

REVIEW

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Bacterial derivatives mediated drug delivery in cancer therapy: a new generation strategy

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Abstract

Cancer is measured as a major threat to human life and is a leading cause of death. Millions of cancer patients die every year, although a burgeoning number of researchers have been making tremendous efforts to develop cancer medicine to fight against cancer. Owing to the complexity and heterogeneity of cancer, lack of ability to treat deep tumor tissues, and high toxicity to the normal cells, it complicates the therapy of cancer. However, bacterial derivative-mediated drug delivery has raised the interest of researchers in overcoming the restrictions of conventional cancer chemotherapy. In this review, we show various examples of tumor-targeting bacteria and bacterial derivatives for the delivery of anticancer drugs. This review also describes the advantages and limitations of delivering anticancer treatment drugs under regulated conditions employing these tumor-targeting bacteria and their membrane vesicles. This study highlights the substantial potential for clinical translation of bacterial-based drug carriers, improve their ability to work with other treatment modalities, and provide a more powerful, dependable, and distinctive tumor therapy.

Keywords Bacterial derivatives, Bacteria-derived membrane vesicles, Drug delivery, Bacteria-mediated cancer therapy, Engineered bacteria

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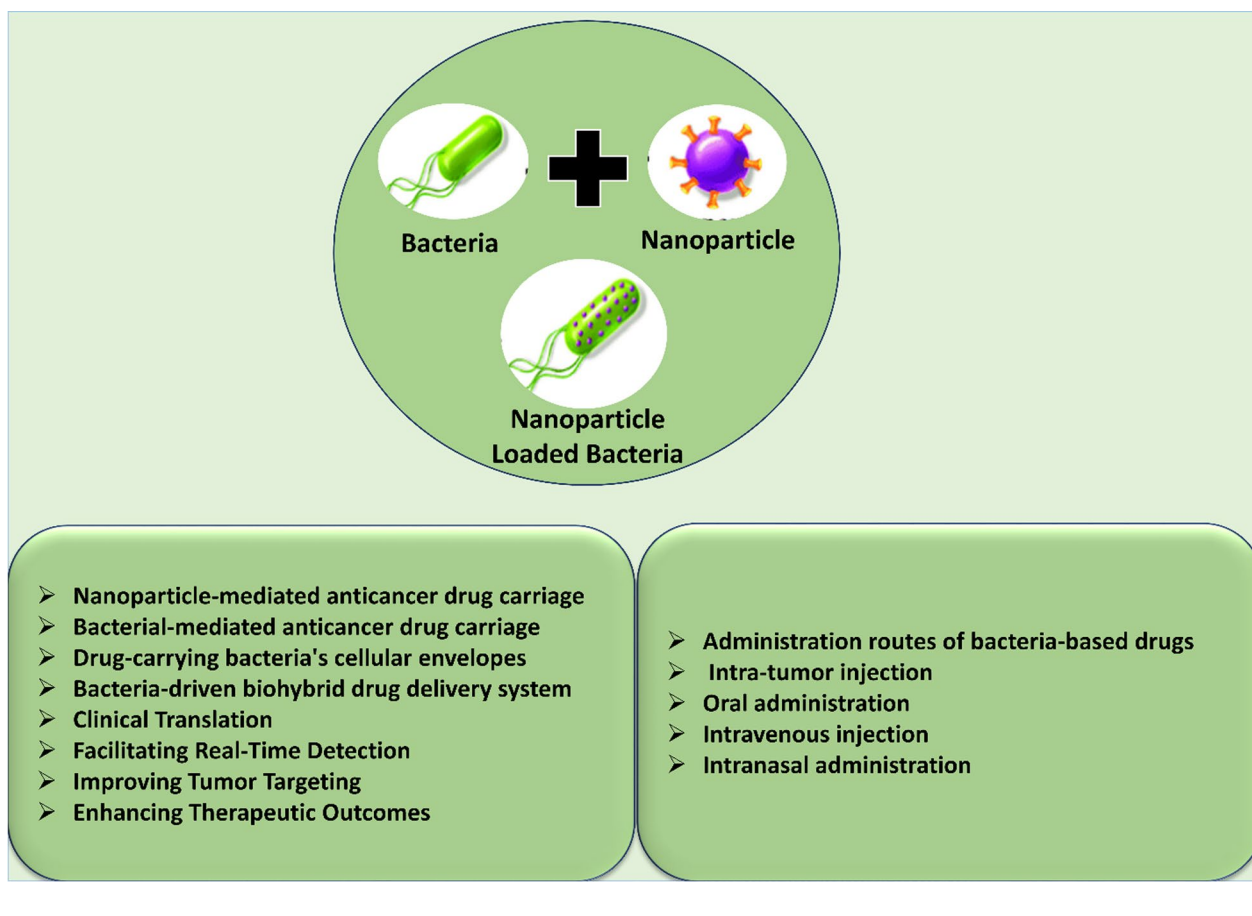
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Graphical Abstract

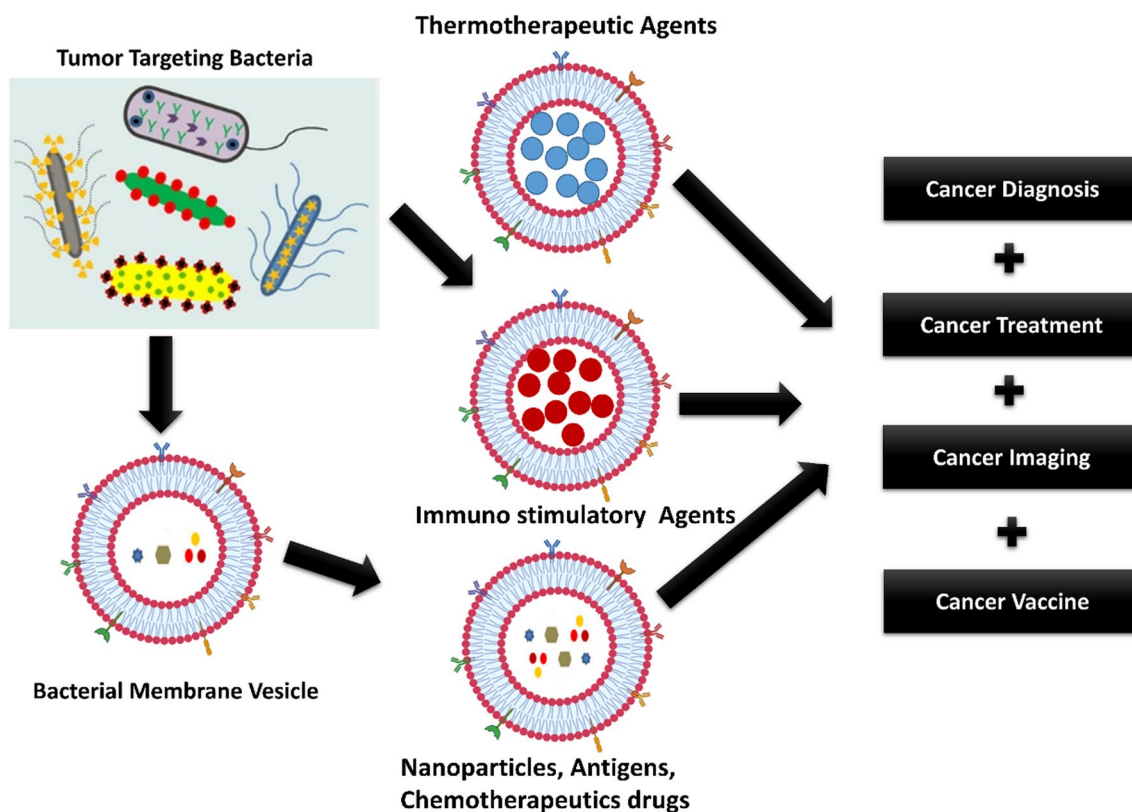


Introduction

Cancer is a complex, devastating disease that invades surrounding tissues and organs, leading to serious health complications. Cancer is a major global health challenge, encompassing various different cancer types. According to the WHO, cancer is a leading cause of death worldwide, with an estimated nearly 10 million deaths in 2020, 20 million new cancer cases, and 9.7 million deaths in 2022 [1]. The most common cancer types include lung, breast, prostate, stomach, and colorectal cancers. In 2022, nearly half of all cases (49.2%) and most cancer-related deaths (56.1%) worldwide were predicted to occur in Asia [1, 2]. Cancers of the lung, prostate, and colorectum in males. The lung, breast, and colorectum in women account for the majority of deaths [2, 3]. Due to limited resources and infrastructure, cancer is also a significant health concern in low- and middle-income countries. Early detection and treatment of cancer are vital to improving outcomes for cancer patients.

Researchers have been developing various new methods to improve the outcomes of tumor patients, among that, drug delivery methods have been raised to the forefront of studies due to the efficiency in recent years. Numerous therapeutic agent delivery systems, such as liposomes and nanoparticles, have been developed and extensively tested in animal models and clinical trials because they may increase the effectiveness of treatment by lowering toxic side effects, extending circulation time, and enhancing tumor specificity. Few therapeutic drugs truly reach the cancer site using these kinds of drug delivery systems due to inevitable instability, leakage, inadequate local targeting [4]. As a result, it's critical to create novel drug delivery methods that can get past biological and physical obstacles to transport and reach malignant tissue in sufficiently high quantities.

Bacterial-based drug delivery for cancer is an innovative approach that harnesses the unique properties of bacteria to target and deliver therapeutic agents specifically to tumor cells. By utilizing the natural ability of



Scheme 1. MVs-mediated delivery of diagnosis, imaging, treatment and vaccine agents for cancer therapy

certain bacteria to selectively accumulate in tumors, this method offers a promising strategy for enhancing the efficacy and reducing the side effects of cancer treatment [4–6].

Bacteria show great promise as medicinal, bioimaging, and diagnostic agents due to their distinct features. First of all, under the correct circumstances, bacteria can be easily genetically altered and grown in vast quantities. It's interesting to note that different bacterial species can colonize different parts of mammalian hosts' bodies, such as the skin or the stomach, as well as disease locations like tumor tissues and abscesses. Furthermore, bacteria are naturally able to interact with living things [4, 5, 7]. For example, they can adhere to host epithelial cells and decorate their surface with synthetic materials like different kinds of nanoparticles and medications that carry cargo. Furthermore, bacteria have the capacity to produce outer membrane vesicles (MVs) either actively or passively, and these MVs have demonstrated a great deal of promise as a form of effective and safe drug carrier [5, 6, 8]. Recently, bacteria-derived membrane vesicles have been successfully used to load different kinds of diagnosis, imaging, treatment and vaccine agents with various structures, hydrophobicity, charges and solubility, for cancer therapy (Scheme 1).

The use of bacteria and bacterial derivatives bacteria has been shown to significantly influence the fabrication of controlled targeted medication delivery systems to combat the spread of cancer. Bacteria and their derivatives are unique medication carriers for cancer therapy compared to most other conventional drug delivery methods. They have the ability to penetrate physical barriers, gather in tumor tissues, and trigger immune responses that are anti-tumorous. Moreover, they may be chemically and genetically altered to generate and transport anticancer drugs into tumor tissues, improving the safety and effectiveness of cancer treatment while reducing the harmful effects on healthy cells.

This article reviews recent developments of drugs delivery systems mediated by bacteria, and it also discusses the advantages and present obstacles of using these to treat cancer. In particular, concentrating on several therapeutic approaches within the framework of bacterial-mediated drug delivery systems, which fuse bacteria with nanoparticles to combat cancer. This work also describes the genetic engineering used to genetically modify a variety of pathogenic and non-pathogenic microorganisms in order to promote tumor regression. Drug delivery systems based on microorganisms have proven to be a reliable and efficient approach for tumor theranostics. Finally,

we discuss the clinical translation and future prospects of bacterial derivatives-based cancer treatments.

Bacterial-mediated anticancer drug carriage

Since the late nineteenth century, researchers have been exploring the potential use of bacteria and spores as agents to combat tumors, inspired by observations that bacteria could infiltrate tumor cells and impede their growth [9–12]. This pioneering approach involves injecting various therapeutic agents, including potent proteins and low-molecular-weight drugs, directly into tumors. Several anaerobic bacterial species have demonstrated the capacity to overcome physiological barriers that often impede the effectiveness of conventional chemotherapeutics. They are well-suited for precise localized tumor targeting due to their ability to thrive in the hypoxic conditions inside tumors [13, 14]. However, challenges persist, including dosage-dependent bacterial side effects and issues related to limited therapeutic efficacy, as the reticuloendothelial system can clear bacteria before they reach their specific targets [15, 16]. A cooled charge-coupled device detector detects and tracks the light released by Lux-expressing bacteria. The administration of these microorganisms to animals offers an extremely useful method for producing sensitive whole-body pictures with a very low backdrop. With many mouse tumor models, this imaging method should allow for the robust measurement of bacterial migration into primary and metastatic tumors in real-time [16].

To mitigate these challenges, extensive research has focused on reducing systemic toxicity through genetic and chemical modifications of microorganisms. For

instance, the deletion of crucial virulence factor genes has been utilized to attenuate various bacterial species, including *Salmonella*, *Clostridium*, *Escherichia coli*, and *Listeria* (Table 1) [17–23]. In recent years, Phase I/II clinical studies have shown progress in various bacterial treatments. These treatments typically involve the use of modified microbes containing cytotoxic proteins, cytokines, angiogenesis inhibitors, antigens, and antibodies as anticancer agents [24–26]. Modified bacteria have leveraged their intricate sensory systems to enable self-guided movement in response to gradients of oxygen, pH, temperature, and various attractive chemical compounds. Because of their biological functions and biocompatibility, modified bacteria can administer anticancer treatments within in vivo settings, capitalizing on their unique capabilities [27]. For instance, their chemotactic migratory abilities propel bacteria toward preferred nutritional environments, while their anaerobic and hypoxia tropism traits direct them to the hypoxic regions of disease lesions (Fig. 1).

