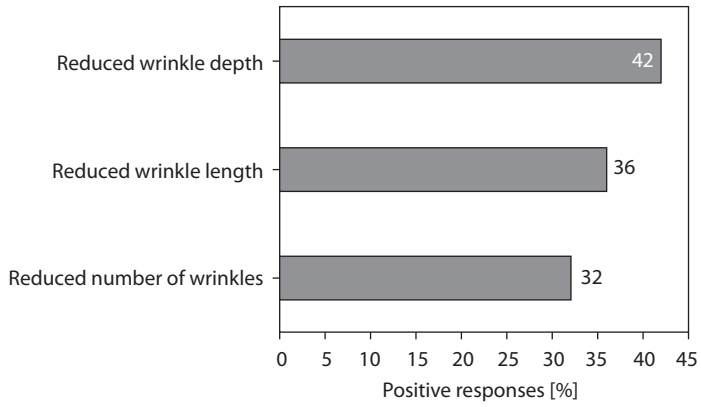

Figure 8. Patient satisfaction survey results regarding perceived changes in wrinkle parameters

Overall outcomes

In the group of 55 participants who completed the study, a reduction was observed in both facial and static wrinkles, including "crow's feet" and lower-eyelid wrinkles, as confirmed by the documented clinical cases. These regions are particularly challenging to treat with botulinum toxin, underscoring the effectiveness of the tested formulation. Among a subset of volunteers with upper-eyelid ptosis, a noticeable lifting effect was recorded. This outcome may be attributed to relaxation of the orbicularis oculi muscle, which lies very close to the skin and is nearly fused with it. Several participants who had previously struggled with this condition demonstrated clear improvement.

It is important to emphasize that the eye area is one of the most difficult regions to treat aesthetically due to its thinner epidermis, reduced subcutaneous tissue, and lower muscle mass compared to, for example, the forehead musculature. Despite these challenges, a significant improvement was also observed in static wrinkles in other facial areas, including the nasolabial folds and marionette lines. The most pronounced effect, however, was noted in the periocular region, with 47% of all volunteers reporting visible wrinkle reduction.

A cumulative effect of the cream was evident in a subset of participants who experienced enhanced skin texture and smoothness with continued use. Additional benefits included evening of skin tone and a reduction in erythema. These findings may suggest supplementary properties of the conotoxin ingredient, potentially analogous to botulinum toxin in its mechanism of modulating nerve-mediated vasodilation. Participants with oily or combination skin reported improvements in sebum control: 49% noted reduced skin shine, while 44% reported better regulation of sebum production. Representative images of the clinical outcomes are shown in Figure 9.

Importantly, the formulation demonstrated an excellent safety profile. No adverse dermatological reactions, irritation, or discomfort were reported during the four-week study period, confirming the good tolerance and suitability of the product for routine facial use.

Discussion

In this study, we developed and characterized recombinant forms of conotoxin μ-TIIIA and its mutant TIIIAlaMut using a bacterial *E. coli* expression system. The use of a thioredoxin fusion enabled the production of soluble peptide forms, representing a significant improvement over earlier approaches in which incorrect folding of conotoxins in *E. coli* limited production yields (Becker and Terlau 2008; Klint et al. 2013). The recombinant peptides were efficiently cleaved from the fusion partner using CNBr, and the reaction conditions were optimized to minimize peptide degradation. Electrophysiological studies on *Xenopus laevis* oocytes expressing Nav1.4 channels demonstrated that both TIIIA and TIIIAlaMut retained their ability to inhibit Nav channel conductance, comparable to the synthetic CnIIIC standard, although with lower affinity. Similar findings have been reported for other μ-conotoxins, which exhibit high selectivity for Nav1.4 channels and show therapeutic potential in modulating skeletal muscle excitability (Green et al. 2014; Pei et al. 2024). Importantly, peptide activity was also confirmed after extraction from a completed cosmetic formulation, indicating structural stability and resistance to degradation during formulation and storage.

The 55-participant application study demonstrated a clear antiwrinkle effect, particularly in the periocular

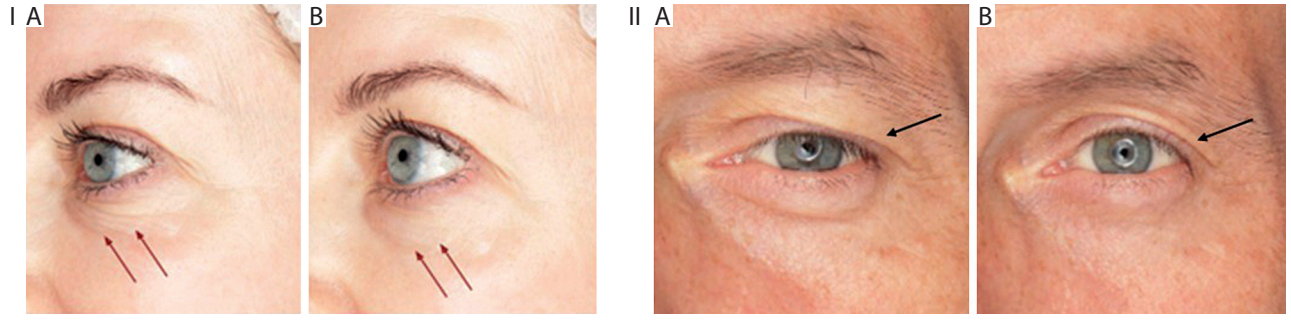

Figure 9. Representative before/after images of study participants. **I**) Visible reduction of periocular wrinkles, smoother skin texture, and softened crow's feet. **II**) Reduction of upper-eyelid ptosis. **A)** Before treatment. **B)** After 28 days of using the cream containing conotoxin TIIIA

region, where 47% of participants reported visible improvement. These observations are consistent with the known myorelaxant properties of μ-conotoxins, which block sodium channels in muscle tissue, promoting muscle relaxation and subsequent smoothing of the overlying skin (Zou et al. 2024). The observed lifting effect of the upper eyelid may be clinically relevant, particularly for individuals with mild eyelid ptosis, where traditional botulinum toxin injections can be less effective or may carry a higher risk of adverse outcomes (Musharbash and Chakra 2024).

The results of the study demonstrated a high level of participant satisfaction and excellent usability of the tested cream formulation. The mean satisfaction level reached approximately 71%, and 78% of participants declared their willingness to continue using the product after completing the study. The cream also exhibited very good cosmetic properties, with an overall approval rate of 88.6% across all evaluated sensory parameters, including texture, spreadability, absorption, and pleasantness of application. Participants reported noticeable improvements in general skin condition, particularly in hydration and softness, which were rated positively by at least 70% of users. Subjective assessments showed visible wrinkle reduction in approximately 30–40% of participants, supporting the antiaging potential of the formulation.

Additional benefits – such as improved skin tone, reduced erythema, and sebum regulation in individuals with oily skin – suggest that conotoxins may exert effects beyond muscle relaxation. It is plausible that these peptides modulate neurogenic signalling within the skin's microvasculature or sebaceous glands, a hypothesis previously proposed in the context of botulinum toxin type A (Rahman et al. 2024; Dayel et al. 2024). An important aspect of our findings is the potential for synergistic use of TIIIA conotoxin with botulinum toxin in aesthetic applications. Previous literature indicates that agents with similar mechanisms but different sites of action within the motor unit can prolong the duration of clinical benefits (Coleman and Carruthers 2006). This raises the possibility of developing cosmetic products that support or extend the effects of injectable treatments.

The main limitations of this study include the relatively small sample size and the short, 4-week application period. Further randomized clinical trials with control groups and longer follow-up are needed to assess the durability and long-term safety of the effects. Moreover, although the *in vitro* and *in vivo* results were consistent, a full understanding of the mechanism of action of conotoxins in the skin requires additional molecular studies, including analyses of their interactions with ion channels in keratinocytes and fibroblasts.

