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Bacteriophages as nanocarriers for targeted drug delivery and enhanced therapeutic effects

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The effective delivery of therapeutic agents is as important as the active therapeutic agent because it may make or mar the outcome. Traditional/conventional drug delivery systems (DDSs) face many limitations, including poor bioavailability, poor specificity and targeting, inconsistent drug adsorption, short half-life, rapid drug clearance, instability, varying and suboptimal drug effects, uncomfortable administration, limited drug loading into delivery vehicles, and poor treatment adherence and compliance, opening the way for innovations, including optimising the use of nanocarriers. Nanocarriers encapsulate and deliver drug agents using nanosized vehicles to enhance drug effectiveness, bioavailability as well as specific, targeted, and controlled drug release. Examples of such nanocarriers include micelles, microemulsions, nanoemulsions, lipid nanoparticles, liposomes, niosomes, dendrimers, and exosomes, which are formulated using different vehicles. Virus-like nanoparticles are emerging, with most involving bacteriophages—the environmentally ubiquitous, abundant, and diverse group of nanosized structurally simple viral particles with the intrinsic and inherent ability to invade bacteria. Studies involving different bacteriophages, their nano drug formulation, and their application against some diseases exist; however, no current review aggregates the advances made so far, which could be attributed to the recency of the research areas. Such a review is vital because it highlights and precipitates milestones and can provide necessary basis for further advancements. Thus, this study aims to evaluate the advancing potential use of bacteriophages as nanocarriers for targeted drug delivery and their potential for enhanced therapeutic effects.

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1. Introduction

Therapeutic agents for medical interventions are delivered to the body to achieve desired pharmacological effects using different systems that enhance therapeutic output and are referred to as drug delivery systems (DDSs).[1] They are primarily aimed at improving therapeutic efficacy by providing the optimal or sustained release of bioactive agents; reducing administration frequency; ensuring continuous drug supply at therapeutic levels; achieving the targeted/specific delivery of drugs to the site of action; enhancing drug localisation; reducing off-target effects, minimising side effects; enhancing safety *via* the minimisation of systemic exposure; protecting drugs against enzymatic degradation, pH and other environmental conditions; improving drug stability and bioavailability; and increasing patient and treatment compliance and adherence.[2–6]

Traditional/conventional DDSs include the most common and convenient ones: oral DDSs, involving the use of capsules, tablets, suspensions, and syrups adsorbed *via* the gastrointestinal tract (GIT) into the bloodstream;[7] parenteral DDSs, involving the intramuscular, intravenous, or subcutaneous liquid drug passage, bypassing the GIT and rapidly reaching the bloodstream for fast pharmacological activities;[8] transdermal/topical DDSs, involving the direct topical application of therapeutic agents, including creams, lotions, ointments, gels, and patches on the skin barrier to reach the bloodstream;[9] pulmonary DDSs, involving the localised and rapid administration of therapeutic inhalable particles, such as powders, aerosols, and nebulisers into the lungs;[10] nasal DDSs, involving the administration of nasal drops and sprays through the nasal cavity into the bloodstream;[11] and recta and vaginal DDSs, involving the respective localised administration of drugs *via* the rectal and vaginal routes.[12] Although these DDSs have been studied and applied extensively, they face many challenges bordering on

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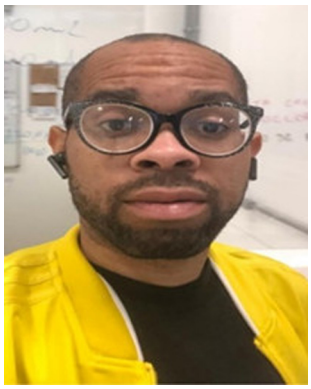
their efficacies, safety profiles, and patient compliance. Some of these exhibit poor bioavailability owing to factors such as low solubility, liver-related first-pass metabolism, poor permeability, and poor specificity and targeting, leading to increased potential side effects and toxicities, inconsistent drug adsorption owing to varying systemic conditions, including food interactions, pH and intestinal motilities, short half-life leading to frequent drug administration, impacting patient drug regime compliance, rapid drug clearance as seen with hepatic or renal pathways, physical and chemical instabilities while responding to environmental conditions, varying drug effects owing to variations in genetics, metabolism, and physiological states, suboptimal drug effects due to non-uniform drug distribution, uncomfortable administration routes as seen with parenteral DDSs, which also increase the chances of infections, limited drug loading into delivery vehicles, and as already mentioned, poor treatment adherence and compliance.[13–17] These challenges leave room for interventions and innovations, including using nano drug carriers for more efficient drug delivery and enhanced therapeutic outcomes.

Nanocarriers are nanosized (size < 500 nm) colloidal systems whose emergence has revolutionised drug delivery technologies and encapsulate and deliver drug agents using nanosized vehicles to enhance drug effectiveness, bioavailability, and specific, targeted, and controlled drug release.[18] Micelles, micro- and nanoemulsions, lipid nanoparticles, liposomes, niosomes, dendrimers, and exosomes are the most common nanocarriers and have been developed and delivered using diverse, relevant materials and particles, including natural dwelling life forms.[18] One of the emerging advances in nanocarrier technologies is virus-like nanoparticles, including bacteriophages.[19]

The environmentally ubiquitous, abundant, and diverse group of nanosized structurally simple viral particles with the intrinsic and inherent ability to invade bacteria, making them serve as biological hosts for their propagation through various mechanisms, including the manipulation of the host bacterial



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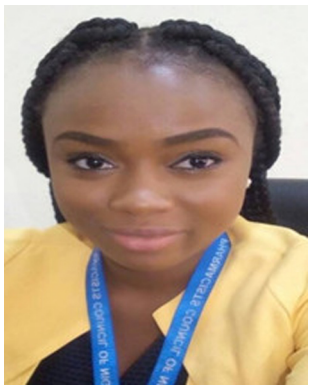

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replication, the use of the same, and the release of specific associated proteins, such as endolysins and holins, are known as bacteriophages and are abridgedly termed phages.[20–22] Phages have distinguishing and associated characteristics, including their small size and ability to invade and propagate with only one or limited but usually related species of bacterial hosts.[21,23] This property is one of the essential features of categorising bacteriophages into different host range profiles and has been exploited for research and medicinal purposes.[24,25] Bacteriophages, with polyvalent ligands, display scaffolds,[26] and have found many applications, including their use in the therapeutic management of disease in different delivery systems and routes of administration, environmental monitoring of pathogenic bacteria for specific purposes, and their use as the basis for the delivery of bioactive agents for specific therapeutic benefits.[24,27,28] Alongside the efforts to utilise phages as active therapeutic agents and alternatives to antibiotics in managing diseases, there are also advancing studies in their use as nanocarriers of therapeutic agents in different applications owing to their already-mentioned desirable properties. Different studies involving different bacteriophages, their nano drug formulation, and their application against some diseases exist; however, limited current reviews are aggregating the advances and could be attributed to the recency of the research areas. Such a review is vital because it highlights and precipitates the milestones and can provide the necessary basis for further advancement of the research area, including comparing research methodologies and findings. Therefore, the study aims to evaluate the potential use of bacteriophages as nanocarriers for targeted drug delivery and the potential for enhanced therapeutic effects. Consequently, we described the nature and characteristics of bacteriophages, the basic concepts of nanocarriers in drug delivery, the application of bacteriophages as nanocarriers for drug delivery, possible enhancement effects of bacteriophage-mediated drug delivery, specific application of bacteriophage-mediated drug delivery, and finally the challenges and future directives.

2. Drug delivery systems and nanocarriers

This section examines conventional drug delivery systems, their drawbacks, and novel drug delivery approaches. We also try to describe different nanocarriers and their benefits and limitations as drug delivery systems.

2.1 Overview of drug delivery systems

Drug delivery systems refer to approaches, technologies, and formulations for transporting bioactive compounds in the body safely and efficiently.[18] Conventional drug delivery systems include solutions, mixtures, suspensions, tablets, capsules, powders, suppositories, paste, ointment, and aerosol. They face the problems of the uncontrolled and wide distribution of drugs in the body, leading to a high frequency of administration and systemic side effects.[29] Consequently, conventional drug delivery systems are associated with low efficacy, increasing toxicity, poor patient compliance, and failure in therapeutic outcomes. Over the past few decades, researchers have developed different delivery systems with the potential for controlled and sustained drug release and the ability to target cargo to specific tissues or cells. A novel delivery system can significantly improve drug pharmacokinetics, efficacy, and safety. An old drug can be reformulated and given a new life *via* a novel delivery system.

Novel drug delivery systems include nanoparticles, niosomes, nanoemulsions, dendrimers, liposomes, exosomes, and micelles. Researchers have employed several strategies to improve the controlled release and site-specificity of delivery systems. These methods include stimuli-responsive and ligand-modified target drug delivery strategies. In stimuli-responsive

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drug delivery systems, the release of the cargo is triggered by external or internal stimuli, including pH, temperature, enzymes, and a light magnetic field. Using enzymes, Radhakrishnan *et al.*[30] facilitated the delivery of an anticancer agent into cancer cells by the dual trypsin or hyaluronidase degradation of nanocapsules bearing anticancer agents. Ligands, such as folic acid, sugars, hyaluronic acid, and aptamers, interact with receptors and biomarkers found in target tissues; hence, they can be applied in targeted drug delivery systems.[31] Red blood cell membrane-coated nanocrystals were modified with tumour-targeting peptide c(RGDyK) by Chai *et al.*[32] and resultantly presented a superior tumour accumulation and therapeutic efficacy in subcutaneous tumour and orthotropic glioma-induced mice. These strategies ensure that the drugs are released only at the target sites, thereby reducing off-target side effects and increasing drug accumulation at the target site.