Furthermore, substantial efforts are dedicated to expanding bacteria-powered biohybrid microswimmers. These biohybrids enhance the capabilities of microswimmers designed to transport synthetic vehicles, such as liposomes, NPs, and hydrogels for drug delivery. They achieve this by incorporating the self-directed biotic momentum and detection skills of microbes. An illustrative example is the utilization of magneto-aerotactic bacteria in a micromotor system to deliver drug-loaded liposomes to tumor locations, as demonstrated by Felfoul et al. [28] Bacteriabots, with synthetic microparticles attached to bacterial surfaces through biotin-streptavidin

Table 1 The delivery of anticancer medications through bacterial mediation is an innovative approach that holds promise in cancer treatment

Bacteria name	Therapeutic mediator	Adaptation approach	Therapy outcome	Ref
<i>Salmonella typhimurium</i>	Paclitaxel-loaded liposomes	Biotin-streptavidin	Chemotherapy	[168]
<i>Salmonella typhimurium</i> YS1646	DOX loaded-low-temperature sensitive liposome	Biotin-streptavidin	Chemo-immunotherapy	[169]
<i>Escherichia coli</i> MG1655	Doxorubicin and Fe ₃ O ₄ NPs	Surface charge and non-covalent interactions	Chemotherapy	[170]
<i>Escherichia coli</i> MG1655	DOX and SPIONs loaded soft red blood cells	Biotin-avidin-biotin	Chemotherapy	[171]
<i>Listeria monocytogenes</i>	188-Rhenium	Listeria-binding antibodies	Radiotherapy	[172]
<i>Listeria monocytogenes</i>	32-Phosphorus	Metabolic labeling	Radiotherapy	[173]
<i>Salmonella typhimurium</i> VNP20009	Polydopamine	Coating	Photothermal-therapy	[15]
<i>Salmonella typhimurium</i> Ty21a	Gold NPs	Encapsulation	Photothermal-therapy	[27]
<i>Escherichia coli</i> MG1655	Fe ₃ O ₄ NPs	Covalent linker	Photothermal-therapy	[174]
<i>Salmonella typhimurium</i> YB1	Indocyanine green	Biotic/abiotic cross-linker	Photothermal-therapy	[163]
<i>Escherichia coli</i>	CD47 nanobodies	Genetic modification	Immunotherapy	[21]
<i>Salmonella typhimurium</i>	FlaB	Genetic modification	Immunotherapy	[175]
<i>Salmonella typhimurium</i>	NY-ESO-1	Genetic modification	Immunotherapy	[176]
<i>Salmonella typhimurium</i>	Cytolysin A	Genetic modification	Protein-based biotherapy	[177]

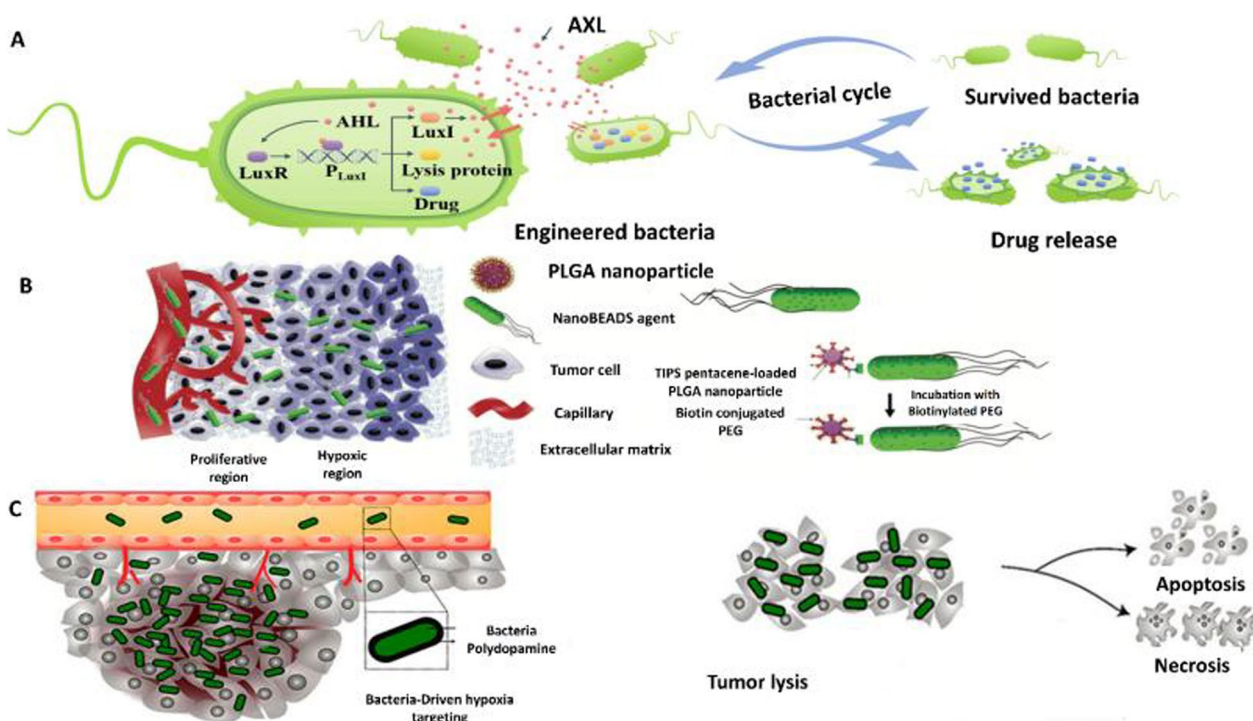


Fig. 1 **A** Genetic engineering. Through the bacterial cycle, plasmid-transfected bacteria may generate the medicine constantly and accomplish pulsed release of the medication. **B** Grafting of linkers. Biotin antibody-modified and streptavidin-modified NP “drug-linker-bacteria” is formed by *salmonella*. Reproduced from ref. [9]. MDPI, Copyright 2023. **C** The killing of tumor cells produced by photothermal treatment based on pDA-VNP enhances bacteria-mediated biotherapy much further, Reproduced from ref. [166]. ACS Nano, Copyright 2018

linkages, exhibit increased adherence to gastrointestinal and urinary system epithelial cells due to the lectin’s preference for mannose molecules on cell surfaces. As shown in Table 1, the delivery of anticancer medications through bacterial mediation is an innovative approach that shows potential in cancer treatment.

Bacterial surface modification strategies and advantages

Bacteria’s distinct qualities, including their capacity to manipulate DNA, grow quickly, colonize well, and exhibit targeted behaviors, have drawn a lot of interest among the biomedical community. But there are several barriers that hinder therapeutic implementation, including the unavoidably harmful adverse reactions of bacteria and inadequate colonization in disease locations [9, 29]. Using the numerous groups of chemicals on the surface of bacteria, surface alterations have been carried out to modify the structure and composition of the bacteria’s surface, reduce their toxicity, or add unique therapeutic components to modify the biological characteristics of the bacteria and give them novel roles. The advancement of such methods will show the prospective role that bacteria could have in medicine, particularly in tumor immunotherapy. Generally, bacterial methods for

modifying surfaces can be classified into three categories: chemical, physical, and biological (Fig. 2).

The bacterial surface consists of polysaccharides, proteins, and lipids which make up the complex biological structure. As an essential part of the bacterial cell wall, peptidoglycan is mostly made up of small peptide chains, *N*-acetylmuramic acid, and *N*-acetylglucosamine. Furthermore, a multitude of chemical locations, including free thiols, amines, hydroxyls, and carboxyl groups are found. These sites have the potential to form permanent chemical interactions with diverse substances.

Mostly in outer membranes of bacteria peptidoglycans and teichoic acids having plenty of hydroxyl groups. These molecules provide the membranes a negative charge that permits surface alteration by electrostatic interactions. At the moment, bacterial surface modification is accomplished using physical techniques including mechanical extrusion and electrostatic adsorption [30].

Biological modification of the surface uses genetic engineering, biosynthesis, and numerous other methods to change the surface structure of bacteria, giving them additional capabilities and improved biocompatibility, physical and chemical methods of modification improve the surface structure of bacteria artificially.

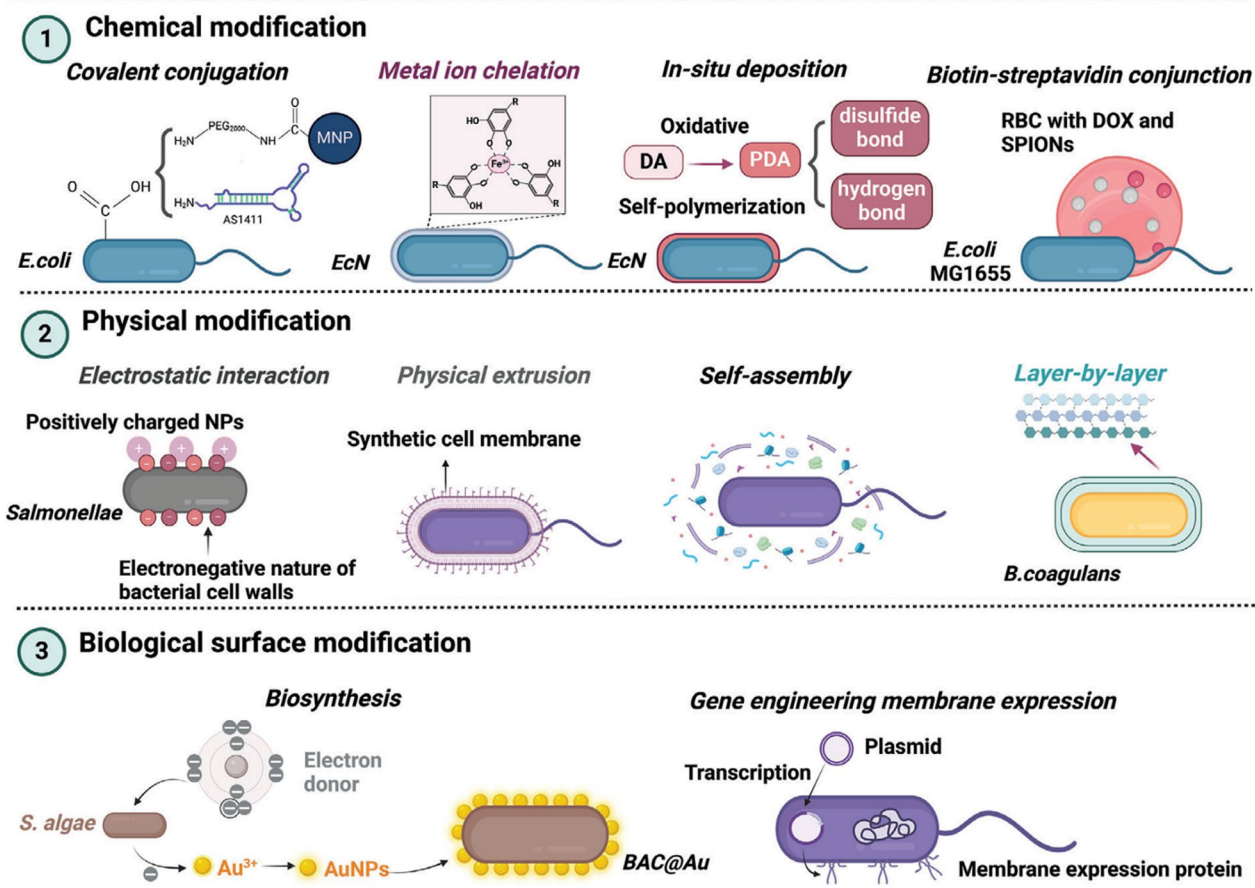


Fig. 2 Diagram illustrating the use of chemical, physical, and biological reactions to modify the bacterial surface. 1) Chemical modification contains the formation of covalent bonds by the many chemical locations on the surface of bacteria, as well as other techniques such metal ion chelation, in situ deposition, biotin-streptavidin conjunction, and in-situ polymerization. 2) Electrostatic contact, physical extrusion, self-assembly, and layer-by-layer techniques are the principal examples of physical interaction. 3) The two primary biological interactions that result in outcomes that are visible on the surface are genetic engineering and biosynthesis. Reproduced from ref. [30] Wiley, Copyright 2024

Drug-carrying bacteria's cellular envelopes

When compared to bacterial cells, cellular envelopes for drug administration offer several advantages, with a key benefit being their inability to colonize vital organs. By genetically modifying living bacterial cells to customize both their inner and outer surfaces, we can harness the potential of cellular envelopes. Importantly, cellular envelopes retain their immunomodulatory qualities by preserving their surface features [31]. Bacterial ghosts (BGs) represent a common type of cellular envelope, essentially the empty cell envelopes of gram-negative bacteria altered by the lysis gene E from bacteriophage X174. Gene E codes for a 91-aa polypeptide. When the simulated gene E is expressed under controlled conditions in gram-positive bacteria, these bacteria quickly perish without undergoing lysis [32]. The expression of protein E, a membrane protein capable of oligomerizing and forming transmembrane tunnel structures,

induces a differential in osmotic pressure between the cytoplasm and its adjoining environment. This leads to the expulsion of cytoplasmic content, microbial cell lysis, ultimately transforming the cell into a lifeless covering, justifying the term “bacterial ghost.” The remarkable stability of these cellular envelopes, which can even be lyophilized, is a noteworthy attribute [33–36].

For over 20 years, these non-denatured cellular envelopes have served predominantly as non-living delivery vehicles for medicines, antigens, nucleic acids, and various physiologically active substances, capitalizing on their intrinsic cellular features [37]. BGs, derivatives of *E. coli* NM522, were biologically modified in 1999 to facilitate the passage of biotinylated substances through their cytoplasmic membrane. To achieve this, the BG creation process commenced before the inner side of the bacterial cytoplasmic membrane produced a streptavidin fusion protein. Exploiting the strong binding affinity

between streptavidin and biotinylated substances, DNA of the plasmid combined with biotinylated poly-L-lysine and biotinylated fluorescent dextran could adhere to the surface of the BG crust. This study demonstrated that BGs, acting alongside streptavidin on the cytoplasmic membrane, might serve as drug delivery vehicles for the site-specific transportation of medications that have been biotinylated [38].

A companion study by Haslberger et al. investigated the immune system activation effects of various bacterial ghost (BG) platforms through in vitro absorption. The findings revealed remarkably effective BG acceptance and suggested that BGs, capable of triggering immunological responses, may hold value as in situ immunotherapy delivery systems. Measurement of IL-12(p70) production and IL-12(p40) mRNA accumulation indicates activation of IL-12. Particular significance lies in this interleukin in the induction of cellular TH1 immunological responses. Electron microscopy could verify the quick absorption of bacterial ghosts in macrophages, occurring within a half-hour to an hour. These sites can change the bacterial surface functionally by forming long-lasting chemical interactions with various substances [39]. Due to the immunosuppressive milieu often present in tumor-draining lymph nodes (TDLNs), oncolytic microorganisms

struggle to efficiently cross-prime tumor-specific T lymphocytes via antigen-presenting cells such as dendritic cells (DCs) in TDLNs. In this context, *Escherichia coli* (EcP) overexpressing pyranose oxidase (P2O) were used, encapsulated in low-concentration photosensitizer NPs and PEGylated mannose, to create a micro-to-nano oncolytic microbial therapy. P2O generated hazardous hydrogen peroxide upon treatment, leading to tumor regression and the release of tumor antigens. By promoting DC maturation and influencing the TDLNs' immuno-microenvironment, the enhanced TDLNs distribution by OMVs improved tumor antigen-specific T cell immune responses. This micro-to-nano oncolytic bacterium shows promise in altering TDLNs and eliminating tumors, as illustrated in (Fig. 3) [40].