Conclusions

In this study, we successfully demonstrated that the *E. coli* expression system can be effectively used for the production of recombinant μ-conotoxin TIIIA and its mutant TIIIAlaMut. The developed methodology provides a scalable and cost-efficient alternative to chemical synthesis, enabling access to functional μ-conotoxin variants. Importantly, the recombinant toxins retained their biological activity not only in electrophysiological assays but also after incorporation into cosmetic formulations, confirming their structural stability and compatibility with formulation processes. The application tests confirmed the potential of TIIIA conotoxin as a biologically active ingredient in cosmeceuticals.

Topical application of a cream containing conotoxin TIIIA resulted in visible antiaging benefits, most notably

in the reduction of periocular wrinkles and improvements in overall skin smoothness and tone. Collectively, these findings indicate that recombinant μ-conotoxin TIIIA is a promising cosmeceutical ingredient with measurable antiwrinkle effects. Its properties suggest potential use both as a stand-alone cosmetic active and in combination with botulinum toxin to enhance or prolong aesthetic treatment outcomes.

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Author contribution

Investigation – DM, AMP, MJ, AM, TC, AS, FB. Methodology – DM, AMP, MJ, AS, AM, FB. Writing – DM, AMP. Review and editing – DM, MJ, AS, AMP, TC.

Competing interests

The authors declare that they have no competing interests

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Nanotechnology in food systems: opportunities and risks for human health

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Abstract

Nanotechnology has emerged as a promising field with the potential to revolutionize several industries, including the food industry. It offers innovative solutions to critical challenges in food, such as safety, nutrition, waste reduction, and sustainability. This study examines the possibilities offered by nanotechnology in the food sector, with a focus on risk assessment, safety evaluation, and regulatory approaches. While nanotechnology in food applications presents many advantages, it also raises concerns about potential health risks. Due to their distinct characteristics, nanoparticles may interact with living organisms in unpredictable ways, creating challenges for risk assessment and management. This review also explores the possible hazards of using nanomaterials in the food system, highlighting the need for comprehensive toxicity studies and effective regulatory frameworks. Addressing these issues requires a multidisciplinary approach involving collaboration among scientists, regulators, policymakers, and stakeholders to balance the benefits and risks of nanotechnology in the food system. As the food sector seeks novel approaches to meet rising global demand, it is crucial to thoroughly assess both the advantages and risks of nanotechnology to ensure its responsible and sustainable application while protecting human health and the environment.

Key words: nanotechnology, food systems, nanomaterials, nanoparticles, health, risk assessment

Introduction

Nanotechnology is one of the most promising fields for enhancing food availability and developing innovative products across diverse sectors, including food, water, agriculture, the environment, medicine, energy, and electronics (Sadeghi et al. 2017). By enabling the precise manipulation of atoms and molecules at their most fundamental level, nanotechnology has led to the creation of novel materials with exceptional properties. The term *nano* originates from the word *dwarf*, signifying something extremely small – one billionth of a meter (10^{-9}), or approximately one nanometer (nm). This scale is roughly 3–5 atoms wide and about 40,000 times thinner than a human hair or comparable to the thickness of a virus (100 nm). Nanotechnology primarily focuses on creating materials ranging from 1 to 100 nm in size (Ullah et al. 2024). In food science, this technology is applied to develop materials with enhanced stability, solubility, and bioavailability (Chudasama and Goyary 2024). Scientists and industry experts have already recognized the vast potential of nanotechnology in nearly every aspect of the food industry – from agriculture and food processing to packaging, safety, and nutrient delivery – promising transformative advancements in these areas (Pathakoti et al. 2017).

Nanotechnology bridges the gap between conventional and quantum mechanics through the utilization of mesoscopic systems – an intermediate domain. In medicine, these mesoscopic systems enable the development of nano-assemblies, including agricultural products, nanomedicine, and nanotools designed to enhance therapies and diagnostics. Nanomaterials (NMs)

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are being widely applied in regenerative medicine, advancing fields such as tissue engineering, cell therapy, and gene sequencing. Research has documented nanoassemblies with properties that support cell adhesion, migration, and differentiation. Nanotechnology is also making notable contributions to microbiology and antiviral research, offering innovative solutions for disease prevention and treatment (Malik et al. 2023).

On September 9, 2003, the United States Department of Agriculture (USDA) released its first roadmap for integrating nanotechnology into the food industry. As a partner agency in the Federal National Nanotechnology Initiative (NNI), the USDA had previously organized a National Planning Workshop, *Nanoscale Science and Engineering for Agriculture and Food Systems*, in November 2002 in Washington, DC. The workshop explored the potential of nanotechnology in transforming agriculture and food systems and identified key opportunities for its application. Its outcome was the development of a scientific roadmap and strategic plan, which included recommendations for launching a dedicated nanotechnology program within the USDA to advance agriculture and food systems (Siddiqui and Alrumman 2021; U.S. Department of Agriculture 2003).

Over the past few decades, nanotechnology has increasingly become a part of daily life through applications in electronic chips, textiles, paints, agriculture, food processing, packaging, wastewater treatment, clinical diagnostics, therapies, and environmental restoration (El-Sheekh et al. 2022). In recent years, substantial evidence from industrialized countries suggests that nanotechnology holds considerable potential for addressing environmental challenges. Its applications include improving drinking water hygiene, detoxifying pollutants such as toxic heavy metals and organochlorine pesticide residues, and reprocessing these materials through advanced techniques like nanofiltration (Taran et al. 2021).

Nanopesticides have been ranked first among the ten chemical innovations identified by the International Union of Pure and Applied Chemistry (IUPAC) as having the greatest potential to shape the future of human civilization. This recognition stems from their ability to minimize negative impacts on the environment and public health while providing effective pest management solutions (Gomollón-Bel 2019). In agriculture, pesticides incorporating nanotechnology benefit from improved penetration, coverage, and absorption facilitated by NMs. The delivery of nano-agrochemicals through NMs holds significant potential to enhance food security while promoting the ecological sustainability of agricultural practices. This is achieved by reducing labor costs, minimizing environmental pollution, and increasing the efficiency of agricultural inputs (Li et al. 2021; Su et al. 2020; An et al. 2022). In the food sector, nanotechnology also contributes to food safety through nanosensors that detect contamination during production, processing, storage, packaging, and transportation (Nile and Kai 2021). Moreover, nanotechnology-enabled processing and packaging have proven highly effective in improving both the efficiency and safety of the food system (Weiss et al. 2006).

Nanotechnology has made significant contributions to food science in several key areas, including enhancing the tracking and tracing of pollutants, extending the shelf life of food products, improving storage, and enabling the incorporation of antibacterial agents and health supplements (Neo et al. 2013). Researchers have developed numerous technologies to improve food quality and safety, with nanotechnology playing a pivotal role in producing food with higher oral bioavailability, improved solubility, and enhanced thermal stability (Semo et al. 2007).

The application of nanotechnology in packaging is often classified based on functionality. Most NPs used in food packaging exhibit antimicrobial properties, act as carriers for antimicrobial polypeptides, and protect against microbial spoilage (Nile et al. 2020). Nanostructures introduced into the food industry serve two major purposes: food ingredients and sensors. Nano-food ingredients have diverse applications in food processing and packaging, offering functionalities such as antimicrobial and anticaking agents, nano additives, nanocarriers, and nanocomposites. Additionally, nanosensors are incorporated into food packaging to monitor and ensure food quality throughout storage and distribution (Sahani and Sharma 2021).