2.2 Nanocarriers in drug delivery

Nanomedicine is a new but rapidly growing field in science in which nanosized materials are employed in diagnosing and treating diseases. Nanotechnology-based drug delivery systems (nanocarriers) are colloidal drug carriers with sizes typically less than 500 nm.[33] They have been applied in the controlled and targeted delivery of drugs, phytoconstituents, chemotherapeutic agents, genes, and imaging agents to specific body parts. Nanocarriers, such as nanoemulsion, liposomes, and micelles, have been used to treat various diseases, including infections and malignancies, while some are still being investigated in preclinical and clinical studies.[4,31,34] Nanocarriers have been reported to offer many advantages over traditional delivery systems. Their unique nanoscale size range, morphology, and biological properties help to improve the solubility, absorption, *in vivo* stability, site-specific delivery, release, toxicity, and cellular uptake of the drug.[35,36] Their compositions, shapes, sizes, and surfaces can be easily modified to achieve the desired therapeutic effects.[37] Surface modification by coating with polymeric materials or attachment of ligands or functional groups enhances their pharmacokinetic and biodistribution profiles. Hence, PEGlation, coating, and surface functionalisation are common with nanocarriers.

The description of common nanocarriers, their advantages, and their limitations as drug delivery systems are presented in Table 1. Some common nanocarriers employed in drug delivery are briefly explained below.

2.2.1 Micelles. Micelles are drug delivery systems formed spontaneously in a polymeric or surfactant solution when the concentration of the polymer/surfactant is above the critical micellar concentration.[38] They can be grouped into normal, reversed, or polymer micelles (Fig. 1). Normal micelles have a hydrophilic moiety as the core and serve as carriers for hydrophobic drugs. The reversed micelles consist of clusters of hydrophobic moieties as the core and are utilised to deliver hydrophilic medicines.[39] Normal and reversed micelles are formed from surfactants, and polymer micelles are formulated using amphiphilic di- or tri-block polymers. Micelles increase the solubility and stability of drugs and sustain their release *in vivo*.[40,41] Some challenges in using micelles as nanocarriers are toxicity, poor drug loading, and lack of scalability.

2.2.2 Microemulsions and nanoemulsions. Microemulsions and nanoemulsions are colloidal dispersions of oil-in-water or water-in-oil. They are stabilised by a surfactant and a cosurfactant.[42] Microemulsion and nanoemulsion have similar components but differ in stability. Although the former is thermodynamically stable, the latter is not thermodynamically stable and exhibits only kinetic stability. The increasing interest in these systems is due to their easy formulation process, ability to be loaded with hydrophilic and hydrophobic drugs, and inexpensive and long shelf life.[43] However, their instability and toxicity due to high surfactant concentrations hinder drug delivery use.[44]

2.2.3 Nanoparticles. Nanoparticles comprise mainly lipid and polymer nanoparticles. However, inorganic nanoparticles, such as gold, silver, mesoporous silica, and iron oxide nanoparticles, are also employed as drug delivery systems. Lipid nanoparticles are colloidal drug delivery systems with a lipid core surrounded by a surfactant shell. Solid lipid nanoparticle is a first-generation lipid nanoparticle similar to nanoemulsion, but the liquid oil used in nanoemulsion is replaced with solid lipid to improve the instability issue of nanoemulsion. Owing to their beneficial properties, SLNs are superior to nanoemulsions, liposomes, and polymeric nanoparticles.[45] The lipid core is suitable for solubilising poorly water-soluble drugs in aqueous dispersion. The use of generally regarded as safe and biocompatible lipids in the formulation of SLN improves the safety profile of the system. The solid-state of the lipid core at body temperature increases the stability of this system. They can be easily modified to control drug release and targeting. The drawbacks of using this system are low drug loading, difficulty in obtaining uniform particle dispersion, toxicity, and drug expulsion during storage.[46] Recent advancements in this system, such as nanostructured lipid carriers, lipid–drug conjugates, and hybrid lipid polymer nanoparticles, are strategies to overcome the limitations of solid lipid nanoparticles.

Nanostructured lipid carriers (NLCs) are an effective alternative to solid lipid nanoparticles. They comprise a lipid core comprising both liquid and solid lipids (Fig. 2). The presence of both lipids leads to a less perfect crystalline structure with more defects/spaces for drug loading.[47] Polymeric nanoparticles are nanosized colloidal systems of polymeric materials with drugs entrapped in the core (nanocapsule) or the polymer matrix (nanosphere). Biocompatible and biodegradable natural or synthetic polymers are employed to formulate polymer nanoparticles. Polymeric nanoparticles have higher storage stability, higher circulation half-life in the biological system, and a better-controlled release profile.[48] The lipid-polymer hybrid nanoparticle is an emerging type of lipid nanoparticle, a blend of polymer and lipid nanoparticles. The polymeric core is surrounded by a phospholipid layer coated with a lipid-PEG shell[49] (Fig. 2). A system's mixture of lipid and polymer results in higher mechanical strength, biocompatibility, high payload for both hydrophilic and lipophilic drugs and controlled drug release.[50]

2.2.4 Liposomes. Liposomes are lipid vesicular drug delivery systems with an aqueous core and one or more phospholipid

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Table 1 Novel drug delivery systems with their advantages and limitations

Drug delivery system	Description	Advantages	Limitations
Nanomicelles	Self-assembled system of amphiphilic surfactants or block polymeric materials	Improved drug solubility Increased drug stability Suitable for drug targeting	Toxicity Poor drug loading Lack of scalability.
Microemulsions Nanoemulsion	Colloidal dispersion of oil and water stabilised by surfactant	Improved solubility Ease of formulation Ability to load both hydrophilic and hydrophobic drugs Inexpensiveness Long shelf life	Toxicity due to the presence of surfactant Low stability
Lipid nanoparticles	A colloidal system with a lipid core and a surfactant shell	Solubilise poorly water-soluble drugs Use of safe and biocompatible lipids Provide controlled drug release Provide targeted delivery Large scalability potential	Challenging to achieve uniform particle size distribution Expulsion of the drug upon storage Poor drug loading capacity Cytotoxic effects associated with the components
Liposomes	Vesicular drug delivery systems with an aqueous core and one or more phospholipid bilayers	Capacity to load both hydrophilic and lipophilic drugs Biocompatible and low-toxicity High membrane permeability	Low stability Low payload Lack of appropriate sterilisation method Leaching of encapsulated drug Expensive production cost
Niosomes	A vesicular system with amphiphilic non-ionic surfactant bilayer	Less toxic Biodegradable Biocompatible	Low drug loading Drug expulsion Instability and aggregation of vesicles High production costs
Dendrimers	Star-shaped hyperbranched structure with numerous surface function groups	Small size Improved drug solubility Capacity to deliver both hydrophilic and hydrophobic drugs High loading capacity Protection of medicine from premature degradation High functionalisation capability High drug targeting potential	Low aqueous solubility Nonspecific toxicity Low scalability potential
Exosome	Extravascular vesicle	Biodegradability Inherent bioactivity High tolerability High drug loading High membrane permeability High tissue targeting and cellar uptake	Instability Low yield Low scalability potential Lack of quality control standards
Virus-like nanoparticles	Self-assembled nanosized viral capsid-based systems with uniform geometry	Biocompatibility High surface functionalisation capability Targeted delivery of their cargo High encapsulation efficiency	Low yield High production cost

bilayers. Based on the number and size of the bilayer, liposomes can be classified into small or large unilamellar or multilamellar liposomes.[51] Liposomal drug delivery systems show biocompatibility and biodegradability properties. They are biocompatible owing to their structural similarity with cell membranes. Both hydrophilic and hydrophobic compounds can be encapsulated in this carrier. Positive charge liposomes interact with the cell membrane through electrostatic interaction. Their short shelf life, low drug payload, drug leakage during storage, and sterilisation issues limit their use in drug delivery.[52]

2.2.5 Niosomes. Niosomes are another vesicular delivery system and are structurally similar to liposomes. However, their bilayer is composed of amphiphilic non-ionic surfactants.[53] They are biocompatible, non-immunogenic, and biodegradable and exhibit low toxicity due to non-ionic surfactants. They show more chemical stability and longer shelf life than liposomes.[54] Some limitations of niosomes are low drug loading, drug expulsion, instability, and high production cost.[44]

2.2.6 Dendrimers. Dendrimers consist of a polymeric star-shaped structure of a core, hyperbranched, and numerous surface function groups.[55] They exhibit unique architectural designs with various shapes, sizes, branchings, and surface functional groups. Therapeutic agents can be entrapped in the dendrimer core or conjugated on the surface.[56] Different generations of dendrimers (G1, G2, G3, G4, and G5) represent different levels of branches added to the initiator core during the formulation process. Their advantages as drug delivery systems include small size, functionalisation capability, drug targeting, and ease of preparation.[57]

2.2.7 Exosomes. Exosomes are bio-inspired vesicular nanocarriers derived from the fusion of multivesicular bodies with the plasma membrane and secreted upon stimulation.[58] They are extracellular vesicles with a hydrophilic core surrounded by a phospholipid bilayer. The host automatically generates exosomes without eliciting immunological reactions. They are superior delivery systems compared to liposomes and other

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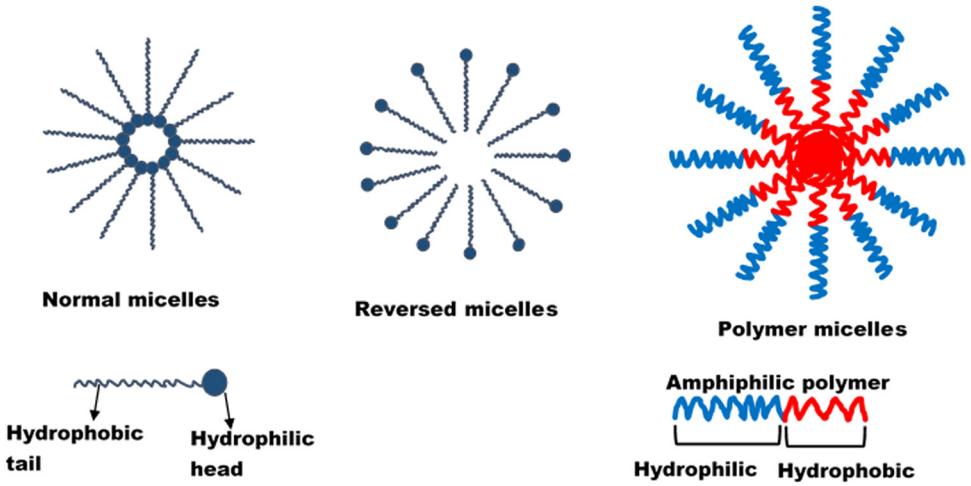