By encapsulating the systemically delivered anticancer drug DOX within bacterial ghosts (BGs) derived from *Mannheimia haemolytica*, they significantly alleviated its severe side effects, highlighting the potential of these engineered BGs for controlled and prolonged drug administration [41]. Through in vivo investigations, these BG systems also demonstrated effectiveness in delivering medications to sites of ocular surface disorders [42]. In a separate study, resveratrol, a well-known polyphenolic substance with immunomodulatory properties,

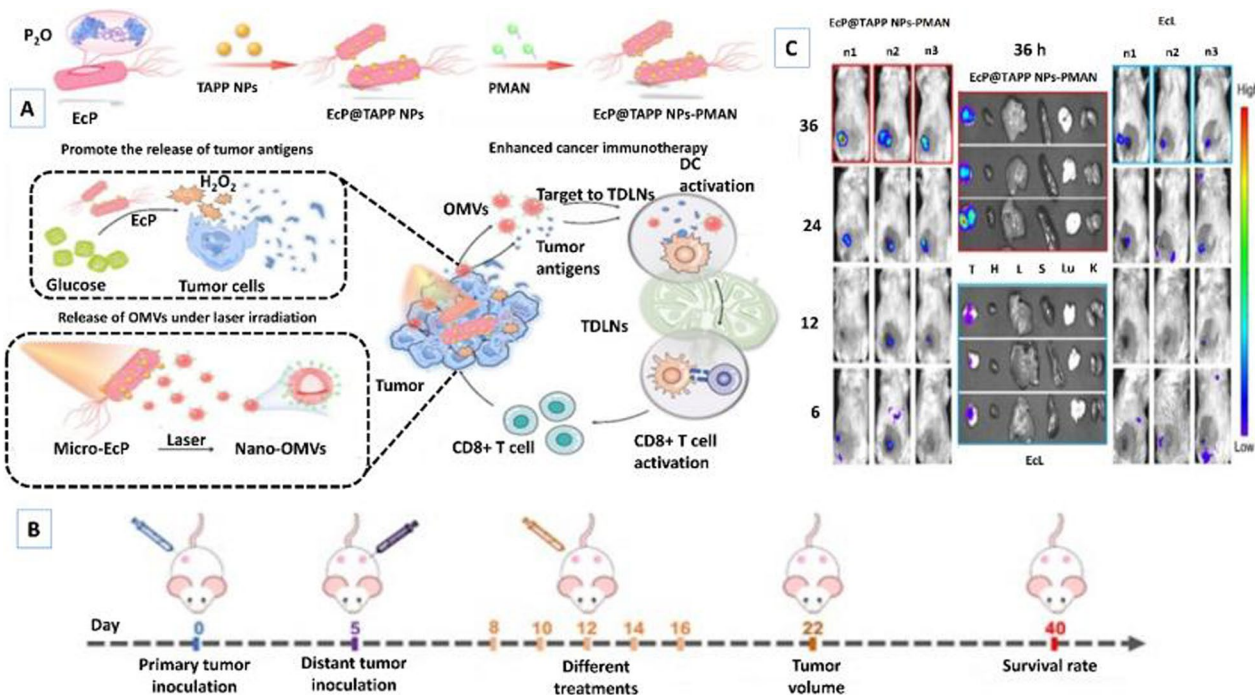


Fig. 3 A Laser irradiation-induced OMV release and immune activation effects in vitro. EcP@TAPP NPs-PMAN preparation diagram. B Diagram illustrating the timetable for creating and treating a bilateral tumor model. C TDLNs remodeling for improved immunotherapy and EcP@TAPP NPs-PMAN for cancer carnage. Following an intravenous injection of Ecl and Ecl@TAPP NPs-PMAN, the primary organs and tumor-bearing 4T1 mice's fluorescent pictures (T: tumor, H: heart, Li: liver, S: spleen, Lu: lung, K: kidney). Reproduced from ref. [40] with permission from Wiley, Copyright 2023

was incorporated into *E. coli* NM522 BGs. These resveratrol-loaded BGs exhibited an enhanced impact on macrophage cells when cultured through murine macrophage cells, emphasizing the potential of BGs as efficient delivery carriers for substances like resveratrol [43].

Bacteria that cause cancer may initiate innate immunity. Attenuated live bacteria provide significant safety hazards, whereas inactivated microorganisms have limited antitumor effectiveness. In this instance, Wang et al. demonstrate that manganese dioxide-coated paraformaldehyde-fixed bacteria administered intratumorally have a capacity to stimulate innate immune reactions, alter the immunosuppressive tumor microenvironment, and elicit tumor-specific and abscopal antitumor responses. In mice, rabbits, and tree shrews, one single intratumoural injection of mineralized *Salmonella typhimurium* inhibited the development of several subcutaneous and orthotopic tumor types and shielded the treated animals against tumor recurrence [44]. Additionally, they demonstrate that orthotopic

liver cancer in rabbits may be treated with mineralized bacteria delivered by arterial embolism (Fig. 4). The results of the study encourage the use of oncolytic calcified bacteria as effective and secure antitumor immunotherapeutics in more translational studies.

Minicells represent a distinct type of non-chromosomal cellular envelope that holds promise as a drug delivery system. Typically ranging in size from 100 to 400 nm, these minicells result from aberrant cell division. They contain minimal to no chromosomal DNA but retain all the RNA and membrane proteins of the parent cell in their molecular form [45, 46]. While minicells can undergo plasmid-directed protein synthesis, they lack the capacity for sexual reproduction. Notably, their ability to express therapeutic compounds encoded by recombinant plasmid DNA at specific target sites is a remarkable feature [47, 48]. In practical applications, siRNA-encoding plasmids have been effectively loaded into minicells, enabling RNA interference to inhibit the

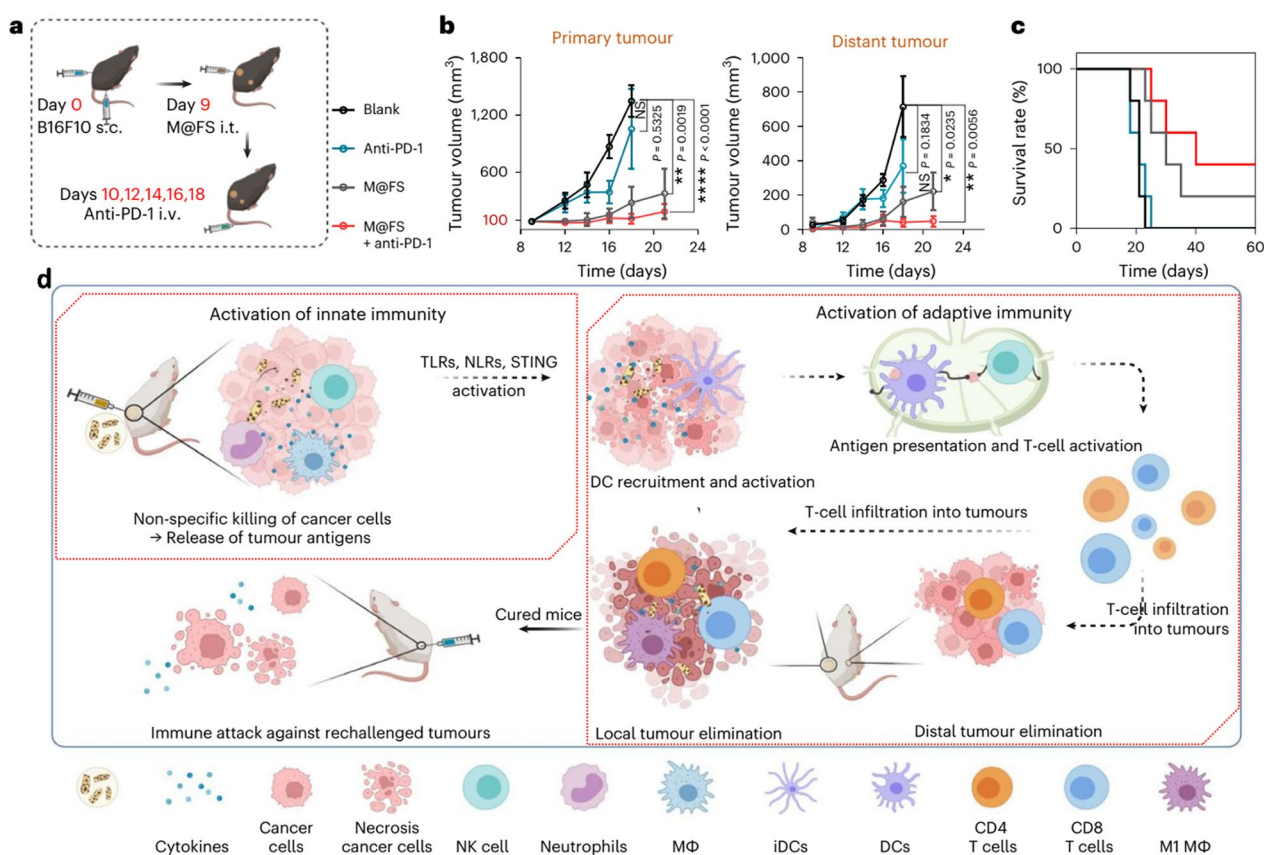


Fig. 4 Mineralized bacteria produce immunological protection and activate the systemic immune response. **a** Building and managing the B16F10 bilateral tumor model for melanoma. The following treatments are shown in the graphs **b**, **c**: normal saline (blank), 15 μg anti-PD-1 (i.v.), 20 μg mineralized FS (i.t.), and 15 μg anti-PD-1 (i.v.) + 20 μg mineralized FS (i.t.) (n = 5). Growth curves of primary tumors and distant tumors are shown in the graphs (**b**) and the survival rates of mice are shown in the graph (**c**). **d** The mineralized bacteria's successive immune activation and modulation methods against tumors. Reproduced from ref. [44] Nature, Copyright 2024

expression of proteins that support tumor development or enhance drug efflux mechanisms [49, 50].

Several envisioned applications underscore the diverse utility of non-chromosomal cellular envelopes in drug delivery. Their production is both straightforward and cost-effective, allowing manufacturing at various scales. Notably, they are characterized by extended shelf life and an inability to revert to pathogenic forms [51]. However, it is crucial to recognize that these cellular envelopes bear the same surface antigens as living bacteria, potentially inducing immunological responses. Despite this, these envelopes provide a pragmatic avenue for designing drug delivery systems, leveraging their internal storage capacity and exceptional internalization properties. Similar to living bacteria, a major challenge for drug carriage methods based on cellular envelopes is ensuring accurate spatiotemporal distribution. Incorporating artificial elements, such as magnetic NPs, into these enclosures may further enhance this controllability.

Generation of bacterial membrane vesicles

In a natural process, bacteria bud their membranes, releasing substances into their surrounding environment. Once separated and purified, bacterial membrane vesicles (BMVs) structures enveloped by lipid bilayers that

remarkably resemble eukaryotic extracellular vesicles (EVs). BMVs may be spontaneously released by bacteria, both Gram-positive and Gram-negative, typically ranging in size from 20 to 400 nm [52–54]. The environment in which these vesicles are released significantly influences various biological activities, including pathogenicity, horizontal gene transfer, metabolite export, phage infection, and intercellular communication [55, 56]. In this section, we discuss various types of BMVs formed by microbes, such as OMV, IMV, and DMV (Fig. 5) [167].

Differentiating between Gram-positive and Gram-negative bacteria is facilitated by their structural differences. Gram-positive bacteria possess a thick peptidoglycan coat but lack an outer lipid membrane, whereas Gram-negative bacteria have both an outer lipid membrane and a thin peptidoglycan layer [57]. Consequently, the type of bacteria influences the construction and development of bacterial membrane vesicles (BMVs) (Fig. 5) [167].

BMVs created by Gram-negative bacteria

Outer membrane vesicles (OMV)

Numerous studies have investigated bacterial membrane vesicles (BMVs) produced by Gram-negative bacteria [56, 57]. The distinctive structure of gram-negative bacteria is characterized by an outer lipid membrane and the

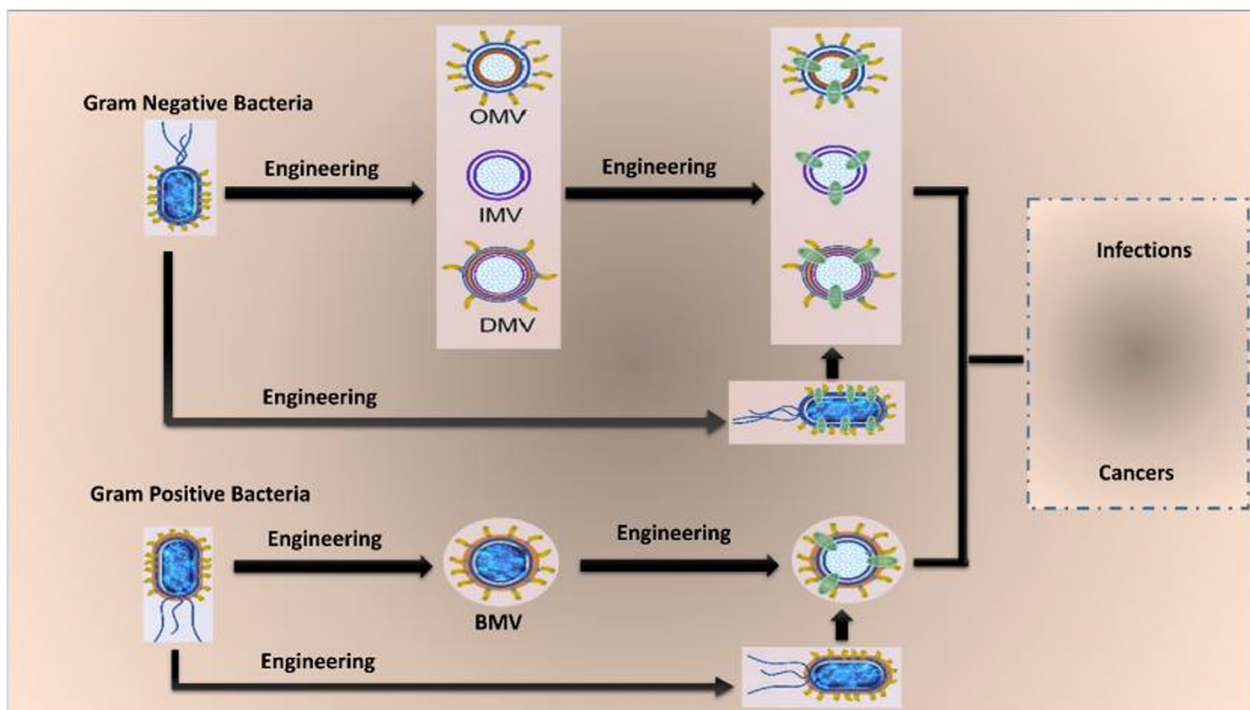


Fig. 5 Diagrammatic depiction of the several BMV types that are manufactured from either Gram-positive or Gram-negative bacteria, as well as their applications in the treatment of infections and cancer. Gram-negative bacteria’s outer membranes give rise to OMVs. DMVs (double-membrane vesicles) and IMVs (inner membrane vesicles) are made by physical or biological methods. Furthermore, BMVs are made using genetically altered Gram-positive and Gram-negative bacteria. Reproduced from ref. [167] with permission from Elsevier, Copyright 2022

presence of lipopolysaccharides (LPS). The outer lipid membrane of these bacteria spontaneously separates, leading to the formation of outer membrane vesicles (OMVs) [58, 59].