A new revolution is underway in the Agri-tech sector, aiming to sustainably meet the growing global food demand. Advances in nanotechnology have led to increased use of NMs, highlighting their potential to address these challenges effectively (Zain et al. 2023). Nanotechnology is a rapidly expanding field with applications spanning energy, medicine, food, and many other industries

(Arpanaei et al. 2024). The market for nanotechnology-enabled products has shown steady growth, currently generating over $1 billion annually. By 2024, it is projected to surpass $125 billion, driven by advancements across electronics, pharmaceuticals, automotive, agriculture, and other sectors (Santos et al. 2024).

The presence of more than a thousand NMs-containing products on the market underscores nanotechnology's status as a rapidly growing multibillion-dollar industry. In the past decade, over 300 nanofood products have been introduced to international markets, reflecting its increasing influence in the global food sector. Figure 1 illustrates *Adoption trends of food nanotechnology: market share (%) by application (2019–2031)* (Mali 2023).

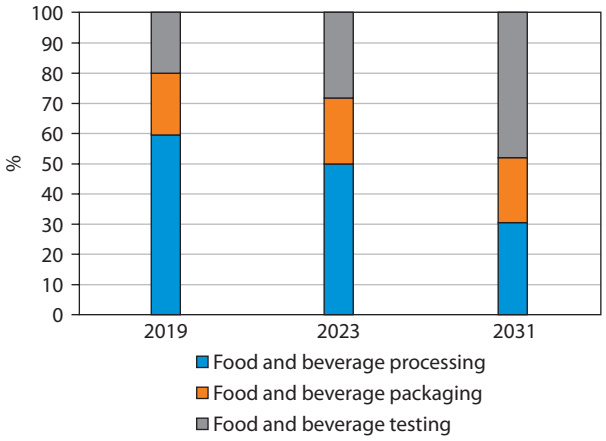

Figure 1. Adoption trends of food nanotechnology: market share (%) by application (2019–2031)

Opportunities in food systems

There are many advantages to employing nanoparticles (NPs) in the packaging sector, as mentioned above. However, the increased toxicity associated with the behavior of particles on the nanometric scale raises serious concerns about the use of these nanostructures. These particles have the potential to harm consumers when they migrate from food packaging into food. When creating any novel food packaging material, it is necessary to examine the component migration behaviors to see if any unwanted or dangerous components are leaking into the food (Gupta et al. 2023).

The European Food Safety Authority's (EFSA) guidelines, the amount of silver that migrates from packaging materials into food must not exceed 0.05 mg/kg in food and 0.05 mg/l in water. The US Environmental Protection Agency states that silver levels in drinking water should not be higher than 0.10 mg/l. A disadvantage of silver is its potential to migrate into food products, thereby increasing the risk of toxicity, even though it can extend the shelf life of food products (Maria et al. 2024).

Despite growing agricultural challenges, combining nanotechnology with conventional methods offers a forward-thinking approach to ensuring food security. The potential of nanotechnology to enhance food security, identify pathogens, treat illnesses, enable effective delivery systems, and produce diverse packaging materials is considerable (Saha et al. 2024).

Recent developments in nanotechnology and biotechnology, such as precision fermentation, are examples of biological technologies. These technologies use biological processes and organisms to produce food ingredients or improve the safety and quality of food. They provide innovative ways to enhance nutritional value, extend shelf life, and promote sustainable food production practices. However, they also raise issues related to consumer acceptance, legal challenges, and the ethics of altering living organisms (Hassoun et al. 2024).

NPs can readily cross tissue and cell barriers due to their small size and large surface area, which may harm biological systems. In addition to an NPs size and shape, its charge and digestibility can also influence its potential toxicity. Numerous studies have shown that nanoemulsions have higher bioavailability, which may cause adverse health effects in some populations when consumed in excess for certain substances. The type and quantity of ingredients used to create nanoemulsions represent another potential source of toxicity. In some cases, small droplet sizes can only be achieved using small-molecule synthetic surfactants, which may be more toxic than natural emulsifiers, and higher levels of emulsifiers are required to stabilize their larger surface areas (Kaur et al. 2024).

Additionally, surface coatings such as polyethylene glycol (PEG) can alter cellular uptake, reduce immunological recognition, and improve biocompatibility and systemic circulation. Because poorly degradable NPs can accumulate and have long-term toxicity, digestibility and biodegradability are also important considerations. These elements influence the biodistribution, cellular interaction, and toxic potential of NPs (Nel et al. 2006). Additionally, the way that NPs interact with cellular membranes and how toxic they are overall are

greatly influenced by their surface charge. Stronger electrostatic interactions between positively charged NPs and the negatively charged phospholipid bilayer of cell membranes can result in increased cytotoxicity and membrane disruption as well as improved cellular uptake. Conversely, neutral or negatively charged NPs may be absorbed less effectively but are typically less harmful. For instance, a study showed that, in contrast to their anionic or neutral counterparts, cationic gold NPs caused increased levels of reactive oxygen species (ROS) and cell membrane damage in HeLa cells. Similarly, a study showed that surface charge influences the overall biocompatibility of the NMs by influencing intracellular localization, immune response activation, and uptake efficiency (Zhao et al. 2011; Fröhlich 2012).

The food industries are looking for innovations in packaging unit operations in this age of technological advancements in every sector because of the many detrimental effects of traditional packaging materials, which are constantly contaminating the environment. In order to enhance the physicochemical and functional qualities of packaging film, the food packaging industry is currently utilizing a number of NMs in the food packaging system (Gupta et al. 2024).

Types of NMs used in food

There are many uses for nanotechnology in the food sector. In these applications, a particular food product is combined with a specific type of NM to provide desirable properties (Bajpai et al. 2018). Table 1 presents the applications and mechanisms of NMs in the food sector (Nile et al. 2020). NMs are defined as materials with sizes ranging from 1 to 100 nm (Dazon et al. 2020). They have developed into a flexible platform capable of offering economical, environmentally friendly, and efficient solutions to global challenges (Singh et al. 2023).

NMs are grouped based on their size, properties, and structure. NMs with a high surface-to-volume ratio are particularly desirable for their physicochemical properties, such as bioavailability, diffusivity, solubility, optics, magnetism, strength, color, toxicity, and thermodynamics (Sahoo et al. 2021). Because of their superior physicochemical properties and antimicrobial activity, NMs are widely employed in water treatment, agriculture, healthcare, food safety, and preservation, as well as against a variety of pathogenic microbes (Baranwal et al. 2018). In addition to their unique physicochemical characteristics, NMs are also easily conjugable (Hu et al. 2020).

One general way to classify engineered NMs used in feed, food, and agriculture is as follows: inorganic (e.g., metal and metal oxide NPs), organic (mostly natural product NPs), or combination (e.g., surface-modified clays) (Peters et al. 2016). Inorganic NMs include metals, metal oxides, salts, and carbon-based materials such as carbon nanotubes, fullerenes, carbon black, and clay (King et al. 2018). Among these, silver NPs are the most widely produced and commercially used due to their antimicrobial activity, whereas gold (Au) NPs are extensively studied for use as sensors and detectors. Titanium dioxide (TiO_2) NPs have also been investigated as a food preservative, primarily as a white pigment and taste enhancer (He et al. 2019).

Nanocomposites based on metals and metal oxides are applied in food coating and packaging. Silver nanocomposites and NPs are among the most widely used antimicrobials in the food industry (He and Hwang 2016). TiO_2 can be incorporated into packaging materials as a coating agent to reduce *Escherichia coli* contamination (Chellaram et al. 2014). The use of NMs in food packaging is expected to reduce challenges associated with traditional packaging materials while lowering waste and conserving valuable raw ingredients (Sozer and Kokini 2009).

Numerous NMs are being developed as functional additives for food packaging, including nano-TiO_2, silver NPs, nanoclay, titanium nitride NPs, and nano-zinc oxide (ZnO) (Pal 2017). To create biosensors for quantification of microbes and other testing for applications related to food safety, NMs such as metal NPs, carbon nanotubes, quantum dots, and other active NPs can be used (Inbaraj and Chen 2016).