Fig. 1 Structure of micelle. Reprinted from Onugwu *et al.*, 2023 with permission from Elsevier.[34]

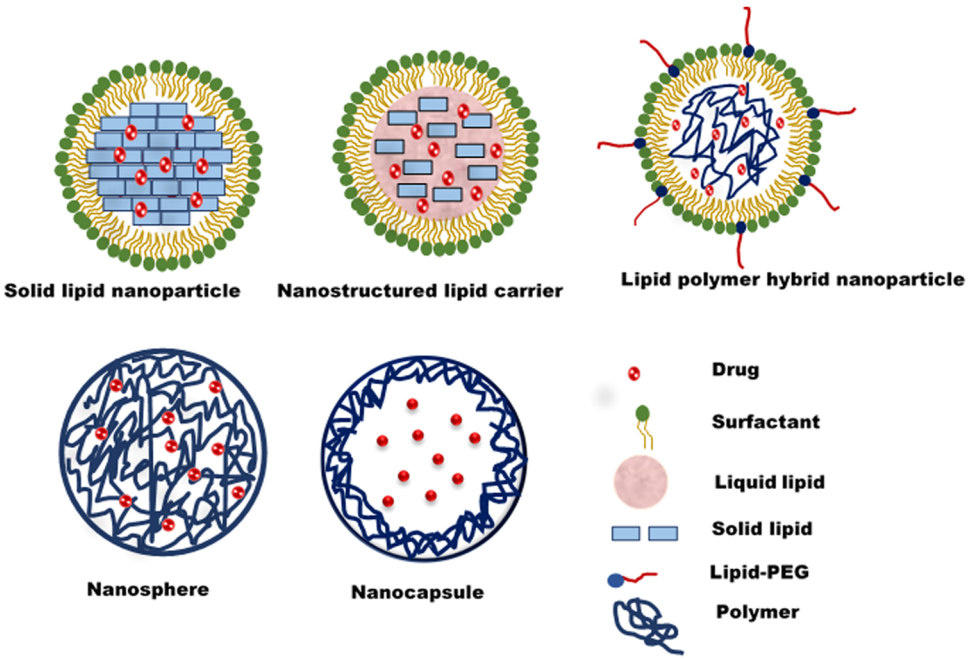

Fig. 2 Different types of lipid nanoparticles.

nanocarriers. They offer the advantages of biodegradability, inherent bioactivity, high tolerability, drug loading, membrane permeability, tissue targeting, and cellar uptake.[59] They have been investigated for the targeted delivery of small molecules, proteins, and genetic materials. Their major setbacks are instability, low yield, scalability potential, and a lack of quality control standards.[60]

2.2.8 Virus-like nanoparticle. Viruses are utilised as carriers in drug delivery. Virus-like nanoparticles are self-assembled nanosized viral capsid-based drug delivery systems with uniform and well-defined morphology. They are made from viral shells without genetic materials. Hence, they are non-infectious. They deliver drugs, genes, vaccines, and imaging agents.[61] Plant, animal, and bacterial viruses (bacteriophages) are employed in this system. Some favourable features of virus-like nanoparticles as nanocarriers are uniform geometry, biocompatibility, and high surface functionalisation capability.[59] The surface of the particles can be easily modified for the targeted delivery of their cargo. They exhibit high encapsulation efficiency of the loaded cargo.[62]

Nanocarriers have shown great potential for revolutionising drug and gene delivery. Studies have reported promising results involving different modifications/advancements in nanotechnology-based delivery approaches. However, relatively few nanocarriers have been translated into clinical use because of some hindrances militating against their large-scale manufacture and

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use. Hence, more studies are needed to introduce more nanocarriers into the market.

3. Bacteriophages

Bacteriophage-inspired nanocarriers have been investigated for the targeted delivery of genes and other therapeutic agents. In this section, we consider the structure and classification of bacteriophages as well as their surface engineering as nanocarriers.

3.1 Structure and classification

Bacteriophages, coined by D'Hérelle from "bacteria" and Greek words "φαγεῖν" (phagein), which collectively means "bacteria eater"[63] and are referred to as phages, are viruses that infect and replicate within bacteria. They are the most abundant biological entities on earth and are essential components of various microbiomes, playing a crucial role in regulating bacterial populations in various ecosystems. Structurally, bacteriophages are diverse; however, most of them share some common characteristics, including possessing a polyhedral head (consisting of approximately 2000 capsomeres) enclosing the genetic material, a short collar, and a helical tail. They are categorised into various families, including the syringe-behaving contractile tail possessing *Myoviridae*, long noncontractile tail possessing *Siphoviridae*, and short noncontractile tail posing *Podoviridae*; these three families belong to the *Caudovirales* class,[64] *Tectiviridae* with a protein-rich internal membrane and linear dsDNA genome enclosed within a flexible spike associated icosahedral protein capsid,[65] *Corticoviridae* with approximately 10 kbp circular DNA and inner membrane within the icosahedral capsid and associated with limited *Pseudoalteromonas* species,[66,67] *Lipothrixviridae* that are filamentous and enveloped, with a single molecule of linear dsDNA within its helical nucleoprotein core and associated with extreme thermophiles,[68] *Plasmaviridae* that are enveloped pleomorphic virions, with approximately 12 kbp circular, supercoiled dsDNA and associated with the *Acholeplasma* species,[69] *Rudiviridae* that are stiff-rod-shaped and unenveloped and possess dsDNA with size in the range of 32.3–35.8 kbp,[70] *Fuselloviridae* with short tail fibres, heterogenous size but lemon shaped containing circular, supercoiled polyamines and virus-coded basic protein associated dsDNA, with size in the range of 14.8–17.8 kbp,[71] *Inoviridae* with flexible filaments and positive sense circular ssDNA within helical array of many copies of a major capsid protein,[72] *Microviridae* associated with enterobacteria are nonenveloped, with ssDNA and $T = 1$ icosaheral symmetry,[73] *Leviviridae* that are spherical with $T = 3$ icosahedral symmetry and containing a molecule of positive sense ssRNA,[74] and *Cystoviridae* that are enveloped with segmented dsRNA and interior protein capsid polymerase

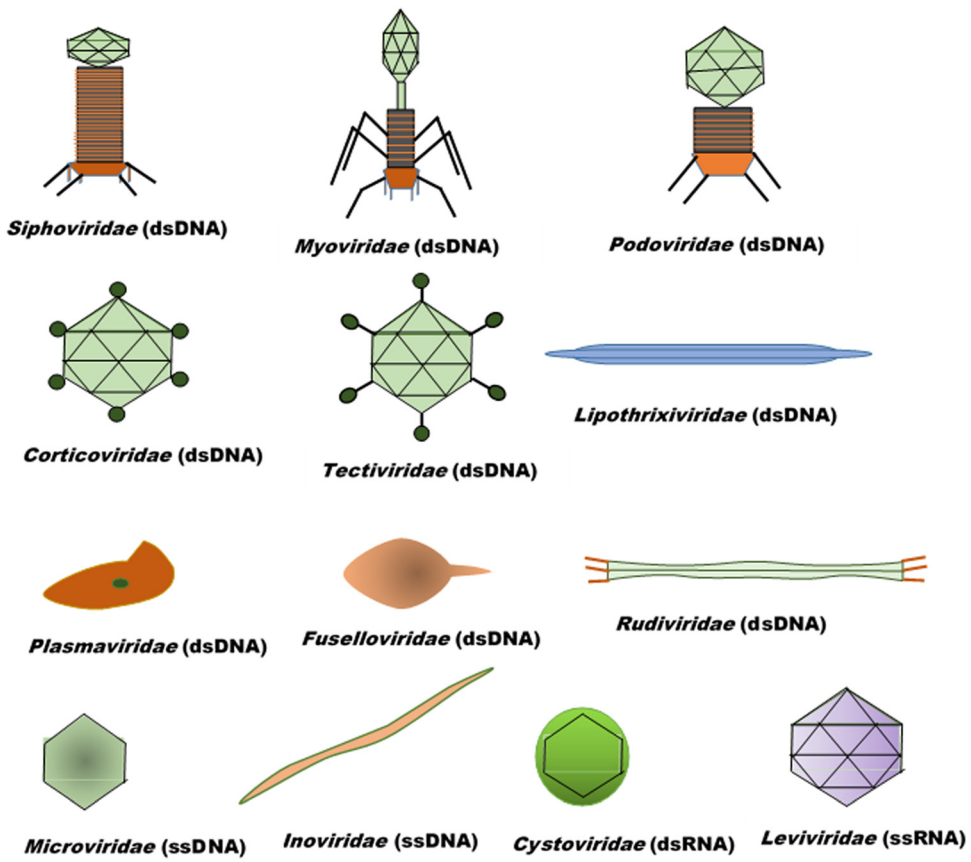

Fig. 3 Basic classes of bacteriophages.