Initially, membrane proteins play a crucial role in connecting the outer lipid membrane to peptidoglycan, forming a stable Gram-negative envelope. However, as outer membrane vesicles (OMVs) begin to form, these proteins may become less stable. This instability can result from either the movement of connecting proteins causing a disruption in the linkage between peptidoglycan and the outer lipid membrane or a direct break in the connection. The disruption of these connecting proteins leads to the release of OMVs [56, 60]. It is important to note that local environmental factors significantly influence the generation of OMVs. Consequently, OMVs can carry a variety of cargoes, including proteins and genetic materials. Additionally, they may contain proteins that facilitate the interaction between peptidoglycans and the outer lipid membrane.

The intricate processes involved in OMV generation result in the production of vesicles with variable sizes and compositions [61]. Among the materials found in these vesicles are phospholipids, proteins, nucleic acids, virulence factors (such as LPS), and occasionally metal ions, signaling molecules, and metabolites [62]. The study of OMV proteins is crucial for understanding tissue targeting and signal transduction, and proteomics has played a vital role in this regard. Various techniques have been employed in proteomic investigations, including direct trypsin digestion of electrophoresis gels followed by liquid chromatography (LC)-MS/MS, 2D electrophoresis followed by *in situ* mass spectrometry (MS), and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [63, 64]. These investigations consistently support the notion that OMVs are predominantly composed of proteins associated with the outer membrane, further substantiating the natural formation of OMVs [65, 66]. Local signals, including stressors and stimulants, significantly influence the formation of OMVs in Gram-negative bacteria. Enhancing the culture medium and adjusting environmental conditions can lead to improvements in OMV production and enable better control over their compositions [67–70].

Inner membrane vesicles (IMVs)

Gram-negative bacteria encounter challenges in spontaneously generating membrane vesicles due to the protective outer membrane and peptidoglycan layer surrounding the inner membrane. Moreover, concerns arise regarding potential immunological toxicity when utilizing outer membrane vesicles (OMVs) in medical applications, primarily due to the presence of LPS in OMVs.

To address these challenges, Kim et al. introduced a technique for producing bacterial protoplast-derived nanovesicles (PDNVs), also known as inner membrane vesicles, from Gram-negative microbes [71].

Protoplasts, lacking the peptidoglycan layer (cell wall) and the toxic outer membrane, are achieved through the action of lysozyme, representing a bacterial state devoid of these components. Subsequently, protoplasts give rise to inner membrane vesicles (IMVs) once the outer membrane has been eliminated. The utilization of a serial extrusion method has proven effective in the production of IMVs, which have found application in the development of a universal adjuvant-free vaccine. The remarkable aspect is that IMVs have demonstrated superior effectiveness and safety compared to vaccinations using outer membrane vesicles (OMVs). Strong humoral and cellular immune responses specific to antigens have been effectively elicited by IMVs. This breakthrough opens new avenues for the development of vaccines that are not only safer but also more effective [72].

Double membrane vesicles (DMVs)

When used as drug delivery systems, both outer membrane vesicles (OMVs) and inner membrane vesicles (IMVs) may face stability and cargo loading challenges as they share the same lipid membrane. Addressing these issues, especially in the context of medicinal applications, necessitates the creation of vesicles that incorporate both membrane linkers made of peptidoglycans and lipid membranes of the bacterium. This is particularly crucial for the development of vaccines [73, 74].

This study has confirmed that double membrane vesicles (DMVs) indeed encompass the complete bacterial membrane and possess the unique property of containing multiple crucial antigens essential for vaccine production. This achievement was realized through the application of cryogenic transmission electron microscopy (cryo-TEM), biochemistry, and proteomics. In comparison to outer membrane vesicles (OMVs) generated from the same bacteria, DMVs significantly increased animal survival in a sepsis mouse model caused by *Pseudomonas aeruginosa*. This superior survival is attributed to DMVs' enhanced adaptive immunity and distinct biodistribution, most likely resulting from the presence of more pathogen-associated molecular patterns (PAMPs) on DMVs [55].

A study used double membrane vesicles (DMVs) to target various cells within tumor microenvironments and highlights the potential of the nitrogen cavitation method for generating DMVs from diverse bacterial sources. The endogenous targeting ligands were produced, and arginine-glycine-aspartate (RGD) peptides were expressed by *Escherichia coli* to construct DMVs. Within tumor

microenvironments, these DMVs demonstrated a spontaneous attraction to neutrophils, monocytes, and endothelial cells. Particularly noteworthy is the finding that DOX can be loaded with remarkable efficiency into DMVs using a pH gradient (12% w/w). DMVs loaded with Dox significantly reduced tumor diameters in a mouse model of melanoma compared to DMVs without the expression of targeting ligands, indicating the innovative and efficacious potential of DMVs as a platform for targeted drug delivery in cancer therapy [75].

BMVs formed by Gram-positive bacteria

Gram-positive bacterial membrane vesicles (BMVs) have emerged as a unique and captivating research area, recently gaining significant attention and evolving into a thriving field of study [76]. BMVs have been observed in Gram-positive bacteria from over 30 different species (Table 2) [77]. These vesicles carry a wide range of cargo molecules, including proteins, lipids, enzymes, poisons, and nucleic acids. Notably, Gram-positive BMVs exhibit substantial differences from Gram-negative outer membrane vesicles (OMVs), primarily in their absence of periplasmic components and lipopolysaccharides (LPS) [78].

The exact process controlling the biogenesis of Gram-positive bacterial membrane vesicles (BMVs) is still under investigation. In contrast to Gram-negative bacteria, which release outer membrane vesicles (OMVs) from their outer membrane, Gram-positive bacteria possess a robust cell wall composed of peptidoglycans, potentially hindering the formation of lipid membranes [79, 80]. According to a prevailing theory, enzymes could potentially degrade the peptidoglycan layer, exposing the lipid membrane and facilitating the development of BMVs [81, 82]. Gram-positive BMVs serve two essential

functions, namely the transfer of various chemicals and the promotion of bacterial survival [83, 84]. Studies have documented the movement of surface receptors in BMVs and the transfer of bacterial chromosomal DNA, particularly in Ruminococcin species. Remarkably, Gram-positive bacteria release BMVs that contain factors promoting nutrient uptake, antibiotic-degrading enzymes such as β -lactamase, and even harmful agents designed to enhance bacterial survival [85]. The size of Gram-positive BMVs typically ranges from 10 to 400 nm [86]. As shown in (Table 2), facilitation of anticancer medication delivery by bacterial membrane vesicles (MVs).

Bacteria-driven biohybrid drug delivery system

The primary objective of a drug delivery system is to transport and release medication precisely at the intended location in the body. This process aims to shield the drug from adverse conditions, including potential immune reactions and encounters with low pH levels, during its journey from the administration site to the site of action. Furthermore, the system should offer protection to healthy tissues against potential drug side effects (Table 3).

Bacterial cells not only function as efficient microswimmers but also act as microsensors, capable of perceiving alterations in the physicochemical properties of their environment, such as pH, oxygen levels, glucose levels, and temperature. They respond accordingly to these changes [87]. However, the effectiveness of bacterial sensing often relies on placing bacterial cells close to the target action site. Additionally, bacterial sensing is typically most efficient at short distances. Therefore, achieving active control over the placement of the

Table 2 Anticancer medication delivery facilitated by bacterial MVs

Parent bacterium of MVs	Therapeutic agent	Type of therapy	Type of cancer	Refs.
<i>Salmonella typhimurium</i>	Tegafur	Chemo-immunotherapy	Melanoma	[164]
<i>Salmonella typhimurium</i>	DOX	Chemotherapy	Brain tumor	[178]
<i>Salmonella typhimurium</i>	Paclitaxel	Chemotherapy	Breast, bladder, pancreatic, prostate and lung tumors	[179]
<i>Salmonella typhimurium</i>	DOX	Chemo-immunotherapy	Glioblastoma, colon cancer (CT26)	[180]
<i>Salmonella typhimurium</i>	DOX	Chemotherapy	Neuroblastoma	[181]
<i>Salmonella typhimurium</i>	DOX	Chemotherapy	Recurrent glioblastoma	[182]
<i>Salmonella typhimurium</i>	Melanin	Photothermal therapy	Breast cancer (4T1)	[183]
<i>Salmonella typhimurium</i> SL1344	Ovalbumin fragment	Immunotherapy	n/s	[184]
<i>Salmonella typhimurium</i>	Ovalbumin fragment	Immunotherapy	n/s	[185]
<i>Escherichia coli</i>	Basic fibroblast growth factor molecule	Immunotherapy	Melanoma (B16)	[186]
<i>Salmonella typhimurium</i>	Melanoma cytomembrane vesicles, PLGA-ICG NPs	Photothermal and Immunotherapy	Breast cancer (4T1)	[187]

Table 3 An overview of biohybrid nanocarriers based on bacteria for the treatment of cancer

Bacteria type	NP-bacteria relations	Bio-consolidate method	Nanomaterial	Therapeutic approach	Refs.
<i>L. monocytogenes</i>	Attached	Antigen/antibody and Avidin/neutravidin	Polystyrene NPs	Gene delivery and protein expression in tumoral cells	[188]
<i>E. coli</i>	Attached	Acid-labile linker	Free drug	Sustained release of drug	[189]
<i>Salmonella</i>	Adsorbed	Electrostatic interactions	PEI NPs	Cancer immunotherapy	[190]
<i>E. coli</i>	Attached	Tetrazine/norbornene click reaction	Polymeric pro-micelles	On-demand release of two drugs	[191]
<i>Salmonella</i>	Attached	Biotin/Streptavidin	PLGA NPs	–	[192]
<i>Magnetospirillum magneticum</i>	Attached	Michael addition to maleimide	Indocyanine green PLGA NPs	Photothermal therapy	[193]
<i>Salmonella</i>	Attached	Oxidation and self-polymerization	Polydopamine NPs	Photothermal therapy	[15]
<i>E. coli</i>	Adsorbed	Electrostatic interactions	Polyelectrolyte multilayer microparticles	Drug delivery with magnetic guidance	[170]
<i>E. coli</i>	Attached	Azide/DBCO click chemistry	MSNs	Transport of high amounts of drug	[194]
<i>E. coli</i>	Adsorbed	Electrostatic interactions	Carbon nitride NPs	Photoinduced in situ generation of cytotoxic species	[195]
<i>Clostridium novyi-NT spores</i>	Adsorbed	Electrostatic interactions	Branched Au NPs	Theragnostic combination therapy	[196]
<i>Bifidobacterium</i> and <i>Clostridium difficile</i>	Adsorbed/Attached	Electrostatic interactions and antigen/antibody	Au nanorods	Photothermal ablation	[197]
<i>E. coli</i>	Adsorbed	Metal-peptide affinity	Au NPs	–	[198]
<i>E. coli</i>	Attached	Carbodiimide chemistry	Fe ₃ O ₄ NPs	Chemodynamic therapy	[174]
<i>E. coli</i> and <i>Salmonella</i>	Engulfed	Incubation and electroporation	Liposomes	Enhanced drug delivery	[146]
<i>E. coli</i>	Attached	Bacterial affinity with glycolipids	SUVs, LUVs, and GUVs	–	[199]
<i>Magnetococcus marinus</i>	Attached	Carbodiimide chemistry	Liposomes	Enhanced drug delivery	[28]

medication delivery device within the body is considered ideal [88–90].

Synthetic mobile microrobots hold the potential to address challenges related to long-range communication and precise control. However, they encounter various technological limitations when operated at small sizes. The biohybrid approach, involving the integration of microbial cells with micro/NPs, aims to overcome these limitations [91]. Through this integration, biohybrids can collaborate and carry out advanced functions that neither the biological nor the synthetic component could perform independently. For instance, when in proximity to the target action site, bacteria can utilize chemotactic sensing and steering. When farther from the target, remote magnetic steering can be employed to guide microbes configured as an imitation microrobot body toward the desired location. This biohybrid technique accelerates the arrival of drug delivery systems at the target location, reducing the threat of sanction by the reticuloendothelial mechanism [92].

One major benefit of the biohybrid method is the “division of labor” between biological and

non-biological systems, which reduces the need for major genetic alterations to bacterial cells [93]. Concerns about the potential reversibility of genetic alterations in microorganisms used within the body have prompted challenges related to control and containment. Therefore, when incorporating bacteria into biohybrid systems, it is preferable to choose non-pathogenic, ideally food-grade, or commensal bacteria with few or no mutations. This choice aligns with the fundamental need for bacteria to actively respond to environmental gradients. While this approach may not fully harness all the advantageous features of bacteria, it simplifies the concept of bacterial drug delivery systems, making it more practical for implementation. Subsequent sections will delve into the essential design criteria for the improvement of biohybrid medication delivery systems [93]. Here, we focus on different routes of administration for bacteria-based drugs, including intra-tumoral injection, oral administration, intravenous injection, and intranasal administration. As shown in (Table 3), the summary of biohybrid nanocarriers based on various bacteria for the treatment of cancer.