By construction, NMs are typically divided into five categories: metal-based, carbon-based, dendrimers, ceramics, and composites (Biswas et al. 2023). TiO_2 is also widely used as a food additive in cake icing, puddings, candy, gum, and white sauces (Kumar et al. 2020). Figure 2 illustrates the types of NMs used in food packaging.

Risks to human health toxicity

Nanotoxicity, or the harmful effects of NPs on biological systems, can involve several undesirable physiological reactions, such as internal and external interactions

Table 1. Applications and mechanisms of nanomaterials in the food sector

S. no.	Nanomaterial source	Category	Mechanism	Technology	Applications
1	Iron	Inorganic nanoparticles	Enhanced bioavailability, improved absorption, controlled release, reduced toxicity, immune function enhancement, and antioxidant effects	Chemical reduction, sol-gel synthesis, green synthesis, spray drying, co-precipitation, and encapsulation technology	Food supplement
2	Silver		Antimicrobial action, anti-inflammatory effects, bioavailability in food supplements, surface disinfection, and synergistic effects	Chemical reduction, biosynthesis, physical vapor deposition, electrochemical synthesis, encapsulation, and spray coating	Food supplements, antimicrobial agent – used in food contact surfaces (cutlery, storage containers, fridges, and worktops)
3	Iridium		Enzyme mimicry, antioxidant properties, cellular protection, anti-inflammatory effects, detoxification, and biocompatibility and uptake	Chemical reduction, biosynthesis, physical vapor deposition, encapsulation, spray drying, and functionalized nanoparticles	Food supplement
4	Platinum		Enzyme mimicry, antioxidant activity, anti-inflammatory effects, detoxification, cellular uptake, and stabilization of nutrients	Chemical reduction, green synthesis, physical vapor deposition, encapsulation, spray drying, and nano-functionalization	Food supplement
5	Zinc		Enhanced bioavailability, targeted nutrient delivery, improved stability, antioxidant properties, immunomodulation, and coloring agent mechanism	Sol-gel synthesis, biosynthesis, physical vapor deposition, nano-chelation, spray drying, and liposomal encapsulation	Food supplement/colorant
6	Liposomes	Organic nanoparticles	Encapsulation, protection, controlled release, targeted delivery, and enhanced bioavailability	Thin-film hydration, ultrasonication, microfluidization, reverse phase evaporation, supercritical fluid technology, and spray drying	Encapsulation and targeted delivery of food components
7	Protein		Re-micellisation process, gelation properties, heat stability, improved emulsification, enhanced solubility, biocompatibility, and controlled release	Ultrasonication, high-pressure homogenization, electrostatic assembly, freeze-drying (lyophilization), enzymatic hydrolysis, spray drying, and controlled pH and ionic strength	Re-micellised calcium caseinate from dairy protein. Increased functionality (gelation, heat stability, and other properties)
8	Polymeric		Stability and encapsulation, release mechanism, surface modification, bioinertness, biodegradation, controlled release, and biocompatibility	Emulsion-solvent evaporation, nanoprecipitation, electrostatic assembly, coacervation, spray drying, and supercritical fluid technology	Non-degradable: polystyrene Biodegradable: PGLA, gelatin, and collagen

Table 1. Continuation

S. no.	Nanomaterial source	Category	Mechanism	Technology	Applications
9	Globular proteins	Nanofibers/ fibrils	Structural properties, thermal stability, increased shelf-life, gel formation, and increased functional properties	Nanoprecipitation, high-pressure homogenization, electrospraying, spray drying, and solvent evaporation	Thermal stability, increased shelf-life. Formation of a transparent gel network for use as a thickening agent
10	Oil in water	Nanoemulsions	Formation and stabilization, delivery of active compounds, extended shelf-life, flavour release, and low-fat products	High-pressure homogenization, ultrasonication, phase inversion temperature, microfluidization, and solvent emulsification	Stabilization of biologically active ingredients, delivery of active compounds; extended shelf-life, flavor release, and low fat products
11	Calcium carbonate	Nanodispersions	Nanoscale size and surface area, enhanced solubility, stabilization in aqueous solutions, increased bioavailability and reduced grittiness, and sensory impact	High-pressure homogenization, ball milling, solvent evaporation, precipitation methods, and microfluidization	Increased solubility of calcium carbonate – can be used at higher addition levels
12	Clay composites	Nanoclays	Intercalation and exfoliation, improved barrier properties, enhanced mechanical strength, thermal stability and durability, and light and UV protection	Melt blending, solution casting, in-situ polymerization, electrospinning, and solvent casting	Used in packaging materials to extend shelf-life, durability, and thermal properties (includes nanolaminates)

with NPs that cause cell disruption. A comprehensive understanding of NMs and the associated risk factors, including the physiological mechanisms of toxicity, plays a pivotal role in their future applications (Sharma et al. 2024). Several NMs have been reported to negatively impact the environment and human health. Table 2 presents various NMs and their toxicity mechanisms with corresponding physiological responses.

Numerous factors, including the extent of migration and the type of packaging matrix used, may influence NP toxicity (Cushen et al. 2012). Ingesting NPs poses growing health risks, as meals containing NPs may be harmful due to high consumption, bioaccumulation, and overactivity, in addition to the associated hazards (Rasmussen et al. 2010). The toxicity of NPs depends on their type, concentration, duration of exposure, and the sensitivity of the individual (Dimitrijevic et al. 2015).

Beyond concentration and mass, other factors influencing NP toxicity include size, quantity, surface reactivity, surface modification, and aggregation (Muthukrishnan 2022). For instance, smaller silver NPs (10 nm), because of their greater surface area and reactivity, have been shown to enter cells more readily and cause greater cytotoxicity than larger ones (50 nm). Surface modifications, such as PEG coating, can reduce NP toxicity by preventing protein corona formation and enhancing biocompatibility. Aggregation of NPs can also alter toxic effects by changing their biodistribution and lowering cellular uptake. Collectively, these physicochemical characteristics determine how NPs interact with biological systems and influence their potential risks (AshaRani et al. 2009). NPs can enter the human circulatory system through ingestion into the gastrointestinal tract (GIT) or through inhalation (intranasal or intratracheal), leading to significant deviations from normal physiological functions.

The initial impacts of NPs on the cardiac system include elevated blood pressure, lowered heart rate, and altered vascular tone and dysfunction (Yu et al. 2016). In animal models, for example, inhalation of TiO_2 NPs has been shown to reduce vascular function and increase arterial pressure (LeBlanc et al. 2009).

When NPs accumulate in various brain regions and alter the expression of genes critical for the development and normal functioning of the central nervous system, they induce cytotoxic effects on neural cells. Accumulated NPs, such as ZnO or silver NPs, can cross the