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complex associated with transcription, replication, and genome packaging,[75] according to morphology and type of nucleic acid,[76] as presented in Fig. 3. According to their replication cycle, phages are also classified as either lytic, lysogenic, or temperate.[77] Following phage adsorption, penetration, and infection of the host bacteria, the lytic phages, the focus of therapeutic phages, utilise the replication mechanism of the bacteria to propagate its progenies, leading to the death of the bacteria; the lysogenic phages integrate their genome into the host chromosome, with only subtle genetic and physiological effects on the host; the temperate phages, however, can switch between the two life cycles.[77]

3.2 Bacteriophages as nanocarriers for drug delivery

Using self-assembling and easily modifiable bacteriophage capsids as nanocarriers presents potential benefits.[78,79] As they can specifically recognise and bind to limited bacterial receptors when engineered to bear the drug payload, including genes, enzymes, nanoparticles, or other therapeutic payloads, phages can potentially deliver these therapeutic agents to bacterial pathogens or disease sites with high precision, thus improving drug specificity and targeting.[78] The capsids, usually with large loading capacities, can protect and increase the bioavailability and stability of therapeutic payloads against immune clearance and degradation.[80,81] They can contribute to therapeutic actions by the initial lyses of pathogens; subsequently, the direct intracellular delivery of loads, minimising off-target effects and immune responses.[82,83] Because they are chiefly biocompatible with human and bacterial cells, they can significantly reduce the chances of toxicity and adverse effects.[84–86] Ineffective antibiotics owing to the development of bacterial resistance can be reprioritised by the initial lysis and compromise of the bacterial pathogen, especially when the bacterial resistance mechanism is inherent in the cell wall.[87,88] Personalisation and combination therapy can also be enhanced with more efficient therapeutic output.[89] Using bacteriophages as nanocarriers in drug delivery often involves surface engineering and the loading of therapeutic agents onto bacteriophages. Loading generally consists of the engineering of phage genetic materials and inserting payloads, surface conjugations, drug capsid encapsulation, electroporation of phage capsids enabling insertion or loading of therapeutic drugs, adsorption *via* electrostatic or hydrophobic interactions, chemical synthesis during phage assembly, or heat- or pH-induced loading.

3.2.1 Surface engineering of bacteriophages. Surface engineering is vital to utilising bacteriophages as nanocarriers and involves the manipulation of the outer surface for desired outputs using different mechanisms.[90] Peptide display utilises phage capsids to display therapeutic peptides that recognise and bind to specific bacteria surface cell receptors, often involving site-specific mutagenesis N- or C-termini peptide extensions and exterior exposed CP loop insertions.[90,91] Antibody display, similar to peptide display, can present antibodies against the cell antigens of specific-infected tissues and cancer cells.[28] The chemical conjugation of phages and molecules (*e.g.*, lipids and polymers) facilitates the attachment of drug molecules and improves phage stability, biodistribution, and pharmacokinetics.[92] Polymer (*e.g.*, polyethylene glycol) coating of phages reduces immune system recognition and uptake and improves bloodstream circulation.[93] In phage capsid manipulations and encapsulation, phages are used as cargo loading for the specific delivery of drug agents within the capsid.[94,95] Attaching multi-targeting ligands/moieties onto phage surfaces enables binding to different receptors.[96,97] Image tagging of drug payload-carrying bacteriophages enables real-time distribution tracking and targeting efficacy in the body and in disease diagnosis.[28] Additionally, phage genome modification involving advanced technologies employs manipulating the genetic material of bacteriophages to enhance the lytic profile.[98] Studies on the surface engineering of bacteriophages for drug delivery are described below and summarised in Table 2.

1. Peptide display. Zhao *et al.*[91] genetically modified *Leviviridae* PP7 capsid protein to display functional polypeptides, including one[1] kDa-sized cell-penetrating peptide, fourteen[14] kDa-sized Fc-binding Z-domain, and dimeric construct. Wild-type PP7 was reconstructed using extensions and insertions by respective C′-terminal extensions *via* polymerase chain reaction (PCR) by flanking with a 3′-sequence base of the primer attached to C′-terminal sequence and 5′-sequence region coding for the extensions and dimer loop insertions (also using similar PCR procedure), after which the construction was expressed and characterised. The result presented a more tolerable and robust system, displaying a linear and extended looped peptide (exogenous loops between the simultaneous presentation of 2 peptides and capsid monomers) and an open room for achieving desirable homogeneous polyvalent particles.

In targeting influenza A virus replication by competitive blocking of the virus and cell carbohydrate interaction, Gallego *et al.*[99] reported the precise position of sialic acid moieties (ligand) utilising the phage (Bacteriophage Qβ) capsid scaffold strategy to achieve the structurally defined positioning of sialic acid matching the binding site of trimeric hemagglutinin of the virus. Following the identification of a capsid residue spaced 5–6 nm on the particle surface, close to the sialic acid binding site of hemagglutinin, the expression in the presence of L-homopropargylglycine introduced a propargyl group using azide-alkyne cycloaddition and different linkers to anchor the sialic acid ligands. The *in vitro* result showed enhanced binding affinity of the Qβ capsid to the virus and potent inhibition of the virus, including outperforming oseltamivir (an antiviral agent). The *ex vivo* and *in vivo* results also significantly reduced viral titre and body weight loss, respectively.

Additionally, Rhee *et al.*,[100] using a modular system, including specifically engineered RNA–protein interactions and involving chemical derivatisation by acylation and azide-alkyne cycloaddition, encapsulated many copies of fluorescent proteins on Qβ VLP, generating high-affinity carbohydrate-based ligands of the CD22 receptor and particles with similar photochemical and structural properties to monomeric analogue with more stability to thermal and proteolytic degradation.

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Table 2 Surface engineering of bacteriophages

Type	Bacteriophage/ VLP	Agent	Target disease	Method	Result	Ref.
Peptide display	*Leviviridae* PP7	C-terminal extensions, dimer loop insertions	—	Wild-type PP7 was reconstructed using extensions and insertions by respective C-terminal extensions *via* PCR flanking with a 3′-sequence base of the primer attached to C′-terminal sequence and 5′-sequence region coding for the extensions and dimer loops insertions (also using similar PCR procedure), after which the construct was expressed and characterised.	A more tolerable and robust system, displaying a linear and extended looped peptide (exogenous loops between the simultaneous presentation of 2 peptides and capsid monomers) and open room for achieving desirable homogeneous polyvalent particles.	91
	Bacteriophage Qβ	Sialic acid	Influenza virus infections	The precise position of sialic acid moieties (ligand) utilising the phage (bacteriophage Qβ) capsid scaffold strategy to achieve the structurally defined positioning of sialic acid matching the binding site of trimeric hemagglutinin of the virus. Following the identification of a capsid residue spaced 5–6 nm on the particle surface, close to the between the sialic acid binding site of hemagglutinin, the expression in the presence of L-homopropargylglycine introduced propargyl group using azide-alkyne cycloaddition and different linkers in anchoring the sialic acid ligands.	The *in vitro* result showed enhanced binding affinity of the Qβ capsid to the virus and potent inhibition of the virus, including outperforming oseltamivir (an antiviral agent). The *ex vivo* and *in vivo* results significantly reduced viral titre and body weight loss, respectively.	99
	Bacteriophage Qβ VLP	Fluorescent proteins	—	The modular system, including specifically engineered RNA–protein interactions and involving chemical derivatisation by acylation and azide-alkyne cycloaddition, encapsulated many copies of fluorescent proteins on Qβ VLP.	Generated high-affinity carbohydrate-based ligand of the CD22 receptor and particles with similar photochemical and structural properties to monomeric analogue with more stability to thermal and proteolytic degradation. Confocal laser microscopy and flow cytometry were used to detect specific cell labelling by the particles.	100
	Bacteriophage P22 VLP	CD_{40} L	Cancer and other immunologically based diseases.	Multivalent modulation display of CD_{40} L on the exterior of bacteriophage P22 VLP.	Indication of higher potency in immunomodulation, following the significant decrease in the half maximal effective concentration (EC_{50}) compared to the control CD_{40}	101
	RNA bacteriophage MS2	V3 and ECL2 loop peptides of HIV gp120 and CCR5 coreceptor	HIV vaccine development	Engineering and display of V3 and ECL2 loop peptides of HIV gp120 and CCR5 coreceptor, respectively, on RNA bacteriophage MS2.	Through interrupted protein folding, it was effectively suppressed by the genetic fusion of two identical polypeptides of the VLP coat protein into a single-chain dimer, which supported the display of diverse immunogenic peptides.	102
Antibody display	Bacteriophage T4	Type 3 secretion system low-calcium response V antigen (plague), protective antigen and mutated capsular antigen F1 (anthrax)	Anthrax and plague	Engineering of the bacteriophage T4 120- by 86 nm capsids through the multivalent presentation of the *Y. pestis* 56 kDa-sized type 3 secretion system low-calcium response V antigen and anthrax 83 kDa-sized protective antigen and mutated capsular antigen F1 fused to the 9 kDa-sized Soc protein.	Potentiation of the *in vivo* clearance of implicated bacteria through the enhanced elicitation of broad T-helper 1 and 2 immune responses. The resulting vaccine was also reported to be highly stable, requiring no extra adjuvant.	104
		Anthrax toxin antigens, lethal factors and their domains, and protective antigens	Anthrax	Multiple anthrax antigens, including up to 1662 anthrax toxin antigens, lethal factor and their domains, and protective antigens, were displayed on N and C termini of the small outer capsid (Soc) protein and the Hoc protein of bacteriophage T4 capsid.	Presented an effective vaccine model against anthrax	105
			Anthrax	Bacteriophage T4 capsid engineering, multiple display and expression of anthrax toxin, lethal	Elicitation of potent antigens-specific antibodies against anthrax	103

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Table 2 (*continued*)