Nanoparticle-mediated anticancer drug carriage

Nanoparticles (NPs) are extensively employed in tumor treatment due to their outstanding drug-loading capacity, ease of fabrication, and biocompatibility. Specifically, organic NPs are favored for drug delivery because of their numerous advantageous characteristics, including biocompatibility, biodegradability, and adaptability. The rapid advancements in nanotechnology have facilitated the incorporation of various therapeutic substances into NPs, such as liposomes, silica-based porous constituents, polymeric structures, and micelles [94]. A pivotal milestone in cancer treatment was the introduction of the first nano-delivery system, a liposome containing doxorubicin (DOX) with a diameter of approximately 100 nm. This innovation enhanced pharmacokinetics and drug distribution, thanks to the passive accumulation of nano-materials within tumors facilitated by the EPR effect. Drug-loaded NPs exhibited a higher tumor uptake rate and reduced systemic toxicity compared to drug-free NPs [95, 96] (Fig. 6).

Nanoparticle drug delivery systems have revealed great potential in cancer therapy, but they also come with certain drawbacks [8]. Nanoparticles may face challenges in penetrating deep into tumor tissues, which can affect

the distribution and efficacy of the drugs. Nanoparticles can be recognized and cleared by the immune system, reducing their circulation time and effectiveness in delivering drugs to cancer cells. Some nanoparticles may exhibit toxicity or induce inflammatory responses in healthy tissues, leading to adverse effects [97]. Controlling the release of drugs from nanoparticles can be challenging, affecting the therapeutic efficacy and potential side effects. Nanoparticles may require specific storage conditions and have limited stability, which can impact their shelf life and practicality for clinical use [98]. It is important to address these disadvantages through further research and development to optimize nanoparticle drug delivery systems for effective and safe cancer treatment [99].

Although each of these variables possess a significant impact upon the effectiveness of the nano-drug delivery process and, consequently, govern the efficacy of therapy, the NPs employed in medical therapy often have certain sizes, shapes, and surface properties. Nanoparticles (NPs) with diameters in the range of 10 to 100 nm are commonly deemed appropriate for cancer therapy due to their capacity to efficiently transport medications and provide an increased EPR effect. NPs larger than 100 nm

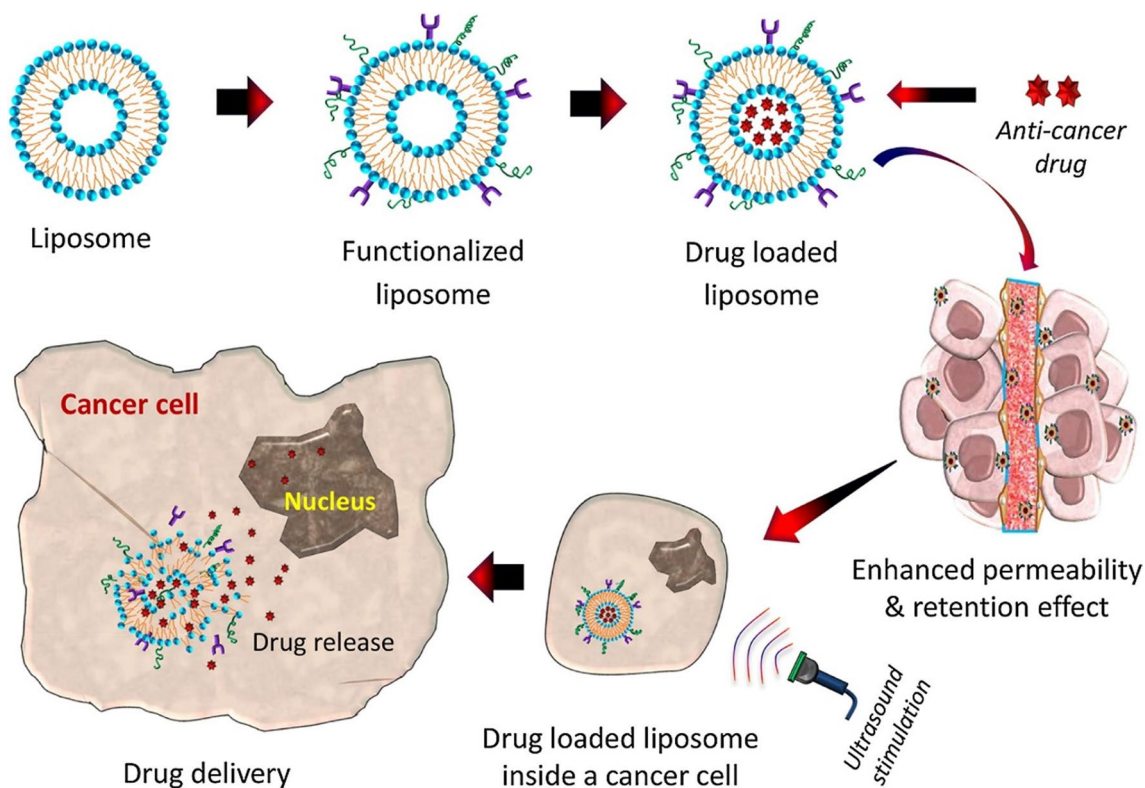


Fig. 6 An example of a liposome-based smart medication delivery system for cancer treatment in steps. Reproduced from ref. [7] Elsevier, Copyright 2019

will probably be removed from circulation through phagocytes, while smaller NPs may escape from the normal blood vessels and are swiftly filtrated through kidneys [99, 100].

Bacterially mediated drug delivery for cancer therapy does offer certain advantages compared to other nanoparticle-based drug delivery systems [100]. Bacteria have the ability to actively target and penetrate deep into tumor tissues, which can enhance the efficacy of drug delivery to cancer cells. Additionally, bacteria can be engineered to release therapeutic agents specifically within the tumor microenvironment, minimizing off-target effects and reducing systemic toxicity [101]. The inhibition of cancer mediated by bacteria involves several pathways, including immune system activation. Furthermore, some bacteria have inherent tumor-seeking properties, making them ideal vehicles for targeted drug delivery in cancer therapy. Overall, the unique capabilities of bacteria in targeting and delivering drugs to cancer cells make them a promising approach in cancer therapy [102].

Administration routes of bacteria-based drugs

Bacteria, whether wild-type or genetically modified, can enter the body through various routes. The choice of the administration route is influenced by several variables, including the target site, pharmacological properties, and ease of application. Apart from considerations related to patient comfort, the selected route of administration significantly impacts the efficacy of the treatment approach and the likelihood of adverse effects [103–105]. It is essential to understand that the design of the medication delivery system is strongly influenced by the chosen method of administration.

Intra-tumor injection

The most effective method for delivering medications to the targeted site is through direct injection into or in proximity to the affected region. Anaerobic bacteria such as *Clostridium novyi*, well-known for their exceptional capacity to target tumors, do not demonstrate the same predilection when given systemically in larger animals with a substantial blood volume [9]. In contrast, compared to systemic injection, intratumor injection of *S. typhimurium* results in a significant improvement in tumor suppression with fewer side effects [106].

For anatomical regions that are challenging to access via the circulatory system, such as the blood–brain barrier (BBB)-protected central nervous system, direct injection is the preferred technique [107, 108]. Bacterial targeting through direct injection into brain tumors has shown considerable promise [109]. In addition to enhanced efficacy, intratumoral injection of therapeutic

microorganisms leads to reduced systemic toxicity. Germs introduced via this method can be eliminated with antibiotics after treatment, thereby reducing the risk of genetically altered organisms being released into the environment. It is crucial to recognize that intratumoral injection is typically highly invasive and, as a result, extra multifarious.

Intravenous injection

Intravenous injection is an extremely effective method of medication delivery, especially for tumors with a richer blood supply compared to surrounding tissues [110, 111]. Although systemic injection of bacteria may result in severe systemic infections, experiments conducted in vivo with several strains of bacteria, including *S. typhimurium* and *Bifidobacterium bifidum*, have not been discouraged [22, 112]. Although systemic injection of bacteria may pose a risk of severe systemic infections, several strategies have been developed to mitigate these risks and harness the therapeutic potential of bacteria, particularly in cancer therapy. Bacteria can penetrate and thrive in hypoxic and necrotic regions of tumors where traditional therapies are less effective. In most cases, bacteria were able to traverse from the circulatory systems of mice to tumors and establish themselves within the core of the tumor. However, the feasibility of applying this approach in larger animals is currently under investigation [113, 114].

Oral administration

Oral administration is a widely adopted drug delivery method due to its convenience, adaptability, non-invasiveness, and greater patient amenability. It proves particularly beneficial for treating specific gastrointestinal (GI) tract diseases, such as inflammatory bowel syndrome. Researchers have shown a keen interest in oral medication administration, especially for oral immunizations delivered through the intestinal mucosa [115, 116]. To be effective in this role, the drug delivery system must traverse the intestinal epithelial barrier and interact effectively with the complex GI environment, which includes factors like significant pH changes, digestive enzymes, and the commensal bacteria population [117, 118].

One approach to address these challenges is using gastrointestinal (GI) commensal bacteria, such as lactic acid bacteria (LAB). Notably, *Lactococcus* and *Bifidobacterium*, commensal gut bacteria, have garnered significant attention [119]. For example, systemic IL-27 injection revealed to be less successful than oral administration of genetically modified *Lactobacillus lactis*, which produces immunosuppressive interleukin-27 (IL-27), to mice for the treatment of colitis [120, 121]. Additionally, there has been promise in treating type 1 diabetes with

this approach, reducing diarrhea caused by *C. difficile*, and avoiding hemolytic-uremic syndrome caused by *E. coli* O157:H7 [122, 123]. In another application, orally administered genetically engineered *B. longum* expressing alpha-melanocyte-stimulating hormone has been employed to treat ulcerative colitis, develop a vaccine against the Hepatitis C virus, and manage myocarditis using the anti-inflammatory cytokine IL-12 [124, 125]. Other bacterial strains under investigation for oral medication administration include *S. typhimurium*, *E. coli*, and *L. casei* [126–128]. Another method for the oral administration of medicinal microorganisms is encapsulation in protective materials [129]. This approach has been shown to significantly enhance the acid survival time of various bacterial strains, including *Lactobacilli*, *Bifidobacterium*, and *E. coli* [130, 131]. While oral bacterial treatment has demonstrated promising results in delivering bacteria to non-GI tract malignancies in mice [132, 133], it is essential to note that delivery efficiency may vary in humans, where bacterial escape from the stomach into the circulation is less common [134–136]. Additionally, it's worth mentioning that confining orally delivered microorganisms may pose challenges regarding potential environmental discharge [137–139].

It is important to remember that most lactic acid bacteria (LAB) employed as delivery vectors are food-grade, innocuous strains of bacteria obtained from fermented foods rather than true commensal bacteria. The physiology of these food-grade bacteria could make it difficult for them to survive in the gastrointestinal system of humans. Therefore, greater research into human commensal bacteria is becoming more important to develop drug delivery vectors that should last and function effectively within the gastrointestinal tract for an extended period of time [140, 141].

Intranasal administration

The nasal route was employed in earlier applications of bacteria-based medicine carriage, specifically in fecal microbiota transplants. However, due to the accessibility of mucosal surfaces, the intranasal administration of bacteria-based treatments is primarily utilized for immunization [142]. For instance, *Streptococcus gordonii* recombinant strains expressing specific antigens from *Mycobacterium tuberculosis* have been administered intranasally to activate CD4+ and CD8+ T cells and provide immunization against *Neisseria meningitidis*, a major cause of meningitis [143]. *Lactobacillus pentosus* has demonstrated the ability to stimulate an immune response against the influenza virus in the respiratory system [144]. Notably, several lactic acid bacteria elicited significantly stronger immune responses when administered intranasally as opposed to intragastrically.

Drug loading inside bacteria

Direct drug introduction into bacteria is also an option, as is the use of gene editing methods that make use of nucleic acids to help integrate pharmaceuticals into bacterial cells. Anticancer medications can be produced constantly by genetically engineered bacteria, whereas the direct loading technique allows for a single drug release after bacterial lysis. Advances in genetic engineering and synthetic biology have made it possible to design highly controlled and safe bacterial therapies. Researchers can precisely tailor bacterial strains to maximize their therapeutic benefits while minimizing risks.

Electroporation

A method employed to enhance cell membrane permeability is electroporation, which involves delivering brief, high-voltage electrical pulses to cells. This process induces temporary holes in the cell membrane surface, enabling the movement of substances that would otherwise struggle to pass through [145]. Reversible electroporation, characterized by brief electric pulses to facilitate the absorption of medicines or liposomes by bacteria, is utilized to maintain the biological activity of the bacteria. It is important to note, however, that electroporation does cause some damage to the bacteria. Electroporated bacteria may exhibit varying degrees of decreased biological activity compared to untreated control microorganisms.

Zoaby et al. investigated the incubation procedures of electroporation and direct incubation to enhance the delivery of DOX liposomes into *S. typhimurium* [146]. The findings revealed that the liposome absorption rate by bacteria was less than 5% when treated directly for over 4 h, while electroporation resulted in 62% of the bacteria absorbing the liposomes. Treated bacteria, compared to untreated controls, showed approximately a 20% reduction in growth. In a separate study, Xie et al. utilized gold nanorods to modify the surface of *E. coli* and employed electroporation to introduce 5-fluorouracil (5-FU) and zoledronic acid (Fig. 7) [147]. These modified bacteria exhibited a lowered existence ratio and crusade hustle to 87% and 88%, respectively, while loading 8.8% 5-FU and 10.5% ZOL. Upon exposure to near-infrared (NIR) light, gold nanorods generated heat, leading to the destruction of both bacteria and tumor cells. Subsequently, the medication was released from the deceased bacteria, enhancing its efficacy against tumor cells.