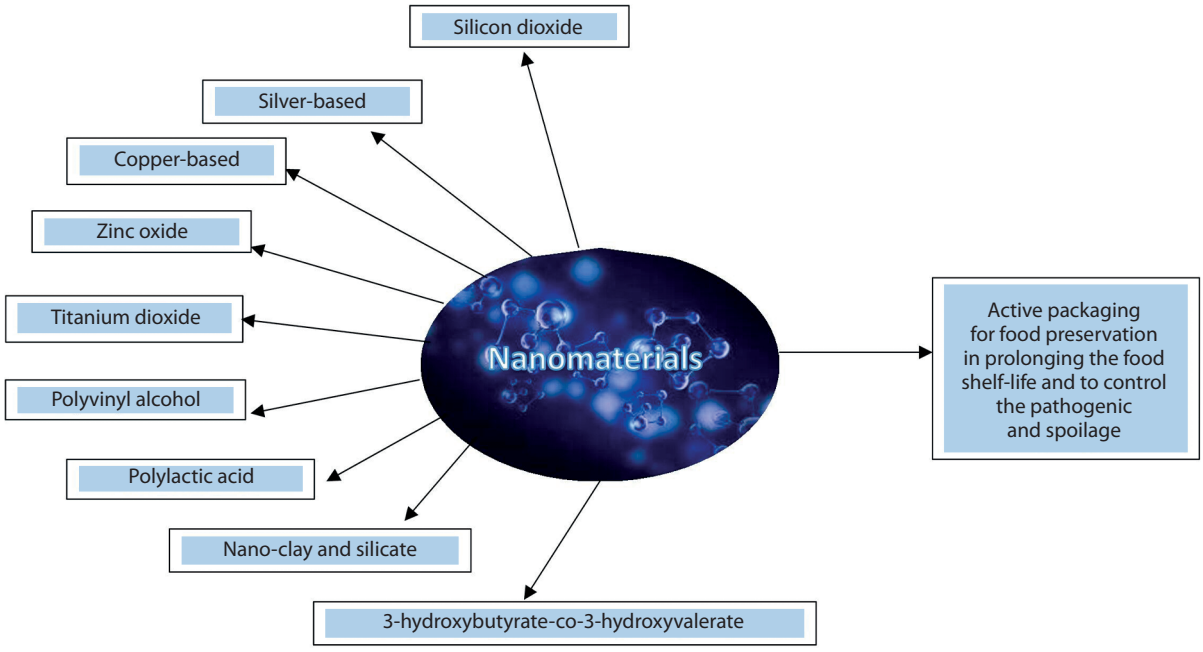

Figure 2. Types of nanomaterials used in food packaging

blood-brain barrier and modify the expression of genes related to neurodevelopment and synaptic signaling, ultimately leading to cytotoxicity and neuronal damage (Tang et al. 2009). ZnO NPs have been reported to decrease cell viability, induce apoptosis, alter the cell cycle, and cause oxidative deoxyribonucleic acid (DNA) damage (Yang et al. 2010; Valdiglesias et al. 2013).

A study demonstrated that administering 150 μg/kg of iron oxide NPs to male rats created an imbalance by increasing triiodothyronine (T_3) hormone levels while decreasing thyroid-stimulating hormone (TSH). Furthermore, palladium NPs have recently been shown to act on hormone receptors, initiating overstimulation and terminating signaling cascades (Jiang et al. 2019; Leso et al. 2018). Excessive NP accumulation has also been identified as one of the main contributors to the present spike in cases of infertility. According to an investigation animal on a high-fat diet, when administered silica NPs which are frequently encountered around workplaces decreased sperm levels and motility rates and elevated sperm abnormality rates (Zhang et al. 2020). TiO_2 NPs have been found to induce inflammation and cytotoxicity after passing the blood-testis barrier, resulting in genotoxic effects and a substantial loss of sperm DNA integrity (Santonastaso et al. 2019).

NPs frequently aggregate in the proximal convoluted tubules (PCT), where endocytosis may cause tubular cells to internalize the particles after glomerular filtration. A study on T helper type 1 (Th1) cells exposed to inorganic NPs reported DNA damage, and NP-induced nephrotoxicity was also observed (Sramkova et al. 2019). NPs can damage and destroy segments of single-stranded and double-stranded DNA, leading to genetic mutations that manifest as lung cancer and other neoplasms (Sonwani et al. 2021). The introduction, distribution, and absorption of NPs in human systems are directly linked to genotoxicity and cytotoxicity (Stark 2011).

The physicochemical characteristics of NPs – such as their biodistribution, bioavailability, concentration in food products, and levels of consumption – dictate their harmful effects on human organs (Wani and Kothari 2018). Despite their many benefits, nanotechnology in food may negatively affect the environment, society, and human health, as these particles can enter ecosystems through agricultural pesticide use or the processed food industry, including packaging. This raises concerns regarding their potential toxicity (Kalpana Sastry et al. 2013).

Bioavailability and accumulation

Numerous investigations have explored the use of NMs as delivery systems to improve the bioavailability of bioactive ingredients in dietary supplements (Oehlke et al. 2014). Enhancing bioaccessibility and absorption,

Table 2. Nanomaterials and their toxicity mechanism with physiological response

Nanomaterial	Toxicity mechanism	Physiological response	References
Iron oxide, gold	Internalization and membrane disruption. Highest cellular uptake with the least membrane disruption among all shapes, thus the least shape-dependent toxicity	Cell division dysfunction and disturbed cellular trafficking; Mechanical interference with the mitotic spindle and DNA	Hartig et al. (2007), Schaffazick et al. (2003), Lee et al. (2007)
SWCNT, MWCNT, gold, mesoporous-silica	Internalization and membrane disruption. Severe influence on the initiation of phagocytosis. Blockage of transport channels. The highest distorting force on the cell membrane among all shapes. Smaller aspect ratios lead to faster internalization and less cell membrane disruption	Chronic inflammation due to frustrated phagocytosis, mutagenic events, and mesothelioma formation	Aaron et al. (2011), Alkilany et al. (2010), Andelman et al. (2010), Hsiao and Huang (2011), Peng et al. (2011), Sun et al. (2011), Yang and Cui (2008), Duan et al. (2018)
Gold	Dependent on the average radius of curvature. Disruption of membrane integrity and transport may occur	Toxicity due to chronic inflammation or impaired phagocytosis	Chithrani et al. (2006)
Nickel, carbon black	Aggregation or agglomeration changes the size of particles, thus increasing their visibility to macrophages	Aggregation changes the retention time of particles; changes in size may increase or decrease toxicity	Sager et al. (2007)
ZnO, iron oxide	Aggregation and cell membrane disruption may be dependent on the prevalence of high aspect ratio particles	Combinational effect similar to aggregated particles and fibrous particles	Mercer et al. (2008), Vevers and Jha (2008)
Quantum dots	QD core degradation, free radical formation, chromatin condensation, and membrane blebbing	Apoptosis	Hardman (2005), Su et al. (2007), Mahmoudi et al. (2010), Jung and Choi (2006)
Rare earth oxides: Y_2O_3, La_2O_3, CeO_2, etc. Mostly 18–60 nm	Lysosomal damage	Pulmonary inflammation and fibrosis	Li et al. (2014)
Gadolinium-based MRI agent: gadodiamide	Nephrogenic systemic fibrosis	Renal failure	Marckmann et al. (2006)
Ag	Decrease in cell survival for endothelial cells and lung cells	Cell death with tissue damage	Korshed et al. (2016)
TiO_2	Elevated lipid peroxidation	Disruption of cellular respiration	Erdem et al. (2015)
Al_2O_3	Disruption of cell membranes	Cell growth inhibition	Ye et al. (2018)
CdO	Endocrine disruption	Death of implanted blastocyst, abnormal foetal dimensions, and diminished growth	Blum et al. (2012)
GO	Immune cell infiltration thickness of 0.93 nm, size of 150–250 nm	Strong inflammation and granuloma formation, cytotoxicity	Yue et al. (2012), Chong et al. (2014), Duch et al. (2011), Ou et al. (2016)

as well as modifying any molecular structural changes that may occur during digestion, are strategies for maximizing the bioavailability of bioactive substances. Particle size variation can increase the surface area-to-volume ratio, improving solubility and enhancing bioaccessibility. For instance, because coenzyme Q10 (CoQ10) is poorly soluble in water and lipophilic, its bioavailability is relatively low (Kommuru et al. 2001). Using nanotechnology, foods of exceptional quality can be produced in a more practical way, increasing the bioavailability of nutrients (Dasgupta et al. 2015).