Type	Bacteriophage/ VLP	Agent	Target disease	Method	Result	Ref.
		Anthrax toxin, lethal and oedema factors, functional domains and protective antigens		and oedema factors, functional domains and protective antigens on all the binding sites (155) of the N-terminal hexahistidine-tagged Hoc.		
		Nef, P24-gag, and modified gp41 C peptide trimer	HIV vaccine development	Multiple displays of human immunodeficiency virus (HIV) antigens, including Nef, P24-gag, and modified gp41 C peptide trimer on bacteriophage T4 capsid Hoc sites	The study finds potential applications in HIV vaccine development. The *in vivo* evaluation of the engineered capsid bearing the HIV antigens demonstrated elicitation of strong p24-specific and Th1 and Th2 cellular antibodies and responses in mice, even without an adjuvant.	106
Phages and molecules' chemical conjugation	Tobacco mosaic virus	Amines and oxime formation sequence/diazonium coupling	—	Respective capsid interior and exterior attachment of amines to the glutamic side chain *via* carbodiimide coupling and oxime formation sequence/diazonium coupling, installing many thousand copies of material on the tobacco mosaic virus, demonstrating the associated orthogonalities.	Presented functional application of VLP nanoscale materials.	107
	Bacteriophage M13	Chlorin e6 (Ce6)	SKOV3 and COV362 ovarian cancer cell lines	Conjugated the bacteriophage M13 capsid with chlorin e6 (Ce6) assayed killing effectiveness against SKOV3 and COV362 ovarian cancer cell lines	Generated reactive oxygen species (ROS) *via* the type 1 mechanism, increased uptake of the resulting M13-Ce6 within the mitochondria, targeting the epidermal growth factor receptor (EGFR), and inducing autophagy against the cancer cell lines.	108
Polymer coating	Bacteriophage M13	Poly(caprolactone-*b*-2-vinyl pyridine) and folate	Cancer	Poly(caprolactone-*b*-2-vinyl pyridine) and folate (conjugated to phage surface *N*-terminal and lysine residue) to form a nanocore shell that encapsulated doxorubicin (an antitumor agent)	Increased release and cellular uptake of the doxorubicin, cytotoxicity *via* receptor-mediated endocytosis, and higher selective uptake in tumour cells.	26
	CN8 bacteriophages	Polyvinyl polymers with ether, alcohol, and pyrrolidone functional groups	Goss's witt	Stabilised CN8 bacteriophages specific against *Clavibacter michiganensis* subsp. nebraskensis associated with Goss's witt, leading to yield loss in maise seeds using polyvinyl polymers with ether, alcohol, and pyrrolidone functional groups.	Increased stability of the CN8 with the polyvinyl alcohol providing the highest stability, allowing long-term storage (four (4)–seven (7) months) under different temperature conditions combined with whey protein isolate and with no negative impact on the seed germination, significantly reduced bacterial loads in the contaminated seedlings.	121
	Phages of methicillin-resistant *S. aureus* (MRSA)	Linezolid, hydroxypropyl methylcellulose	Methicilin-resistant *S. aureus* infection	Employed phages of methicillin-resistant *S. aureus* (MRSA) implicated in orthopaedic device-related infections, linezolid, and polymer (hydroxypropyl methylcellulose) in the coating of wires implanted into the intramedullary canal of the femur of experimental animals in a simulation of surgical implantations, after which methicilin resistant *S. aureus* ATCC 433000 was inoculated within the implantation area.	Phage-linezolid-biopolymer coating maximally reduced bacterial adherence and joint inflammation, prevented the appearance of resistant mutants, and increased motor function of the limb and resumption of locomotion.	109
	T7 phage	Cysteine constrained heptapeptide	Cystic fibrosis	Following the screening of peptide–presenting phage and high throughput sequencing techniques, identified T7 phage-displayed cysteine-constrained heptapeptide serving as phage coating.	Enhanced drug penetration through the mucus, uptake into the epithelial cells of the lungs, distribution uniformity, and retention in the lung airways in treating cystic.	110
	Bacteriophage MS2	PEG chain surface coating, fluorescein	—	Dual modification using orthogonal coupling, including the site-specific PEG chain surface coating and interior surface conjugation with	Achieved the intact assemblage of the capsid following the extensive modifications and 90% reduction/blockage of the interaction of the	111

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Table 2 (*continued*)

Type	Bacteriophage/ VLP	Agent	Target disease	Method	Result	Ref.
				several copies of fluorescein (fluorescent dye) on bacteriophage MS2 capsid was reported by Kovacs *et al.*[111]	phage capsid surface and polyclonal antibodies. Additionally, the polymer-attached biotin group retained the streptavidin binding ability even though it was placed close to the PEG layer.	
Phage capsid manipulations and encapsulation	Bacteriophage T3	Gelstar, bleomycin	Cancer cells	Loading of Gelstar and bleomycin incubated with the phage capsid at the appropriate temperature (45 °C). The confirmation of loading was determined using the band fluorescence produced by the compounds in dry native gel electrophoresis.	Loaded DNA emitting greenish colouration as expected. The engineering could adequately protect the loaded compounds through the blood/plasma and present the potential for the specific monitoring and delivery of anticancer drugs (often susceptible to blood metabolic interruption) to tumour cells.	112
	Phage T4	Ethidium, bleomycin	Cancer cells	Loading of ethidium and bleomycin	Presented the gating and protective potential of phage T4, which at 4 °C for a month and 37 and 42 °C for 6 days, maintained the integrity of the loaded compounds within the loaded phage capsids. The engineering could adequately protect the loaded compounds through the blood/plasma and present the potential for the specific monitoring and delivery of anticancer drugs (often susceptible to blood metabolic interruption) to tumour cells.	113
	PRD1	Chlorpromazine	Antipsychotic conditions	The mode of binding and organisation of chlorpromazine complexed with PRD1	The PRD1 membrane enabled the ejection of the phage DNA. The injection of the drug was achieved, showing the accumulation of the drug at specific locations in the PRD1 capsid while maintaining the stability of the virions. The accumulation was attributed to the formation of micelles, polar *N*,*N*-dimethyl propylamine moiety associated with the negatively charged residues at the capsid protein P3 jellyrolls, and the formation of turreted morphology, leading to the packed and specific hexagonal lattice formation. Additionally, the drug formed aromatic rings between the guanidium group of arginine and the indole ring of the tryptophan side chain.	79
Attachment of multi-targeting ligands/ moieties	Filamentous phage	Phage proteins (pVIII), paclitaxel, 1,2-distearoyl-*sn-glycero*-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-2000	Breast cancer	MCF-7 cancer cell-specific filamentous phage proteins, pVIII, were used in the preparation of phage-paclitaxel micelles after phage self-assembling with the drug and 1,2-distearoyl-*sn-glycero*-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-2000 conjugates.	The directed targeting of breast cancer cells showed *in vitro* and *in vivo* a dramatic tumour reduction and necrosis and apoptosis, respectively. The therapy was also considered safe following the absence of hepatoxicity or pathological tissue changes.	114
	Phage clone library	121 phage-displayed neuroblastoma-binding peptides	Neuroblastoma	Using molecular targeting and a phage clone library, identified a total of 121 phage-displayed neuroblastoma (childhood tumour)-binding peptides.	26, 15, 57 and 23 of the peptides specifically targeting the primary tumour, metastatic mass, and specific microenvironments. These were also demonstrated and validated *ex vivo* and *in vivo* following the coupling of some of the peptides with doxorubicin-loaded liposomes with a corresponding consequential significant reduction and enhancement of tumour volume and survival rates.	115
Image tagging		Folic acids, fluorescent molecules				116

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Table 2 (*continued*)

Type	Bacteriophage/ VLP	Agent	Target disease	Method	Result	Ref.
	Bacteriophage M13		Human cancer cell	Using bacteriophage M13 capsid surface and through conjugation with folic acids targeting cancer cells and small fluorescent molecules, they modified three (3) reactive groups, including the amino group of lysine, carboxylic acids groups of aspartate, and phenol group of tyrosine residues.	A highly fluorescent bacteriophage M13 possesses significant binding affinity to human cancer cells, enabling targeting and probing.	
	Filamentous phage	Ketones, alkoxyamine groups (fluorophores)	Breast cancer	The N-terminal amines of the proteins were converted into ketones, which served as the chemo-specific handles for attaching alkoxyamine groups (fluorophores) *via* oxime formation and many 2 kDa poly(ethylene glycol) molecules.	There is no effect on the resulting single-chain antibody fragment (displayed in the phage coat proteins) binding to the human epidermal growth factor receptor 2 and epidermal growth factor receptor. The phage modification potentially enabled breast cancer diagnostic applications.	117
	Bacteriophage M13	Isothiocyanate of fluorescein	Multiple myeloma	Most suitable M13-pVIII phage display clones were explicitly labelled with isothiocyanate of fluorescein.	Provided fluorescent signals that separately identified multiple myeloma from individuals related to different CDs and presented a sensitive and rapid detection of the disease-associated immunophenotype subtypes and the disease status characterisation	118
Genome modification	Phage T4	Cytosine, glucosyl-hydro methylcytosine	—	Blocking the attack of CRISPR-Cas9 nuclease complexes, an adaptive immunity effector in bacteria, Bryson *et al.* edited and covalently replaced cytosine with glucosyl-hydro methylcytosine in Phage T4.	Reported less susceptibility of modified Phage T4 (replacement of cytosine with glucosyl-hydro methylcytosine) to Cas9 nuclease attack compared to the unmodified phage T4 genome	119 and 120

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Similarly, Goodall *et al.*[101] utilised multivalent modulation to display the CD_{40} ligand (CD_{40} L), an immuno-stimulating ligand of the tumour necrosis factor superfamily with a trimeric quaternary structure on the exterior of bacteriophage P22 VLP, which results in the indication of higher potency in immunomodulation, following the significant decrease in the half maximal effective concentration (EC_{50}) compared to the control CD_{40} and finding application in the management of cancer and other immunologically based diseases. Confocal laser microscopy and flow cytometry were used to detect the specific cell labelling by the particles. Specific sequences were displayed on RNA bacteriophage MS2 (a VLP) by Peabody *et al.*,[102] with potential for application in HIV vaccine development. The results showed improved potent immunogenicity of the VLP, following the engineering and display of V3 and ECL2 loop peptides of HIV gp120 and CCR5 coreceptor, respectively. Though interrupted by protein folding, it was effectively suppressed by the genetic fusion of two identical polypeptides of the VLP coat protein into a single-chain dimer, which supported the display of diverse immunogenic peptides.