Genetic engineering

One technique used to modify bacteria's genes is the transfection of DNA fragments carrying anticancer medicines in the form of plasmids. Through genetic

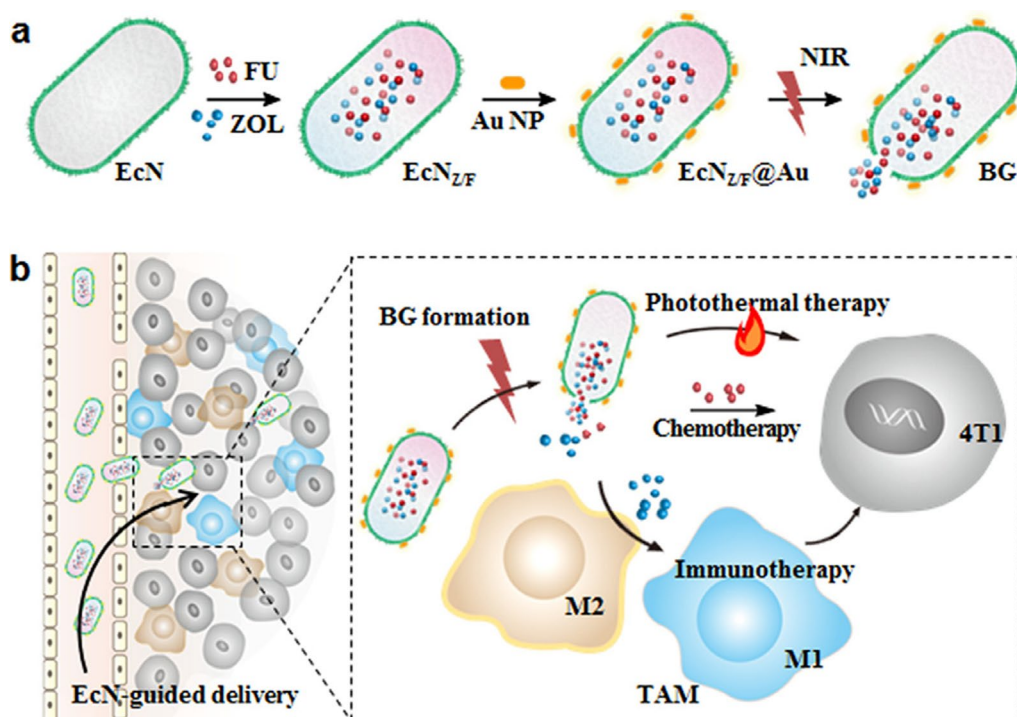


Fig. 7 EcNZ/F@Au/NIR preparation and treatment mechanism. **a** EcNZ/F@Au are created by electroporating FU and ZOL into EcN and decorating the EcN surface with Au NPs. **b** EcN's self-guided motility encourages EcNZ/F@Au to extravasate blood vessels, accumulate in tumor tissues, and engage with tumor cells. Tumor cells have photothermal effects under NIR light, which triggers their conversion to BGs. While ZOL's local release of BGs increases TAM polarization toward the M1 phenotype for immunotherapy, FU's release from BGs has a chemotherapeutic impact on tumor cells. Reproduced from ref. [147] Elsevier Copyright 2021

alteration, bacteria gain the ability to continuously produce various compounds associated with cancer prevention, including immunological factors, tumor antigens, cytotoxic agents, and more. This approach ensures that bacteria within the tumor can sustain their therapeutic actions. For instance, Nguyen et al. developed a tempered strain of *S. typhimurium* capable of expressing cytolysin A (ClyA) [148]. An L-arabinose is integrated into the bacterial ClyA gene, which specifically activates ClyA in the presence of L-arabinose, preventing damage to normal tissue cells. Upon introducing L-arabinose to the tumor site, the promoter activates, allowing the bacteria to consistently produce ClyA, targeting and destroying tumor cells. In another study, Chou et al. suggested that to overcome the immune tolerance to auto-antigens prevalent in liver cancer cells, a plasmid containing the Alpha-fetoprotein (AFP) gene was introduced into an attenuated strain of *S. typhimurium* [149]. Genetically modified bacteria expressing unique AFP associated with liver cancer triggered an immune response, involving T cells, to eradicate and eliminate the tumor (Fig. 8).

Yoon et al. developed a *Salmonella* strain incorporating IFN- γ to combat tumors in the context of utilizing bacteria for cytokine production in tumor therapy [150].

When administered subcutaneously, this genetically modified *S. typhimurium* significantly inhibited tumor development compared to unaltered phosphate-buffered saline, enhancing the survival of mice with tumors. In a distinct approach, Din et al. introduced an innovative bacterial drug delivery system employing genetic manipulation to synchronize, pulse, and repeat drug release [151]. This represents a novel method for periodic drug delivery, deviating from the conventional engineering of bacteria for the continuous expression of antitumor substances. The system's three essential components, regulatory protein LuxR, AHL synthesis protein LuxI, and the pointer molecule Acyl-homoserine lactone (AHL), collaborate to control the microbial cycle.

When the quantity of bacteria is low, most of the AHL that the bacteria make exits the cell and accumulates relatively little. Conversely, intracellular AHL reaches a threshold level when population density rises, significantly enhancing lysin protein synthesis. This, in turn, results in the release of medicine through the lysis of a substantial percentage of bacteria. The periodic cycling technique has the potential to take advantage of circadian rhythms in host-microbe interactions by controlling the recurrence rate with the generosity of these inhabitant

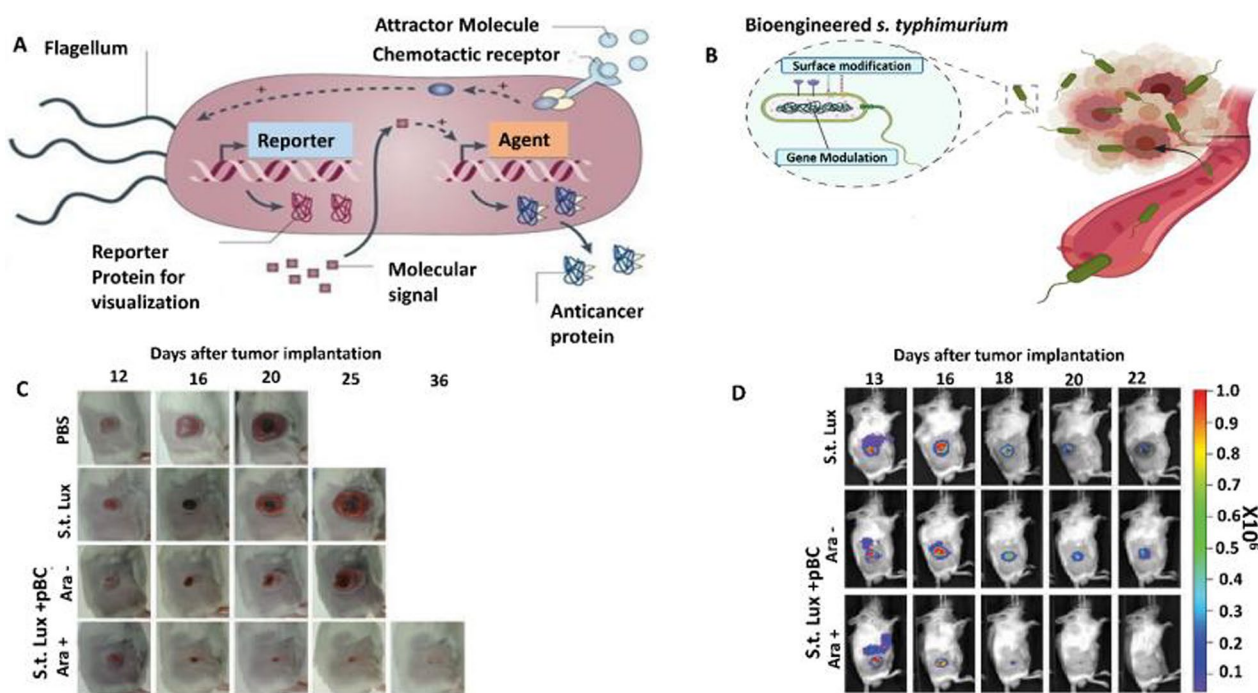


Fig. 8 **A** The biological mechanisms that bacteria use to perform these tasks include machinery for gene translation, which produces anticancer proteins (dark blue); flagella, which performs chemotaxis; specific gene promoter regions, which respond to molecular signals (red squares); chemotactic receptors, which produce blue; and machinery, which produces red molecules. **B** Bioengineering of *S. typhimurium*. **C** ClyA-expressing *S. typhimurium*: imaging and therapeutic implications in tumor-bearing mice. Hep3B2.1-7 or CT-26 cells were administered subcutaneously (s.c.) into five mice per group. Tumor-bearing mice were given PBS, untransformed *S. typhimurium* (S.t.Lux), or transformed *S. typhimurium* [S.t.Lux + pBC (Ara⁻)] once tumors reached 130 mm³ in volume. After day 4 of injection with transformed *S. typhimurium* [S.t.Lux + pBC (Ara⁺)], 60 mg of l-arabinose was given intraperitoneally (i.v.) daily into a different set of tumor-bearing mice (n = 5). **D** Pictures of typical mice with subcutaneous tumors. Bacterial bioluminescence in vivo imaging using noninvasive methods in the typical animals. Reproduced from ref. [148] with permission from Cancer Research, Copyright 2010

cycles. This might lead to a more efficient administration of bacterial medicine [147].

Bacteria are capable of being genetically modified to generate powerful therapeutic gadgets, including cytotoxic drugs, immunomodulators, cytokines, prodrug converting enzymes, small interfering RNAs, and nanobodies, while acting as a tumor-targeting drug carriage. Through their interactions with immune cells, tumor cells, and other TME constituents, these cargoes and the bacteria cooperate to rewire the TME. Immune cells are brought in, activated, and their cytokines and chemokines produced in order to complete this conversion [152]. As a result, the bacteria anticancer effect is higher (Fig. 9).

Challenges and limitations of using bacteria

Using bacteria for cancer therapy, though promising, comes with several challenges and limitations that need to be addressed to ensure safety and efficacy. Systemic injection of bacteria can lead to severe systemic infections, posing a significant risk to patients. This necessitates careful control and monitoring of bacterial growth

and spread within the body. Certain bacteria can produce toxins that may cause harm to normal tissues and organs. Managing these toxic effects is crucial to ensure patient safety. The human immune system is designed to eliminate bacterial infections. This immune response can reduce the effectiveness of bacterial therapy by rapidly clearing the bacteria before they can exert their therapeutic effects. Bacterial therapies can trigger inflammation, which, while potentially aiding in tumor destruction, can also cause collateral damage to normal tissues and exacerbate adverse effects. Tumors are heterogeneous, and not all cancer cells may be equally susceptible to bacterial infection or the effects of bacterial toxins. Ensuring that bacteria selectively target and kill cancer cells without affecting healthy cells is challenging. Ensuring that bacteria localize to the tumor site and do not spread to other parts of the body is essential to prevent unwanted infections and side effects. This requires advanced delivery systems and precise control mechanisms. While bacterial therapy for cancer holds significant promise, addressing these challenges and limitations is essential for its successful development and clinical application. Advances in

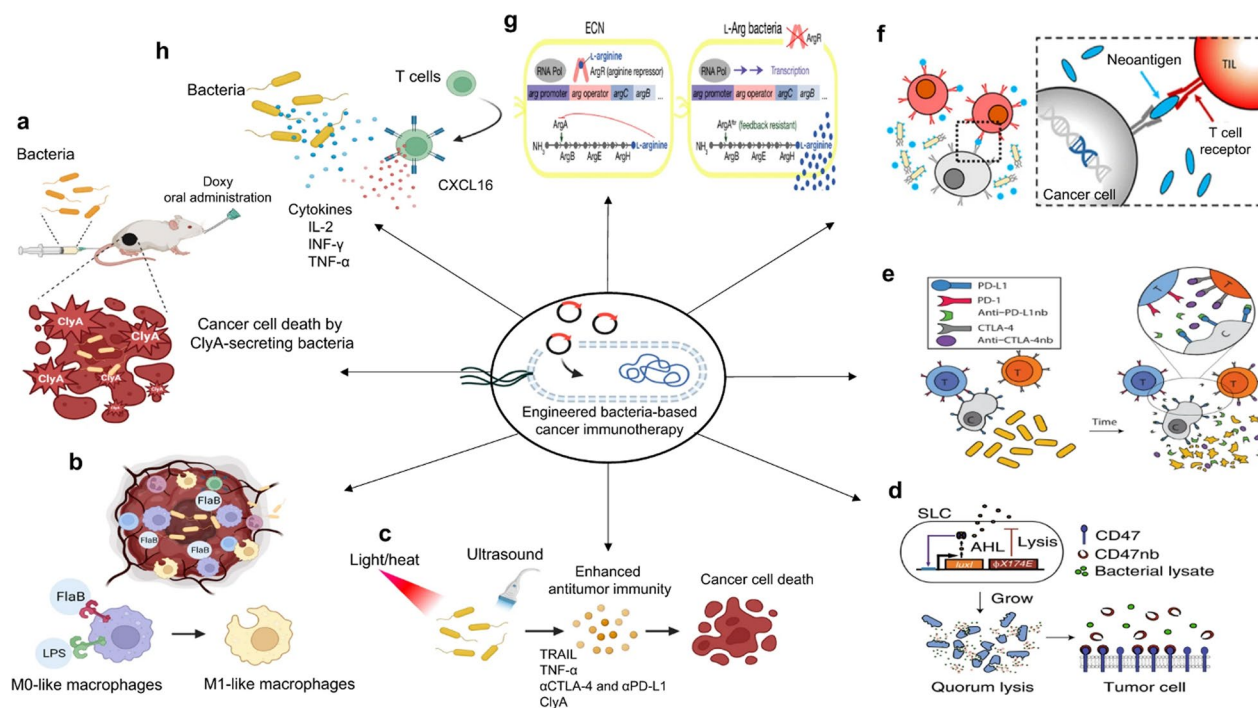


Fig. 9 **a, b** When chemical compounds like Doxy or L-arabinose are present, bacteria release ClyA, which kills cancer cells, or FlabB, which reprograms the TME and increases the recruitment of M1-type macrophages that fight tumors by activating TLR-4 and TLR-5 signaling. **c** Other possible strategies include the use of physical stimuli like heat, light, or targeted ultrasound to encourage the delivery of immunotherapeutics into the TME by microorganisms. **d, e** The QS system, which in this instance is based on the AHL autoinducer, has been used to genetically modify bacteria. This system produces AHL through the luxI promoter and regulated lysE expression, which leads to quorum-mediated lysis and intratumoral release of therapeutics like PD-L1 and CTLA-4-blocking or CD47-blocking nanobodies. **f** Cloned neoantigens activate tumor-infiltrating T lymphocytes, which then identify and eliminate tumor cells. **g, h** By secreting cytokines, chemokines, or other immunomodulatory payloads to attract and activate TILs in the TME, engineered bacteria can also elicit adaptive immune responses against malignancies. Reproduced from ref. [152] Nature, Copyright 2023

genetic engineering, immune modulation, and targeted delivery systems are helping to overcome some of these hurdles. Ongoing research, rigorous clinical trials, and collaboration between scientists, clinicians, and regulatory bodies are critical to unlocking the full potential of bacterial therapies for cancer treatment.