NMs based on trace elements (TEs) also have potential as bioactive agents. According to a report by the World Health Organization (WHO), 21 TEs are essential for sustaining the body's metabolism and functionality, including selenium (Se), iodine (I), molybdenum (Mo), iron (Fe), copper (Cu), zinc (Zn), chromium (Cr), cobalt (Co), and manganese (Mn). These elements participate in fundamental biological processes as components of proteins, enzymes, and cofactors. Examples include antioxidant defense (e.g., Se in glutathione peroxidase), oxygen transport (e.g., Fe in hemoglobin), and hormone synthesis (e.g., I in thyroid hormones). Mo acts as a cofactor for enzymes such as sulfite oxidase, Cu plays a role in redox reactions and mitochondrial respiration, Zn is involved in over 300 enzymes including DNA polymerase, Cr enhances insulin sensitivity, Co is a component of vitamin B_{12}, and Mn contributes to enzyme activation and bone formation. At the nanoscale, these elements often show enhanced bioavailability and targeted interactions, making them promising for medical and nutritional applications.

Currently, four potential pathways of NM metabolism have been identified: elimination via the hepatobiliary and renal systems, interception by the mononuclear phagocyte system (MPS), biodegradation and utilization in the liver, and eventual excretion from the body following gradual breakdown (Cao and Chen 2022). The small size of nanoemulsions also contributes to an extensive surface area, enabling significant interaction with bioactive compounds absorbed in the digestive system. Additionally, nanoemulsions provide more binding sites for digestive enzymes such as lipase and amylase in the intestinal tract, further increasing bioavailability (Gasa-Falcon et al. 2020).

Furthermore, it has been demonstrated that food and humans can bioaccumulate NMs such as nanosilver originating from nanopackaging or from plants and animals (Jovanović 2015). When NPs are exposed to plant or animal tissues, they are more likely to accumulate and persist in those tissues, potentially leading to adverse effects (Speranza et al. 2013). The heightened risk associated with nanoengineered particles arises from their stronger reactivity and the greater bioavailability of smaller particles in the human body, which may cause long-term pathological effects.

By being directly incorporated into novel foods in the form of nanoemulsions, nanocapsules, and nano-antimicrobial films, NMs can enter the food chain. They may also be introduced through nanolaminates and nanosensors used in food production, processing, preservation, and monitoring. Human exposure to NPs varies considerably depending on the specific application and concentration in the food industry, with the highest risk occurring when NMs are directly applied to food products as carriers of novel ingredients. Ongoing research continues to investigate how food packaging materials contribute to NP migration and how these particles behave once inside the body (Magnuson et al. 2011).

Chronic stage effects

Nanotoxicity can cause DNA damage, apoptosis, cytotoxicity, uncontrolled cell stimulation, alterations in cell motility, and the development of cancer (Fu et al. 2014). One way to assess the possible risks associated with nanostructured materials is to examine the invasion site, deposition, and migration of NMs throughout the body (Chau et al. 2007). Humans can experience NP accumulation in the kidneys, stomach, lungs, liver, spleen, small intestine, and other major organs of distribution. Moreover, a single oral dose of ZnO NPs can cause complications such as lung damage, kidney disorders, and hepatic injury. The GIT provides a pathway for NP ingestion, as the particles can easily cross biological barriers and enter the circulatory system (Esmaeillou et al. 2013).

NMs also have the potential to cause significant structural damage to mitochondria and DNA mutations, which may result in cell death (Qiao et al. 2024). Carbon nanotubes, commonly used in food packaging, are hazardous to human skin and lungs (Mills and Hazafy 2009). The deliberate integration of intelligent and active food packaging components, compared with conventional packaging materials, presents new challenges

for safety assessment. The primary concern with food contact materials (FCMs) is the migration of potentially harmful substances from packaging into food at levels exceeding established safety limits (Dainelli et al. 2008). NMs also interact with the immune system, with the ability to stimulate or, in some cases, suppress immune responses (Boraschi et al. 2017).

Environmental impact

NPs can enter the body through cutaneous contact, ingestion, or inhalation. Significant concern arises from the large quantities of NMs used in food packaging because of their potential release into the environment or into contaminated food (Han et al. 2018). As the use of nanoproducts increases, concerns regarding environmental and human health are also growing due to the unique physicochemical properties of NMs (He et al. 2018). Public concern continues to rise about the potential toxicity of NPs in biological systems. Current research is focusing on the possible harm NMs could cause as a new source of environmental contaminants (Moore 2006). Chemically produced NMs are not environmentally friendly, as they require a long time to completely degrade. Consequently, the dose and degree of environmental exposure to nanoproducts determine the extent of toxicity to living systems (Yadav et al. 2023). Once discharged, NPs are almost impossible to retrieve. Airborne NPs can rapidly infiltrate groundwater and soil, eventually spreading into vegetation, crops, and the water cycle (Mitter and Hussey 2019). The use of NPs as nanopesticides, nanoherbicides, nanofertilizers, and, less commonly, immobilized nanosensors has raised concerns about environmental health. Once absorbed, NPs may become potentially hazardous to plants directly or indirectly through the release of toxic ions during NP disintegration. Reported effects include reduced germination, biomass, and root and leaf growth (Shen et al. 2010; Hong et al. 2014). For example, changes in photosynthetic indices were observed in cucumbers exposed to 200 mg/l of CeO_2 and copper peroxide (CuO_2) NPs (Hong et al. 2016). The release of NMs into air, water, or soil could therefore have harmful environmental consequences (Cardoza et al. 2022).

Emerging technologies for food safety and quality

With its numerous applications in food processing, security, and safety, as well as in enhancing nutraceutical value, prolonging shelf life, and reducing packaging waste, recent advances in nanotechnology have significantly transformed the food industry (Wesley et al. 2014). The food sector applies nanotechnology in many different ways. Table 3 illustrates the diverse applications of nanotechnology in food systems.

Food and beverage manufacturers can efficiently incorporate β-carotene using nanoemulsions (Mehmood et al. 2021). Nanonutraceuticals are developed through nanoformulation techniques to produce functional foods, bioactive compounds, vitamin and mineral supplements, and herbal products. Numerous delivery systems – such as liposomes, cubosomes, microemulsions, single-layered structures, biopolymeric NPs, microgels, and fibers – are employed to transport nutraceuticals via nanotubes, nanofibers, fullerenes, nanosheets, and nanowhiskers (He et al. 2019; Nile et al. 2020).

Nanotechnology has also been applied in smart distribution, packaging, and protection (Chen and Yada 2011). Several types of biosensing tools are employed in the food sector, including those for bioscience research, environmental research, and applications involving graphene, reduced graphene oxide, and graphene-based graphene oxide (Taniselass et al. 2019). Packaging applications are often antimicrobial in nature, serving as carriers of antimicrobial polypeptides and protecting against microbial degradation. Bacterial growth can be inhibited by packaging materials consisting of starch colloidal outer shells loaded with antimicrobial agents, which release controlled amounts into the packaged product.

Nanofilters are used to extract lactose from milk and replace it with other sugars, making it suitable for individuals with lactose intolerance. NM-based nanosieve filters are also employed to eliminate bacteria and filter milk, beer, and water (Agriopoulou et al. 2020; Nile et al. 2020). Edible nanocoatings, with a thickness of around 5 nm, act as gas and moisture barriers in baked goods, fast foods, cheeses, fruits, and vegetables. Multiple bakery products on the market already use edible nanocoated antimicrobials. These coatings may include nanostructured gelling agents such as gelatin NPs, cellulose nanocrystals, chitosan films with nano-silicon dioxide (SiO_2), nanosilica-chitosan coatings, and nanolaminate coatings composed of lysozyme and alginate. Such technologies have been applied to preserve fresh foods for extended periods (Singh et al. 2017).