2. Antibody display. In antigen-antibody display, bacteriophage T4 has been extensively employed, most involving the two[2] highly antigenic outer capsid (Hoc) and small outer capsid (Soc) proteins that bind to localised capsid sites.[103]

In the advancement of vaccine research against the highly pathogenic *Bacillus anthracis* and *Yersinia pestis*, respectively, implicated in severe anthrax and plague, Tao *et al.*[104] reported the potentiation of the *in vivo* clearance of implicated bacteria through the enhanced elicitation of the broad T-helper 1 and 2 immune responses, following the engineering of the bacteriophage T4 120- by 86 nm capsids, through the multivalent presentation of the *Y. pestis* 56 kDa-sized type 3 secretion system low-calcium response V antigen and anthrax 83 kDa-sized protective antigen and mutated capsular antigen F1 fused to the 9 kDa-sized Soc protein. The resulting vaccine was also reported to be highly stable, requiring no extra adjuvant. Similarly, Li *et al.*[105] successfully presented multiple anthrax antigens, including up to 1662 anthrax toxin antigens, lethal factor and their domains, and protective antigens were displayed on N and C termini of the small outer capsid (Soc) protein, and the Hoc protein of bacteriophage T4 capsid and also presented an effective vaccine model against anthrax.

In addition, Shivachandra *et al.*[103] showed using mice the elicitation of strong antigen-specific antibodies against anthrax following bacteriophage T4 capsid engineering, multiple display and expression of anthrax toxin, lethal and oedema factors, functional domains, and protective antigens on all the binding sites[155] of the N-terminal hexahistidine-tagged Hoc. Sataliyawala *et al.*[106] also utilised the Hoc-capsid interaction in the multiple displays of human immunodeficiency virus (HIV) antigens, including Nef, P24-gag, and modified gp41 C peptide trimer on bacteriophage T4 capsid Hoc sites, sufficiently saturated. The study finds potential application in HIV vaccine development, with the *in vivo* evaluation of the engineered capsid bearing the HIV antigens using mice showing, in the absence of adjuvant, the elicitation of strong p24-specific and Th1 and Th2 cellular antibodies and responses, respectively.

3. Chemical conjugation of phages and molecules. Schlick *et al.*[107] reported the respective capsid interior and exterior attachments of amines to the glutamic side chain *via* carbodiimide coupling and oxime formation sequence/diazonium coupling, installing many thousand copies of material on the tobacco mosaic virus, which demonstrates the associated orthogonalities and presents a functional application of VLP as nanoscale materials. The SKOV3 and COV362 ovarian cancer cell lines were photodynamically targeted using bacteriophage M13 by Bortot *et al.*[108] They conjugated the phage capsid with chlorin e6 (Ce6), which on irradiation synergistically generated reactive oxygen species (ROS) *via* the type 1 mechanism, increased uptake of the resulting M13-Ce6 within the mitochondria, targeted the epidermal growth factor receptor (EGFR), and induced autophagy in cancer cell lines.

4. Polymer coating. Suthiwangcharoen *et al.*[26] used cancer cells assembled with bacteriophage M13, poly(caprolactone-*b*-2-vinylpyridine) and folate (conjugated to the phage surface *N*-terminal and lysine residues) to form a nanocore shell that encapsulated doxorubicin (an antitumor agent), which on characterisation showed increased release and cellular uptake of doxorubicin, cytotoxicity *via* receptor-mediated endocytosis, and higher selective uptake in tumour cells. Kimmelshue *et al.*[93] used polymers to stabilise CN8 bacteriophages specific against *Clavibacter michiganensis* subsp. *nebraskensis* associated with Goss's witt, yielding loss in maise seeds. The polymers were polyvinyl polymers with ether, alcohol, and pyrrolidone functional groups. The study showed increased stability of CN8, with the polyvinyl alcohol providing the highest stability, which allows for long-term storage (four (4)–seven (7) months) under different temperature conditions combined with whey protein isolate, and without a negative impact on seed germination, significantly reducing bacterial loads in the contaminated seedlings.

In an animal model, Kaur *et al.*[109] employed phages of methicillin-resistant *Staphylococcus aureus* (MRSA) implicated in orthopaedic device-related infections, linezolid, and polymer (hydroxypropyl methylcellulose) in the coating of wires, which were implanted into the intramedullary canal of the femur of experimental animals in a simulation of surgical implantations, after which methicilin-resistant *S. aureus* ATCC 433000 was inoculated within the implantation area. Compared to the control, the phage-linezolid-biopolymer coating maximally reduced bacterial adherence and joint inflammation, prevented the appearance of resistant mutants, and increased the motor function of the limb and resumption of locomotion.

To enhance drug penetration through the mucus, uptake into the epithelial cells of the lungs, distribution uniformity, and retention in the lung airways in treating cystic fibrosis, Leal *et al.*,[110] following the screening of peptide – presenting phages and high-throughput sequencing techniques, identified T7 phage-displayed cysteine-constrained heptapeptide serving as

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phage coating, which *ex vivo* among the already stated and achieved objectives increased mucus penetration up to a 600 fold. Dual modification using orthogonal coupling, including the site-specific PEG chain surface coating and interior surface conjugation with several copies of fluorescein (fluorescent dye) on bacteriophage MS2 capsid was reported by Kovacs *et al.*[111] and achieved the intact assemblage of the capsid, following the extensive modifications and 90% reduction/blockage of the interaction of the phage capsid surface and polyclonal antibodies. Additionally, the polymer-attached biotin group retained its streptavidin binding ability, even though it was placed close to the PEG layer.

5. Phage capsid manipulation. Murine blood persistent bacteriophage T3 capsid, with gating capacity, was utilised by Serwer *et al.*[112] in the loading of fluorescent compounds gelstar (a dye) and bleomycin (an anticancer agent). The compounds were incubated with the phage capsid at the appropriate temperature (45 °C). The loading confirmation was determined using the band fluorescence produced by the compounds in dry native gel electrophoresis, with the loaded DNA emitting greenish colouration as expected. Moreover, using similar procedures, Serwer and Wright[113] achieved the loading of ethidium and bleomycin (also a fluorescent compound), presented the gating and protective potential of phage T4 at 4 °C for a month and 37 and 42 °C for 6 days and maintained the integrity of the loaded compounds within the loaded phage capsids. These studies showed that engineering could adequately protect the loaded compounds through the blood/plasma and present the potential for the specific monitoring and delivery of anticancer drugs (often susceptible to blood metabolic interruption) to tumour cells.

Additionally, the mode of binding and organisation of chlorpromazine, an antipsychotic amphipathic medication, complexed with PRD1 A (an icosahedral membrane-containing and double-stranded DNA phage) capsid protein P3 (43 kDa), in trimeric units, was reported by Duyvesteyn *et al.*[79] The results showed that although the PRD1 membrane enabled the ejection of phage DNA without biochemical or genetic modifications, the injection of the drug was achieved following specific mixing procedures with PRDI, with subsequent cryo-electron microscopy, crystallography, and molecular and biochemical dynamic techniques showing the accumulation of the drug at particular locations in the PRD1 capsid while maintaining the stability of the virions. The accumulation was attributed to the formation of micelles, polar *N*,*N*-dimethyl propylamine moieties associated with the negatively charged residues at the capsid protein P3 jellyrolls, and the formation of turreted morphology led to the packed and specific hexagonal lattice formation. Furthermore, the drug formed aromatic rings between the guanidium group of arginine and the indole ring of the tryptophan side chain.

6. Attachment of multi-targeting ligands/moieties. Multi-targeting phage protein micelles with specific interactions with tumour and vasculature surrounding cells ligated with paclitaxel, utilised in treating metastatic breast cancer, were reported by Petrenko and Torchilin.[114] To achieve this, MCF-7 cancer cell-specific filamentous phage proteins, pVIII, were used in the preparation of phage-paclitaxel micelles after phage self-assembling with the drug and 1,2-distearoyl-*sn-glycero*-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-2000 conjugates. The directed targeting of breast cancer cells showed a dramatic tumour reduction and necrosis and apoptosis *in vitro* and *in vivo*. The therapy was also considered safe following the absence of hepatoxicity or pathological tissue changes. In addition, Loi *et al.*,[115] using molecular targeting and a phage clone library, identified 121 phage-displayed neuroblastoma (childhood tumour)-binding peptides with 26, 15, 57 and 23 of the peptides specifically targeting the primary tumour, metastatic mass, and specific microenvironments. These were also demonstrated and validated *ex vivo* and *in vivo* following the coupling of some of the peptides with doxorubicin-loaded liposomes with a corresponding consequential significant reduction and enhancement of tumour volume and survival rates, respectively. These results open room for rapid diagnosis and targeted treatment of these diseases.

7. Image tagging. Li *et al.*[116] showed the potential application of phage display through chemical modification in cancer cell imaging and probing. Using bacteriophage M13 capsid surface and through conjugation with folic acids targeting cancer cells and small fluorescent molecules, they modified three reactive groups, including the amino group of lysine, carboxylic acid groups of aspartate, and phenol group of tyrosine residues, producing a highly fluorescent bacteriophage M13 that possessed significant binding affinity to human cancer cells, which enables targeting and probing. Carrico *et al.*[117] utilised multicolour fluorescence to allow for the characterisation of breast cancer cells by modifying a filamentous phage with approximately 4200 coat proteins. The N-terminal amines of the proteins were converted into ketones, which served as the chemospecific handles for attaching alkoxyamine groups (fluorophores) *via* oxime formation and many 2 kDa poly(ethylene glycol) molecules, and this did not affect the resulting single-chain antibody fragment (displayed in the phage coat proteins) binding to the human epidermal growth factor receptor 2 and epidermal growth factor receptor. These receptors are prevalently associated with breast cancer, and phage modification potentially enabled breast cancer diagnostic applications.

Similarly, De Plano *et al.*[118] utilised a phage probe for different clusters of differentiation as a diagnostic marker for multiple myeloma. The CDs enable the selection of aberrant and normal myeloma plasma cells associated with multiple myeloma.[118] To achieve discrimination, the most suitable M13-pVIII phage display clones were explicitly labelled with isothiocyanate of fluorescein, in which *ex vivo* provided fluorescent signals that separately identified multiple myeloma from individuals related to different CDs. The study presented a sensitive and rapid detection of the disease-associated immunophenotype subtypes and the disease status characterisation.