Clinical translation

Several significant challenges impede the practical implementation of modified bacterial treatments. Firstly, the creation of aesthetically pleasing bacteria is a crucial prerequisite. These modified bacteria should not only possess the ability to detect latent tumors and metastases in patients but also enable real-time tracking of bacterial development and spread throughout the body. Another major challenge involves enhancing the precision of genetically modified microorganisms in accurately targeting tumors. Overcoming this obstacle is pivotal for obtaining regulatory approval and ensuring patient safety [153]. Ineffective tumor targeting can result in robust bacterial growth in healthy tissues and the potential

dissemination of infection, posing a particular challenge for immune-compromised patients with advanced cancer. Despite the utilization of nonpathogenic bacterial agents in clinical studies, therapeutic benefits have often been negligible or nonexistent. Fortunately, viable solutions exist to address these issues through the integration of synthetic biology and nanotechnology. These advancements hold the potential to significantly improve the safety and effectiveness of modified bacterial medicines. In this context, we highlight real-time detection, improved tumor targeting, and enhanced therapeutic outcomes [154].

Facilitating real-time detection

Monitoring microbial settlement over time is essential for the advancement of therapeutic applications, as it serves two main purposes: first, to monitor proliferation in target locations and other organs to prevent confrontational events or damage to healthy tissue; second, to evaluate the efficacy of bacteria-based cancer therapy in localizing and growing within the tumor. To detect

bacterial colonization in tumors, various techniques have been employed, including bioluminescence, fluorescence, magnetic resonance imaging (MRI), and positron emission tomography (PET) [153, 154]. Plasmids carrying the luxCDABE operon from *Photobacterium leiognathi* are utilized to create bioluminescent bacteria such as *E. coli* and *S. typhimurium* [155]. GFP-carrying plasmids transform bacteria to emit fluorescence. Additionally, fluorescence imaging can be conducted using bacteria loaded with NPs, even in the absence of GFP. These optical imaging techniques, demonstrated through whole-body imaging, have shown excellent effectiveness in detecting cancers in animal models. However, their clinical use is challenging due to the limited penetration of visible light in human tissue. PET and MRI techniques are anticipated to replace optical imaging due to their greater sensitivity and deeper tissue penetration. Magnetic NPs can be employed to directly modify bacterial strains for use in MRI, or they can be genetically transformed to produce magnetosomes [156]. For instance, an external magnetic field can be applied to precisely image tumor locations using *Magnetococcus marinus* strains laden with magnetosomes. Additionally, the use of metabolic 2-nitroimidazole-based PET and 18F-fluorodeoxyisotriazole enables the imaging of *E. coli* to observe their colonization within tumors [157].

There is evidence to support the great therapeutic effectiveness of bacteria-initiated cancer treatment. Nevertheless, the therapeutic impact and result are compromised by the unintended therapeutic efficaciousness and the systemically produced inflammatory maelstrom. Wang et al. presents the rational design and engineering of a thermally-activated living nanomedicine, Sa@FeS, based on reactive biohybrid. The goal is to improve hydrogen sulfide (H₂S)-combined chemodynamic oncotherapy by biomineralizing ferrous sulfide nanoparticles (FeS NPs) onto the surface of a *Salmonella typhimurium* strain (*Sa*) without lowering microbial activity. Owing to *Sa* extensive penetration capacity, FeS NPs promote a photothermally-enhanced catalytic Fenton reaction, which, when exposed to near-infrared light, breaks down endogenous H₂O₂ into deadly hydroxyl radicals within tumor tissues [158]. In the meantime, *Sa* bacteria continue to generate H₂S continuously inside the tumor, resulting in H₂S-induced intracellular acidosis which encourages the cooperative production of reactive oxygen species (Fig. 10).

Improving tumor targeting

Tumor-targeting capabilities aim to provide a safer and more potent cancer treatment. Examples of obligatory anaerobes, such as *Bifidobacterium* and *Clostridium*, demonstrate modest invasion of healthy tissues and

relatively strong tumor selectivity. In contrast, facultative anaerobes like *Listeria* and *Salmonella* could multiply in oxygen-rich conditions, posing a risk to healthy tissues [159]. However, genetic modification of these facultative anaerobes can mitigate their detrimental effects on healthy tissues and enhance their ability to target tumors. For improved safety and precision in targeting, "obligate" anaerobic *Salmonella* strains, like YB1 and ST4, exclusively colonize the necrotic portions of tumor tissue. Nevertheless, these strains may face challenges efficiently targeting early metastatic tumor cells that typically have sufficient oxygen.

To address this issue, auxotroph mutant strains can be engineered for tumor targeting. For example, mutant *Salmonella* strains lacking specific nutrients may be designed to flourish only in tumor microenvironments rich in amino acids, such as Leu- and Arg-rich regions. The use of bacteria-driven microswimmers for drug delivery, responsive to external stimuli, has gained popularity. Bacteria exhibit various taxis mechanisms, including pH taxis, magnetotaxis, and chemotaxis. Notably, *Serratia marcescens* bacteria, displaying efficient chemotaxis, can be observed in microswimmers moving towards L-serine gradients. Fe₃O₄ nanoparticle-modified natural *Spirulina platensis* can be employed for magnetically targeted accumulation in malignancies. Further enhancing these bacteria's on-site adherence to affected tissues may further improve targeting efficiency. Despite advancements in exploiting bacterial taxis capabilities, various processes, such as thermotaxis, phototaxis, and galvanotaxis, remain undiscovered [160].

According to Zhang et al. A number of bacteria are suitable towards cognitive bio-hybrid robots because they have built-in motility and sensing capabilities enabling taxis-based autonomy. Bacteria-based robots that incorporate active nano-hybrids may act as cognitive drug delivery vehicles, reacting to a variety of simulated signals like magnetotaxis or chemotaxis to reach desired locations. The creation, propulsion, imaging, and treatment of bacteria-based bio-hybrid magnetic robots for a range of illnesses have advanced significantly in the past few decades. Therapeutic genes and gene reporters for tumor treatment and in vivo imaging are additionally expressed by genetic alteration [161]. Several peritrichous flagellad bacteria such as *Escherichia coli* might be bioengineered into microrobots for targeted administration that is noninvasive in physiological conditions (Fig. 11).

Enhancing therapeutic outcomes

Since William Coley's pioneering use of live infectious agents, specifically *Streptococcus pyogenes*, for cancer treatment in 1891, extensive research has been conducted

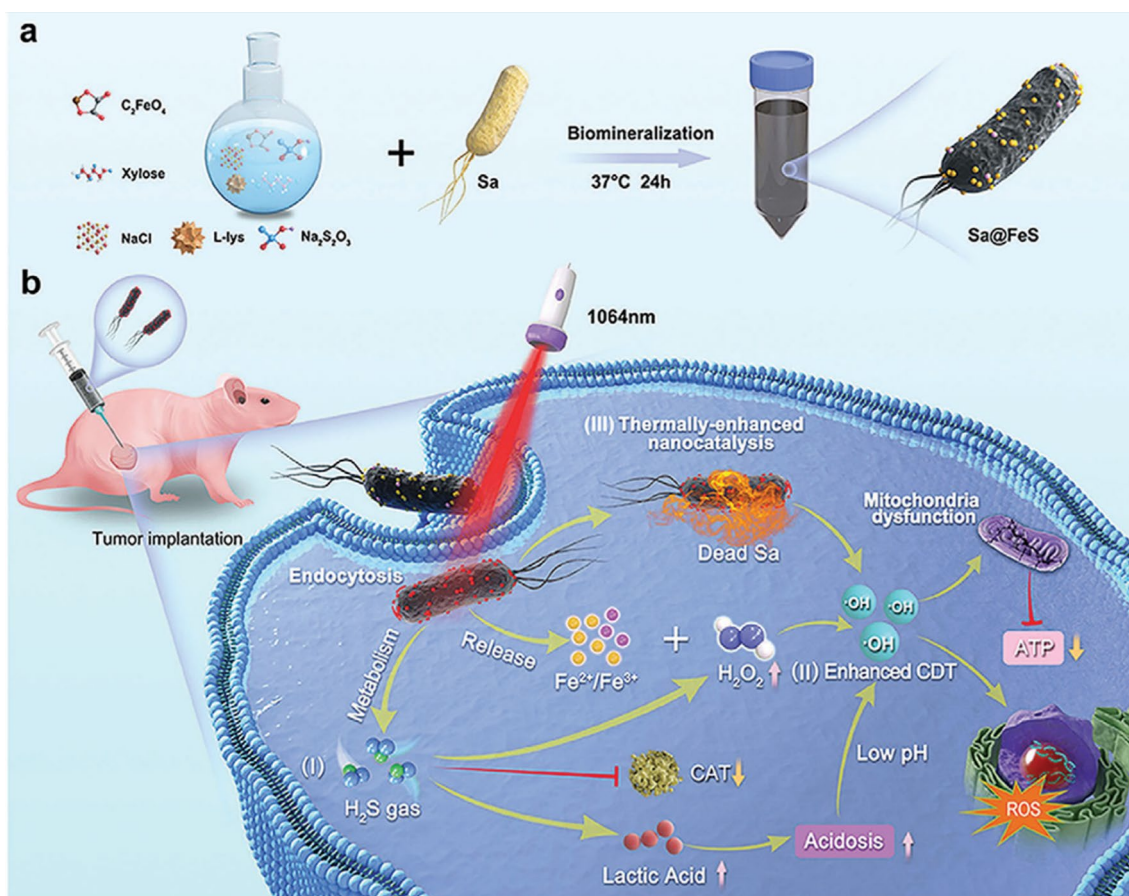


Fig. 10 Systemic demonstration of Sa@FeS, a living nanomedicine, as comprehensive anticancer therapy. **a** The Sa@FeS preparing process. Ferrous iron and exogenous thiosulfate ($S_2O_3^{2-}$) were added into liquid media containing bacteria and sterilized at a high temperature using an inductive cooker. To create the live nanomedicine Sa@FeS, the bacterial colony was subsequently merged with the medium and cultivated anaerobically for a full day. **b** Sa@FeS for all-in-one tumor treatment using targeted therapy. I Intrathecal administration of H_2S gas generated by bacteria has the potential to stimulate CDT by inhibiting CAT activity, which raises H_2O_2 levels, and building up lactic acid, which causes intracellular acidosis, which lowers TME pH. II Cytotoxic ROS against tumor cells may be produced via the Fenton reaction, which is mediated by released Fe^{2+}/Fe^{3+} . III FeS nanoparticles on the surface of bacteria may enable substantially improved CDT and photothermal treatment following exposure to a 1064 nm laser. The above-mentioned treatment routes operate in concert to induce tumor cancer cells' mitochondrial respiratory failure, which in turn blocks the flow of ATP and ultimately causes cellular death or necrosis to prevent cancer. Reproduced from ref. [158] Wiley, Copyright 2024

(See figure on next page.)

Fig. 11 Diagram illustrating the creation of bio-hybrid magnetic robots using bacteria and its uses in specific therapy. **a** A TEM picture of MC-1 and MC-1 that has been liposome-decorated, allowing for the loading of medicines. **b** Transverse MC-1 tumor segments following targeted and liposome attachment. Utilizing a fluorescent optical microscope, pictures of every slice were taken. The photographs demonstrate an excellent dispersion among the injected MC-1 cells within the tumor. **c** An illustration of the AMB-1-based microrobots in successive transmission beneath magnetic and optical fields, together with a SEM picture of a typical microrobot. 5 μm is the scale bar. **d** Setting up and characterizing robots according to AMB-1 that can perform consecutive magneto/optics. **e** Diagram of the bio-hybrid robots created using *E. coli* coupled with SPION and nickel nanoparticles (NLs). Doxorubicin (DOX) and indocyanine green (ICG) are put into the NLs. **f** Intellectual drawings showing how magnetic guidance might direct bacterial bio-hybrid robots across permeable microenvironments and into target tissues, such tumors. **g** Diagram showing how to build hybrid magnetically robots utilizing *E. coli* that can sense three different environments: temperature, hypoxic, and magnetic. Reproduced from ref. [161] MDPI, Copyright 2024

on various bacterial strains in clinical settings [162]. Despite these efforts, the outcomes were not consistently associated with significant tumor reduction; instead, they

often resulted in mild side effects and successful bacterial colonization in patients. The collective experience from clinical trials indicates that although modified bacteria

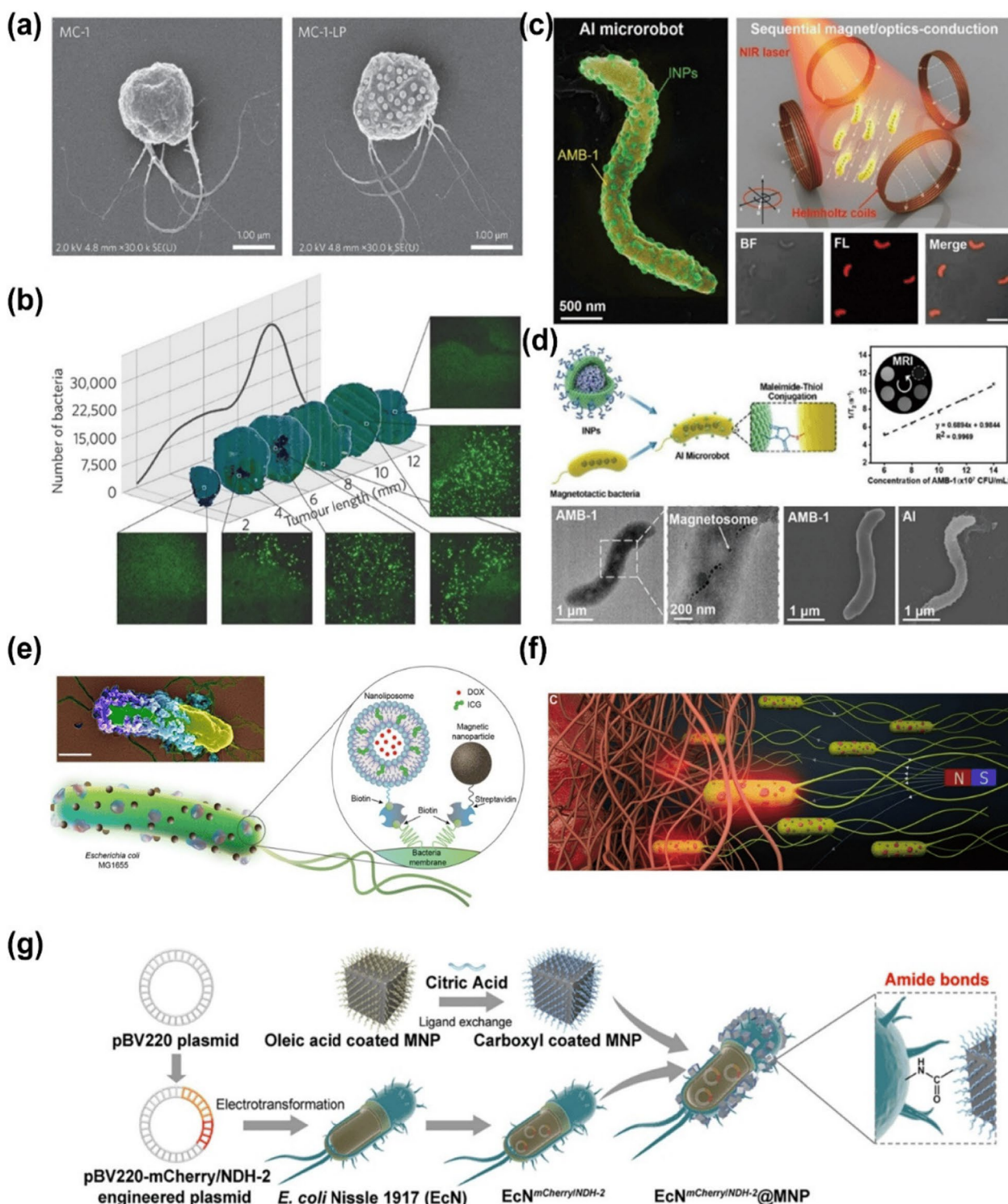


Fig. 11 (See legend on previous page.)

may demonstrate reduced virulence, they frequently fall short of delivering the intended therapeutic effects.