Table 3. Diverse applications of nanotechnology in food systems

S. no.	Nanomaterial	Food product	Applications
1	Nanosized self-assembled liquid structures (NSSL)	Food and beverage	Inhibits the transportation of cholesterol from the digestive system into the bloodstream
2	Nanoselenium	Beverage	Good supplement of selenium
3	Micelles 5–100 nm in diameter	Health drink	Increased lycopene
4	Conversion of vanilla or chocolate into nanoscale	Health drink	Low-calorie diet
5	Liquid-suspended nanoparticle	Food and beverage	Low-calorie diet
6	Nanosized self-assembled liquid structures	Food	Nanocapsules of omega-3 fatty acids
7	Nanodroplets	Food supplements	Efficiency enhancement
8	Silicon	Health supplement	Health and fitness
9	300 nm of iron particles	Beverage	Increases reactivity and bioavailability
10	Nanoscale micelle	Food additive	Increases absorption and effectiveness of nutritional additives and preservatives
11	Nanocochleates as small as 50 nm	Food additive	Effective addition of omega-3 fatty acids
12	< 200 nm synthetic lycopene	Food additive	Potent antioxidant and used in soft drinks
13	Nanoparticles of silver	Food contact material	Potent antibacterial
14	25 nm of silver nanoparticles	Food storage	Antimicrobial protection
15	Plastic	Food storage	Longevity of food products
16	Nanosilver	Food storage	Strong disinfection and storage power
17	Silver	Food storage	Food storage
18	Nanomicelle	Sustain beverage	Introduce antioxidants into food and beverage products
19	Nanocolloidal silicate mineral and Hydracel®	Nanosized powders	Neutralize free radicals, lower the surface tension of drinking water, and increase solvent properties
20	Liposomal nanospheres	Supplements	Health application
21	Silver nanoparticle	Fortified Jambu Juice	Rich in 22 essential vitamins and minerals
22	Silver NPs	Supplemented functional drink	Antibacterial and antifungal effects as a surface disinfectant
23	Silver hydrosols	Supplemented functional drink	Sterilization and quality control
24	Silver NPs	Supplemented functional drink	Antibacterial activity and sterilization effect
25	Silver NPs	Supplemented functional drink	Highest bioavailability
26	Silver NPs	Supplemented functional drink	Supports the immune system and defence for natural healing
27	Colloidal silver	Supplemented functional drink	Sterilization
28	Colloidal silver consists of small nanoparticles of metallic silver	Food supplement	Colloidal silver particles can be excreted
29	Actively charged nanocolloidal silver hydrosol	Food supplement	Safely supports the immune system
30	Silver	Food supplement	Support natural healing

Recent research by Thuong et al. (2020) demonstrated that incorporating silica filler into natural rubber composites significantly enhances their mechanical properties. Tensile strength increased sevenfold, and loss modulus increased twenty-fivefold. Nanotechnologies in food packaging materials with integrated nanosensors can monitor alterations in food processing, whether chemical, biological, or physical (Onyeaka et al. 2022; Pathakoti et al. 2017).

The discharge of metal ions at the surface, from within, or across the cell can alter cellular structure or function (Krzywoszyńska et al. 2020). Recent advances in the targeted delivery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) proteins, messenger ribonucleic acid (mRNA), and single-guide RNA (sgRNA) for crop genome editing – leveraging tissue engineering and engineered NMs – represent a remarkable scientific achievement in nanofarming (Kim et al., 2017; Shang et al., 2019). Nanoformulations containing pesticides have also demonstrated improved controlled release, enhanced efficacy, and higher crop yields (Petosa et al. 2017; Ul Haq and Ijaz 2019).

Risk assessment and safety evaluation

Due to their very small size, NPs have the ability to pass through cell membranes and cause genotoxicity (Fajardo et al. 2022). Assessing the degree of exposure to NPs is crucial to determine the type and extent of damage these microscopic particles may cause to different kinds of viable cells and tissues. The three main exposure routes for NPs are through the skin, the nose and throat, and the gastrointestinal system (Sahu and Hayes 2017).

Exposure of cells to certain NMs has been associated with DNA damage, which may result in interstand and intrastand breaks, single-strand and double-strand breaks, and genomic rearrangements. In addition, it has been shown that transformed bases such as 5-hydroxy-5-methylhydantoin, thymine glycol, and 8-hydroxyguanine can form (Biola-Clier et al. 2017). Regulation of the risks associated with nanofood and the adoption of nanotechnologies in the food manufacturing industry are of paramount importance. Broader civil rights, social, economic, and ethical concerns raised by nanotechnology must also be addressed by federal and state governments. For democracies to maintain control over these technological advancements in food and agriculture, civic engagement in nanotechnology governance is considered essential (Sodano et al. 2016). To assess public opinion and perception of NPs in Switzerland, a convenience sample survey was conducted from August to October 2020 using participants from the Adolphe Merkle Institute and the University of Fribourg's email list. The study revealed that a significant percentage of participants had very limited knowledge about NPs unintentionally incorporated into food products (Rothen-Rutishauser et al. 2021). According to the WHO, the EU, the United Nations (UN), and the Food and Agriculture Organization (FAO), the safety of NPs in food must be further strengthened (Magnuson et al. 2013).

Nanotechnology can extend the shelf life of food packaging and prevent nutrient loss and degradation, thereby ensuring food safety (Graveland-Bikker and de Kruif 2006). Because there is always an unknown risk associated with the use of NMs as pesticides, microbicides, and activation catalysts, risk assessment methods must be strictly followed in food processing (Shi et al. 2013). In *in vivo* toxicological studies using mammalian models such as mice and rats, the quantity of silver NPs tested remains very small (Mao et al. 2016). Public concern continues to grow regarding the possible toxicity of NPs in biological systems. NMs have the potential to represent a new form of environmental pollution, and current research is focusing on determining their potential negative impacts (Shah and Mraz 2019). Guidelines for evaluating the risks associated with the application of nanoscience and nanotechnology in the food and feed chain have been published by the European Food Safety Authority (EFSA) (Hardy et al. 2018).

Labeling requirements for nanotechnology in food

The use of NPs has also enabled the development of "smart" and "intelligent" labeling concepts, as well as functional food contact materials (FCMs) that are more resilient, lightweight, and practical. Conventional food packaging is inert by design, whereas intelligent and active FCMs are specifically designed to interact with food, extending shelf life by preserving or enhancing the condition of packaged products. This can be achieved by transferring or absorbing substances into or out of the food and its environment, such as through a disinfectant agent, or by using a labeling system that indicates food expiry – for example, by changing color

when the maximum storage temperature or shelf life is exceeded (a process known as freshness monitoring). Moreover, according to labeling requirements, the term "nano" must always follow the component name on labels containing NMs (Gottardo et al. 2021).

Consumer awareness and perception

Two guidance documents have been released recently by the EFSA. To ensure consumer protection, one outlines the technical requirements for demonstrating the presence of minute particles or determining whether a product's nanoscale properties persist during use. The other guides scientific risk assessment and appropriate safety testing of NMs (Schoonjans et al. 2023). Although public acceptance and awareness play a significant role, food manufacturers often overlook them. In fact, many manufacturers prefer to develop new products "underground" and keep them hidden from the public, possibly due to competition and trade secrecy (Chun 2009). A case study in Singapore showed that the public's negative perception of nanotechnology is worsened by limited awareness of its harmful implications (George et al. 2014). Businesses should also take note of food product policies that incorporate packaging technologies intended to benefit consumers. While some view such packaging as a useful tool, others regard it as a strategy to promote sales (Siddiqui et al. 2022). To effectively communicate with consumers and influence other stakeholders – such as government bodies regulating NMs in food packaging and industries applying nanotechnology in food systems – the media and publishers play a crucial role (Bumbudsanpharoke and Ko 2015).