8. Genome modification. Attempting to block the attack of CRISPR-Cas9 nuclease complexes, an adaptive immunity

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effector in bacteria, Bryson *et al.*[119] edited and covalently replaced cytosine with glucosyl-hydro methylcytosine in Phage T4, with the results showing the promise of averting bacterial resistance to phages with a resultant intermediate resistance of the Phage T4 mutant against the unmodified cytosine bearing Phage T4 that was sensitive. Similarly, Tao *et al.*[120] reported less susceptibility of modified Phage T4 (replacement of cytosine with glucosyl-hydro methylcytosine) to Cas9 nuclease attack compared to the unmodified Phage T4 genome.

Generally, compared to other nanocarriers, bacteriophage applications as nanocarriers in drug development present desirable advantages over alternative nanocarriers. Specifically, their biological origin makes them biocompatible and stable, thus minimising the risk of immunogenic reactions, as seen in other nanocarriers, such as polymeric nanoparticles, micelles, and niosomes, which are also rapidly released.[122] They can be engineered to target specific bacteria or cells, thus allowing for precision in drug delivery. They have ease of modification, enabling the incorporation of payloads. They also have a high cargo packaging capacity, allowing the carriage of substantial therapeutic agents, a limitation of most polymeric micelles and liposomes with limited cargo loading capacities.[123] They are also less toxic than microemulsions, nanoemulsions, dendrimers, metal and metal oxide nanocarriers, organic, inorganic, hybrid nanocarriers, and carbon-based nanotubes.

3.2.2 Enhanced therapeutic effects of bacteriophage-mediated drug delivery. Bacteriophage-mediated drug delivery systems offer many benefits, including increased drug loading, stability, site-specific targeting, reduced toxicity, synergistic effects with antibodies and improved therapeutic efficacy. These benefits are discussed in this subsubsection.

1. Increased drug loading, stability and efficacy. Despite advancements in modern-day medicine, the issues of controlled and site-specific drug delivery, solubility, and increased bioavailability have remained largely unresolved. Drug hydrophobicity has been associated with decreased efficacy, as only a fraction of the active ingredients is delivered to the site of action.[85] The design of highly targetable and specific carrier systems, such as nanoparticles, nanospheres, liposomes, foams, carbon nanotubes, dendrimers, hydrogels, and other biomaterials, has considerably impacted drug delivery. Ideal carrier systems are expected to have specific physicochemical properties. Also considered are the effects on cellular uptake, intracellular distribution, accumulation, retention, and excretion of these vehicles.[124] While synthetic and semi-organic carriers such as nanoparticles, quantum dots, dendrimers, vesicles, and liposomes all possess these qualities, natural carrier systems such as viruses and virus-like particles, specifically bacteriophages, have been shown to present more advantages in terms of biocompatibility, improved pharmacokinetics, low toxicity and immunogenicity.[62,125] Considering the natural capacity of viruses to invade and infect host cells, advancements in nanotechnology have revolutionised viruses as the safe and efficient delivery of therapeutic and genomic materials.[85]

Bacteriophages have an excellent capacity for packaging therapeutic payloads, including genetic materials, and they are relatively categorised as safe in humans. These advantages make phages great candidates for nanocarriers.[21] It is believed that bacteriophages can control the many adverse effects of conventional therapeutic agents owing to their inherent biocompatible nature, which would, in turn, enhance treatment efficacy. Combining phages and therapeutic compounds could generate hybrid vehicles that serve as highly potent drug delivery systems.[21] Phages are viable biomaterials capable of being manipulated into hybrid bacteriophage-based nanocarriers in combination with various therapeutic moieties. The conjugation of inorganic materials onto the surface of phages displaying ligands introduces novel properties to phage carriers.

Additionally, phage conjugation with therapeutic substances, such as drugs, metabolites, and growth factors, enables the delivery of higher concentrations of therapeutic payloads without any unwanted effects on healthy surrounding tissues and cells. Phage-based nanocarriers could increase the solubility of poorly water-soluble drugs usually through materials on the phage coat that can serve as solubilising and sequestering agents. These delivery systems can equally present improved stability under certain pH and temperature conditions.[126]

In a study conducted by Yacoby *et al.*,[92] a highly toxic antibacterial agent, chloramphenicol, was attached, as a prodrug, to p8 coat protein molecules on the surface of the m13 phage subtype A12C. Upon release of active chloramphenicol against *S. aureus*, partial growth inhibition was observed, which was interpreted as a possible limitation in drug loading primarily owing to the hydrophobic nature of chloramphenicol. This challenge was improved in subsequent studies in which a conjugation method involving aminoglycosides as branched, solubility-enhancing linkers was adopted, resulting in a drastic increase in the drug-loading capacity of the phages. It was noted that over 40 000 chloramphenicol molecules/phages were loaded without disrupting the phage composition. However, about 10 000 molecules/phages were sufficient to produce the desired result, ultimately leading to an improved phage-antibiotic formulation. The results from this study demonstrated an improvement factor of approximately 20 000 compared to the free drug. Another study showed rapid uptake and enhanced antibiotic efficacy of phage-antibiotics upon conjugation of azithromycin to bacteriophage Qβ both *in vitro* and in mouse lungs. The results also indicate the possibility of adopting this formulation to achieve more efficient antibiotic treatment. This strategy could also be explored for pulmonary drug delivery.[127] The enhanced therapeutic effect of phage-mediated drug delivery due to its ability to take up more than one therapeutic material was demonstrated by Stephanopoulos *et al.*[128] by dual modification of the MS2 coat protein, generating a target against Jurkat leukemic T-cell therapeutic nanocarrier. A cell-specific DNA sequence was attached to the phage envelope, and the porphyrins were bound to the phage interior. Upon delivery of the cargo to cells of interest, there was illumination of porphyrins, which resulted in the release of a significant amount of reactive oxygen radicals. This reaction was achieved *via* a photodynamic effect. The generation of oxygen radicals led to the destruction of the target cells within 20 minutes.

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The integration of nanotechnology with phage techniques has led to the development of novel strategies to improve therapeutic activities and reduce the toxic effects of chemotherapeutic agents.[129] A study assessing the anti-tumour activity of MCF-7-specific phage fusion protein-modified liposomal doxorubicin (Doxil) resulted in more rapid and effective antitumor activities than the non-targeted formulations.[130] There was a higher accumulation of phage-Doxil in the tumour cells. Very mild side effects were noted *in vivo*, and hepatotoxicity tests were negative in the phage-doxil-treated mice. Gan *et al.*[131] conducted a study to stop neovascularisation caused by vascular endothelial growth factor (VEGF) and its receptor VEGFR2. By conjugating VEGFR2 to T4 phages, T4-VEGFR2 was obtained. The VEGFR2-phage was then cultured with endothelial cells. There was a significant impairment of neovascularisation due to the inhibition of VEGFR2 phosphorylation and downstream signalling. It was also observed that upon administration of T4-VEGFR2 phages through the intravenous route to experimental animals with lung and colon cancer, there was notable inhibition of tumour growth and vascularisation.

2. Specific targeting and site-specific delivery. Specific barriers affect the permeability of therapeutics. For example, the difficulty in shuttling drugs across the blood-brain barrier (BBB) can impair drug delivery to the central nervous system (CNS). Viruses can intrinsically recognise and attach to specific cell receptors and deliver genes or drugs (Fig. 4). This unique characteristic makes them ideal candidates for targeted delivery systems.[132] The display of tissue-targeting peptides on the phage coat was shown to deliver therapeutic compounds into cardiac myoblasts. Zahid *et al.*[133] modified M13 surface proteins with an APWHLSSQYSRT peptide sequence, enabling the affinity of the phages to cardiac myoblasts. The capsid of M13 has also been modified for breast tissue-specific delivery of therapeutics by adding targeting peptides, such as VOWMEPAYQRFLGGG. This phage capsid-peptide combination was able to recognise breast cancer tissues and neuroblastoma cells.[134] More interestingly, filamentous phages can also penetrate the blood-brain barrier, and this property could make them suitable for use in the treatment of Alzheimer's disease.[135] Rakover *et al.*[136] discovered that the fibrous nature of filamentous phages enables their permeation into the central nervous system *via* nasal administration. They conjugated myelin oligodendrocyte glycoprotein immunodominant epitope (MOG 36-44) on a filamentous phage as an immune-recognising epitope for the treatment of multiple sclerosis. This setup was proposed to function through the inactivation of demyelinating antibodies in the central nervous system. There was significant termination of autoantibodies against MOG and demyelination, which led to reduced inflammation in the CNS and periphery as well as enhancement of overall clinical outcomes in treated mice. The above studies show that there is potential for phages to be loaded with therapeutic agents that can be delivered to target sites, which are challenging to target.

To date, the liver has remained one of the most challenging sites for drug delivery. The option of injecting therapeutic agents into liver tissues has been explored. However, the detection of these agents by Kupffer cells stimulates undesirable immune responses.[137] It has been observed that by labelling the tail fibre protein (P17) of the T7 phage with the asialoglycoprotein receptor (ASGPr), drugs could be successfully delivered into hepatocytes.[138] Wong *et al.*[138] adopted this principle by attaching a targeting ligand composed of 33 amino acids to P17 to transfer siRNA, liposomes, and DNA polyplexes into hepatocytes. Ashley *et al.*[95] established phages as highly reliable cargoes for delivering diagnostic agents, nanoparticles, and siRNA molecules. In their work, MS2 virus-like particles (VLPs) modified with a peptide (SP94) that binds to hepatocellular carcinoma (HCC) exhibited a 10^4-fold higher avidity for HCC than for hepatocytes, endothelial cells, monocytes, or lymphocytes. This modification also enabled the delivery of higher concentrations of therapeutic agents to the cytosol of HCC cells.[139] Vaks and Benhar[140] presented a method involving both the genetic and chemical modifications of filamentous phages to achieve targeted delivery. Through genetic manipulation, phages can display host-specificity-conferring ligands, and by chemical conjugation, cytotoxic drugs can be loaded into the phages. Selective lysis of cancer cells was achieved upon detection of target cells and delivery of therapeutic substances. Scibilia *et al.*[141] reported a different kind of phage-based formulation. Here, a hybrid-nanostructured carrier consisting of an M13 P9b phage clone co-formulated with silver nanoparticles (AgNPs) was developed and used in a therapy targeted against *Pseudomonas aeruginosa*.