Despite these challenges, a substantial number of bacterial strains with significant potential for synergistic

therapy remain untested in human subjects [22]. For instance, studies conducted by Xing et al. showed that AMB-1 combined with laser irradiation successfully reduced tumor cells at temperatures as high as 58 °C

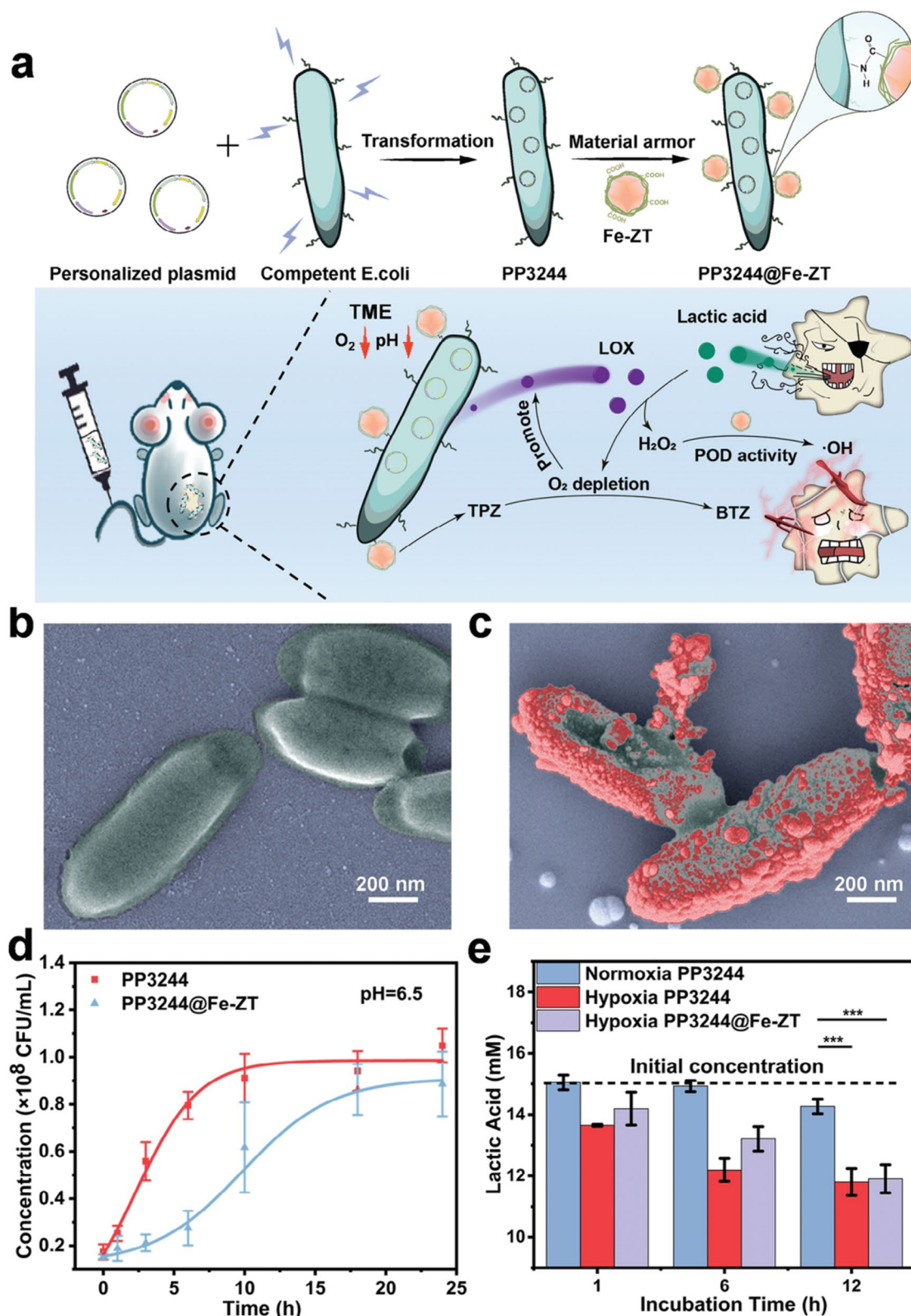


Fig. 12 Features and architecture of PP3244@Fe-ZT. **a** Pictorial representation of PP3244@Fe-ZT production and functions. **b** PP3244 and **c** PP3244@Fe-ZT scanning electron microscopy (SEM) pictures. **d** At pH=6.5 (n=3), growth curves for PP3244 and PP3244@Fe-ZT assuming the identical starting concentration. **e** PP3244 and PP3244@Fe-ZT lactate absorption in various oxygen environments (n=3). Reproduced from ref. [165] Wiley, Copyright 2024

Table 4 Clinical trials with engineered tumor-targeting bacteria

Trial	Bacterial strain	No. patients treated/Cancer type	Treatment/Outcome	Reference/Recruitment Status
Canine trials with tumor-targeting strains	VNP20009	41 patients STS (AUS, FBS, RMS, HPC, MXS), melanoma, carcinomas, OSA, HSA, lymphoma, MCT	IV infusion, 1.5×10^5 – 1×10^8 CFU/kg (dose escalation), 1–19 doses (mean = 3) MTD 3×10^7 CFU/kg; tumor colonization observed in 42% cases; 4 CR, 2 PR	Published [200]
	<i>C. novyi</i> -NT	6 patients HSA, lingual SCC, OSA, nasal ACA, FBS	IV infusion, 3×10^8 spores/kg, 3×10^7 spores/kg, 1 dose DLT (abscess formation) observed at 3×10^7 spores/kg; tumor abscess observed in 3 patients; 4 SD	Published [10]
	<i>C. novyi</i> -NT	16 patients STS (PNST, RMS, FBS, MXS), OSAC, MCT, melanoma, SCS	IT injection, 1×10^8 spores/dose, 1–4 doses Tumor abscess observed in 7 patients; 3 CR, 3 PR, 5 SD	Published [9]
Human trials with tumor-targeting strains	VNP20009	Phase 1 25 patients Melanoma, RCC	30 min IV infusion, 1×10^6 – 1×10^9 CFU/m ² (dose escalation), 1 dose MTD 3×10^8 CFU/m ² ; tumor colonization observed in 3 patients in 2 highest dose cohorts; elevated circulating proinflammatory cytokines detected; objective tumor regression not observed	Published [16]
	VNP20009	4 patients Melanoma	4 h IV infusion, 3×10^8 CFU/m ² , 1 dose Treatment well tolerated; tumor colonization not evident; objective tumor response not observed	Published [201]
	VNP20009	Phase 1 Refractory, superficial solid tumors	IT injection, dose escalation planned	Unpublished, completed [202]
	<i>C. novyi</i> -NT	Phase 1 2 patients Colorectal cancer	IV infusion, 1×10^6 spores/kg, 1 dose	Unpublished, terminated [203]
	SalpIL2 (Salmonella χ 4550 expressing IL-2)	Phase 1 22 patients Liver metastases of solid tumors	PO, 1×10^5 – 1×10^{10} CFU/dose (dose escalation planned)	Unpublished, completed [204]
	<i>C. novyi</i> -NT	Phase 1 5 patients Solid tumor malignancies	IV infusion, 1×10^5 – 1×10^7 spores/kg (dose escalation planned), 1 dose	Unpublished, terminated [205]
	APS001F (B. longum expressing CD)	Phase 1/2 75 patients (estimated) Advanced and/or Metastatic Solid Tumors	APS001F \pm maltose IV infusion/5-FC PO	Unpublished, recruiting [206]
	<i>C. novyi</i> -NT	Phase 1 24 patients Solid tumor malignancies	IT injection, 1×10^4 – 3×10^6 spores/dose (dose escalation), 1 dose	Unpublished, completed [207]

STS: soft tissue sarcoma; AUS: anaplastic/undifferentiated sarcoma; FBS: fibrosarcoma; RMS: rhabdomyosarcoma; HPC: hemangiopericytoma; MXS: myxosarcoma; OSA: osteosarcoma; HSA: hemangiosarcoma; MCT: mast cell tumor; SCC: squamous cell carcinoma; ACA: adenocarcinoma; PNST: peripheral nerve sheath tumor; OSAC: chondroblastic osteosarcoma; SCS: synovial cell sarcoma; RCC: renal cell carcinoma; PO: oral administration; CFU: colony-forming unit; MTD: maximum tolerated dose; DLT: dose-limiting toxicity; SAE: serious adverse event; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; DFI: disease-free interval; OS: overall survival

[153]. NPs gathered in the hypoxic area of the tumor in a research by Chen et al. When the ICG payload was exposed to near-infrared (NIR) light, the oxygenated tumor tissue around it was killed. Significant tumor shrinking was the outcome of this photothermal tumor lysis mechanism, which both provided nutrients and promoted enhanced bacterial penetration into the tumor tissue. Significantly, the mouse survival rate was 100% after 28 days, and the primary organs' hematoxylin and eosin (H&E) staining examination showed no harm [163].

Furthermore, in a study conducted by Chen et al., OMV-coated polymeric NPs exhibited remarkable efficacy, achieving a 70% tumor suppression in a 4T1 xenograft breast cancer model [164]. Additionally, Chowdhury et al. employed genetically modified *E. coli* to produce an encoded nanobody antagonist of CD47. This innovative approach enhanced tumor cell phagocytosis by macrophages, ultimately resulting in complete tumor regression. The method demonstrated promising therapeutic success across various mouse tumor models, including melanomas and triple-negative breast cancers [21].

The microbes inherent traits, such as having non-specific metabolic sites, hazardous contaminants, and unchecked growth, prevent them from being used in therapeutic settings like tumor treatment. Li et al. describe a biohybrid that has been developed to precisely ablate tumors by effectively targeting malignant regions using a pre-established metabolic route. With this approach, lactate oxidase genes and hypoxia-inducible promoters are added to DH5 α *Escherichia coli*, which is then extensively surface-armored using iron-doped ZIF-8 nanoparticles. As response to a hypoxic tumor microenvironment, this bioengineered *E. coli* is able to manufacture and release lactate oxidase, which lowers the quantity of lactate also activates the immune system. Because the nanoparticles have peroxidase-like properties, they may convert hydrogen peroxide (H₂O₂) into very harmful hydroxyl radicals, which is the final result of lactate metabolism. Significant cancer cell iron deficiency being a consequence of this as well as the conversion of tirapazamine-loaded nanoparticles to poisonous benzotriazinyl [165]. This biohybrid when injected intravenously dramatically reduces tumor growth and metastasis (Fig. 12).

Conclusion and future perspectives

The use of bacterial derivative-mediated drug delivery in cancer therapy displays promising potential for targeted and efficient treatment. By harnessing the unique properties of bacterial derivatives, such as their ability to target specific cells and tissues, researchers can develop innovative strategies to enhance drug delivery and improve therapeutic outcomes for cancer patients. Further research and clinical trials are needed to fully explore the benefits

and challenges of this approach, but the initial results suggest that bacterial derivative-mediated drug delivery could be a valuable tool in the fight against cancer. However, there haven't been many clinical trials conducted yet, and the majority of research on bacteria-mediated cancer treatment is still in the pre-clinical stages. Further clinical trials on such microbes are required in the future. Prior research has previously demonstrated that single therapy is not a particularly effective cancer treatment (Table 4) [9, 10, 16, 200–207].

In the future, bacterial derivative-mediated drug delivery for cancer holds great promise for revolutionizing cancer therapy. With ongoing advancements in biotechnology and nanomedicine, researchers can further optimize the design and delivery of bacterial derivatives to enhance their efficacy and specificity in targeting cancer cells. Additionally, the development of personalized medicine approaches, such as utilizing patient-specific bacterial derivatives, could lead to more tailored and effective treatments for individual cancer patients. Collaborations between multidisciplinary teams of scientists, clinicians, and industry partners will be crucial in translating these innovative strategies from the lab to the clinic, ultimately improving outcomes for cancer patients and potentially transforming the landscape of cancer treatment.

Combination therapy leverages the strengths of different therapeutic approaches to provide a more robust, effective, and personalized treatment for cancer. It addresses the complexities and challenges of treating a heterogeneous and adaptable disease, ultimately aiming to improve patient outcomes and quality of life. The strategic use of combination therapy represents a sophisticated and evolving approach in the ongoing battle against cancer.

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Muhammad Ijaz and Ikram Hasan: conceptualization, writing—original draft, methodology, data curation. Tamoor Hamid Chaudhry: visualization, data curation. Rui Huang and Lan Zhang: writing—review and editing. Ziwei Hu and Qingqin Tan: writing—review and editing, supervision, resources. Bing Guo: investigation, writing—review and editing, Project administration, funding acquisition.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Consent for publication

All authors contributed to the article and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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