The dual public voices about nanotechnology in the food industry are assent and altruism (Brown et al. 2015). Consumer food preferences are complex, influenced not only by economic and health concerns but also by social and psychological factors. However, studies on consumer behavior have typically focused on economic and health-related aspects, with social and psychological dimensions receiving less attention (Huang et al. 2020). Today, packaging design is significant because consumers expect it to reflect their aspirations for health and well-being. Packaging can also influence consumption habits and promote healthier lifestyles as a marketing tool (Bou-Mitri et al. 2021). A major challenge for the food industry is meeting consumer demand for food that is safer, more convenient, higher quality, and more natural. The demand for environmentally friendly packaging and products, produced through sustainable and efficient processes, is steadily increasing (Trajkovska Petkoska et al. 2021). Likewise, the need for smart foods with enhanced nutritional value is rising due to changing dietary habits and the rapid pace of urbanization (Nayak et al. 2021).

Future directions

The majority of nations producing NMs lack appropriate laws about nanotechnology. Therefore, comprehensive legislation and regulations, along with stringent toxicological screening procedures, are necessary for the permissible use of nanotechnology (Neme et al. 2021). A clearly defined regulatory objective is also required for the effective control of nanotechnologies in the food industry (Fletcher and Bartholomaeus 2011). Future progress lies with researchers in developing more efficient and adequate nanocarriers with enhanced bioavailability that preserve food's flavor, quality, and appearance during carrier incorporation. When antigen-specific markers are used to create polymer nanocomposite films by incorporating NPs into food packaging, the concept of smart packaging can be fully realized (Hamad et al. 2018). Nanotechnology is also valuable in plant disease prevention and agricultural development (Tripathi et al. 2017). Biosafety regulations are essential for the safe application of synthetic, eco-friendly NPs in the food and agricultural sectors. For example, the Food and Drug Administration (FDA) in the United States regulates food packaging and nano-enabled foods, while the European Union oversees food additives developed using nanotechnology (Gupta et al. 2023).

The next wave of the agri-tech revolution will heavily rely on nanotechnological interventions. Despite its potential, nanotechnology faces several challenges. It has given rise to a number of tools for enhancing the agronomic traits of plants, including those for improving stress tolerance, increasing plant resistance to fertilizers and pesticides, and developing nanosensors for smart agriculture and plant genetic engineering (Kumari et al. 2023). Future food packaging materials could also be transformed by carbon nanotubes, potentially leading to active and intelligent packaging systems (Wang and Irudayaraj 2008).

The global forum for discussing nanosafety issues is the UN Strategic Approach to International Chemicals Management (SAICM). SAICM incorporated new acti-

vities related to NMs and nanotechnologies into its Global Plan of Action, along with a nano-specific resolution (Karlaganis et al. 2019).

With its ability to manipulate matter at the atomic level, nanotechnology holds considerable potential to revolutionize many aspects of medical care, including drug delivery, regenerative medicine, equipment operation, diagnosis, and disease monitoring. It also provides access to advanced research tools that can aid in the development of medications for various conditions. Nanotechnology can be used to deliver drugs to specific body cells, thereby reducing the likelihood of failure or rejection (Haleem et al. 2023). Applications of nanotechnology have enabled earlier disease identification, including the use of carbon nanotubes, Au nanorods, and rapid, cost-effective detection methods. Smart tablets equipped with nanobots designed to target specific cancer cells could be used to diagnose and ensure that affected individuals receive treatment as directed. Tools and processes also improve the safety, efficacy, and physiochemical characterization evaluations of NMs and nanosurfaces in medical device engineering (Haleem et al. 2021; Zhang et al. 2021). NanoFlares are particles engineered to bind to specific genetic targets on cancer cells and illuminate upon detection. Cancer nanomedicine is a relatively new field of study, and regenerative immune sensors represent an emerging area of interest, allowing semicontinuous monitoring and recurring patterns for statistical reliability (Wang et al. 2021; Jurj et al. 2017; Sarmah et al. 2021; Rae and Jachimska 2021).

Conclusions

This review highlights the role of nanotechnology in the food system, summarizing its opportunities and risks for human health while exploring the types of NMs used in food, their regulatory approaches, safety evaluations, and risk assessments. The integration of nanotechnology in food science offers numerous benefits, including improved food quality, enhanced safety, and greater sustainability, and its applications are expected to expand significantly. NPs pave the way for innovation, addressing complex and persistent challenges within the food industry. The incorporation of advanced nanotechnologies is projected to have a transformative impact on food systems, a crucial element of human nutrition. Staying informed about emerging technologies is essential to fully leverage their potential in food science.

Nanotechnology provides innovative solutions to key challenges, such as improving safety, enhancing nutrition, reducing waste, and promoting sustainability, while meeting the rising demand for high-quality, health-focused products. However, concerns about its potential toxicity must be addressed. NPs may accumulate in the body, potentially causing cellular damage, oxidative stress, or inflammation. Moreover, the long-term effects of NP ingestion on human health and the environment remain unclear. Rigorous safety evaluations, regulatory frameworks, and responsible implementation are critical to mitigating these risks.

Despite these challenges, nanotechnology offers a promising path forward, with the potential to provide targeted therapies, improve treatment efficacy, and reduce environmental impact. Ongoing research is essential to fully understand the behavior of NMs and their effects on human health, ensuring a safer and more sustainable future for the food industry. Future research in food nanotechnology should prioritize safety, sustainability, and long-term impacts. Policies must ensure clear labeling, standardized testing, and transparent risk assessments to build consumer trust.

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S.Y. designed the study. J. conducted data analysis and interpretation. S.Y. performed manuscript writing. S.Y. and R.K. edited and reviewed the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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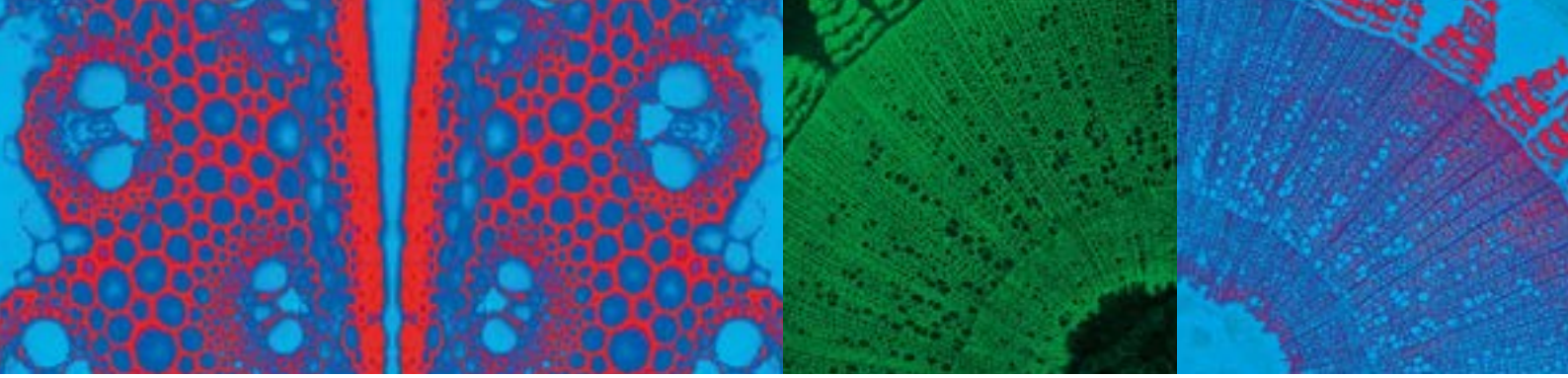

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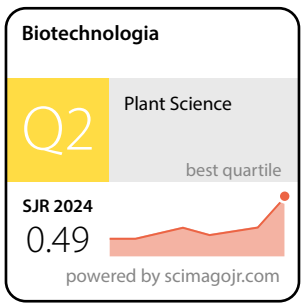

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