3. Synergistic effects of antibiotics. Phages have long been used as nanocarriers for the delivery of antibiotics. The significant antibacterial improvement obtained with chloramphenicol, as reported by Yacoby *et al.*,[92] is because of the increased drug payload from 3000 to 10 000 molecules/phage. Additionally, the synergistic effect was attributed to the improved solubility and possible bioavailability of the entire phage-based antibiotic platform. It was noted that neomycin, used as the aminoglycoside linker, has antibiotic properties. However, the chemical linkage to the phage capsid was caused by a non-labile bond. Therefore, neomycin was not released and did not contribute to the obtained antibacterial effect.

4. Reduced side effects and toxicity. In the study by Vaks and Benhar,[140] an antibody was displayed on the pIII minor coating protein of the f1 phage, with chloramphenicol also chemically conjugated to the phage. This conjugation to a filamentous phage resulted in controlled toxicity and side effects of chloramphenicol at a therapeutic dose. Du *et al.*[142] employed a 12-mer commercial M13-displayed library for the *in vivo* biopanning of xenograft models of BEL-7402 hepatocarcinoma (HCC). Phages were administered intravenously, after which specific tumour-enriched phage clones displaying AGKGTPSLETTP peptides were selected from tumour masses in mice. The therapeutic agent doxorubicin (Dox) was conjugated to the A54 peptide, enabling a strong antitumor effect in HCC tumour-bearing mice. No side effects were observed from this formulation compared to treatment with the drug in its unconjugated state.

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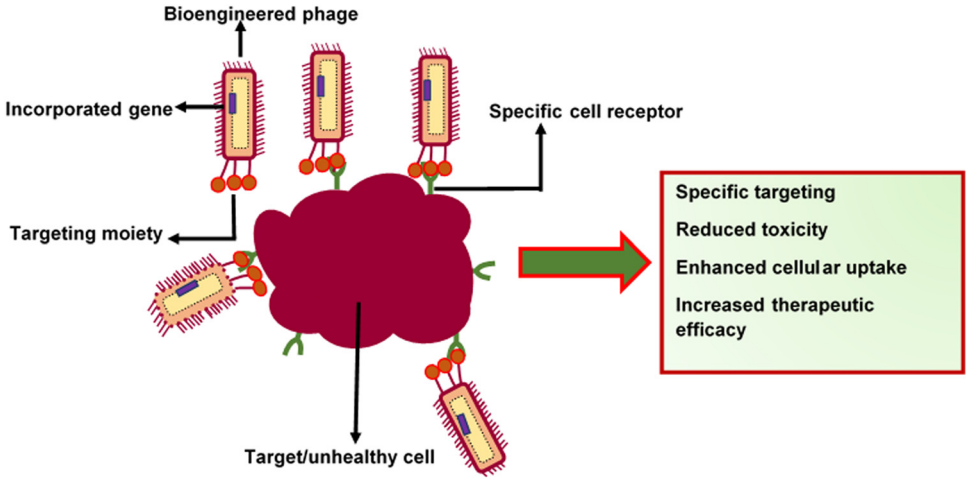

Fig. 4 Specific cell targeting using bioengineered bacteriophages.

4. Applications of bacteriophage-mediated drug delivery systems

As nanocarriers, bacteriophage-inspired drug delivery systems have been applied in cancer therapy, gene therapy, treatment of bacterial infections, vaccination and detection of serological biomarkers, as illustrated in Fig. 5. These applications are described below.

4.1 Cancer therapy

Chemotherapeutic agents delivered using conventional drug delivery systems pose several challenges, such as low drug concentration at the target site, high toxicity resulting from a wide distribution in the body, and the induction of drug resistance. To overcome this problem, the use of bacteriophages as bio-inspired nanocarriers has attracted attention in recent years. Bacteriophages offer many benefits over traditional delivery systems in cancer therapy.[143] Such benefits include their favourable physicochemical properties of nanosized and polyvalent surface morphology. In addition, phages do not exhibit mammalian cell tropism; therefore, they do not affect normal human cells. This allows engineered phages with peptide displays on their surfaces to be targeted to specific tumour cells. Some bacteriophages can avoid clearance by the reticuloendothelial system, thereby exhibiting a long circulation time in the body and increased bioavailability.[144] In addition, bacteriophages are non-toxic, cost-effective, and easy to produce with high scalability potential.[145,146] Phage-based nanocarriers have been investigated in cancer therapy *via* phage display technology, production of DNA vaccines, genes, and targeted drug delivery using MS2, Lambda, T4, M13, T7, and QB bacteriophages.

Bacteriophages can induce immunity and are studied for use as carriers of cancer vaccines. The phages are taken up by antigen-presenting cells, while the peptide (antigen) on its surface is presented *via* major histocompatibility complexes (MHCs). The antigen presentation on MHC leads to the activation of CD8+ T and CD4+ T cells into cytotoxic T lymphocytes and T helper cells, respectively. The former directly kills infected host cells, while the latter aids in producing specific antibodies by B cells.[147] The mechanism of phage-induced immunity is shown in Fig. 6. Iwagam *et al.* (2017) investigated the antitumor effect of lambda (λ) phage vaccine against aspartate β-hydroxylase (ASPH) expressing murine liver tumours. The mice were vaccinated pre- and post-subcutaneous implantation of a hepatocellular carcinoma (HCC) cell line. A significant delay in the growth and progression of HCC was recorded after the prophylactic and therapeutic vaccination. Antigen (ASPH) specific CD4+ and CD8+ lymphocytes, Th1 and Th2 cytokines, were generated by tumour-bearing mice.[148] Similarly, Wang *et al.* (2022) investigated M13 bacteriophages for both prophylactic and therapeutic vaccination against breast cancer. The phage was designed to display the extracellular and transmembrane domains of human HER2 or Δ16-HER2 (a splice variant of HER2) on their surface.[149] These surface peptides aid in the navigation of the bacteriophage and the identification of target molecules. The onset of the mammary tumour was delayed, and the tumour growth rate was reduced in Δ16HER2 transgenic mice. HER2-specific antibody production correlates with the antitumor effect. The suggested mechanisms of this antitumor activity are the impairment of ERK phosphorylation and the reactivation of suppressor retinoblastoma protein function.[149] Therefore, phage-based vaccines impair the onset and progression of tumours and can be a platform for cancer prophylaxis and treatment.

Bacteriophages are carriers of drugs, genes, and imaging agents. Through phage display technology, bacteriophages can be engineered to display functional peptides on their surfaces. Such proteins act as ligands interacting with receptors and biomarkers in the tumour cell microenvironment. This interaction increases active targeting and enhances chemotherapeutic penetration and retention effects. Thus, phage display technology has been widely investigated in targeted gene delivery systems. Chondrosarcoma exhibits treatment resistance using conventional methods of radiotherapy and chemotherapy.[151] Chongchai *et al.*[152] employed bacteriophage display technology to design a phage carrying a human tumour necrosis factor-alpha (TNFα) transgene cassette for targeting chondrosarcoma.

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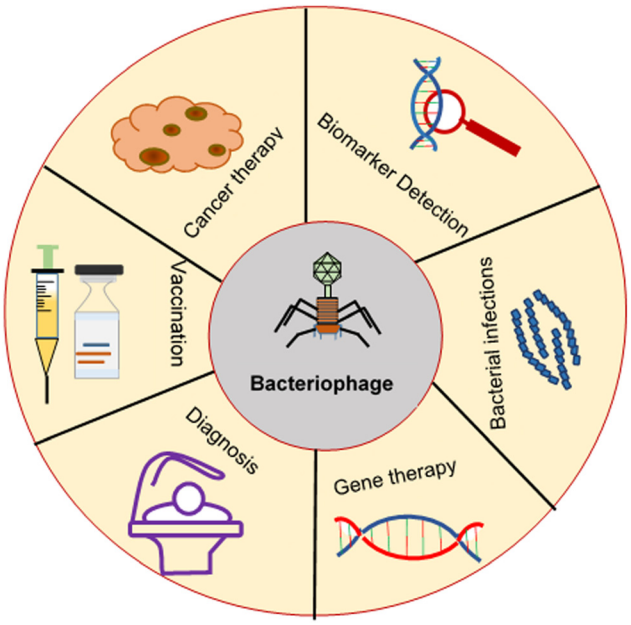

Fig. 5 Applications of bacteriophages.

The phage containing the incorporated gene was designed to display the endosomal escape peptide and RGD4C ligand to enhance gene delivery and specifically bind to integrin receptors (Fig. 7). The tumour cell overexpresses the integrin receptor and human tumour necrosis factor alpha (TNFα) receptors. Therefore, these receptors are targets in drug delivery to tumour cells. The expression of a high amount of TNFα and other apoptosis-related genes after treating the tumour cells with the phage led to significant cell death. Suppression of tumour growth in mice treated with the targeted bacteriophage was also reported.

Another lethal resistant tumour investigated for treatment using bacteriophage-based suicide gene therapy is glioblastoma multiforme (GBM). Przystal *et al.*[153] developed a multicomponent M13 phage system consisting of a recombinant adeno-associated virus genome, a glucose-regulated protein (Grp 78), a chemotherapeutic agent (temozolomide), and a phage display protein (RGD4C). The RGD4C ligand binds to integrin receptors on tumour cells, while temozolomide induces the expression of the Grp78 gene and promotes transgene expression. This combination of gene therapy and chemotherapy has been reported to have a synergistic antitumor effect.

Hwang *et al.*[154] designed a T7 bacteriophage displaying a Pep42 peptide targeting glucose-regulated protein 78 (GRP78) expressed in murine melanoma cells. A mammalian expression cassette of the cytokine granulocyte macrophage-colony stimulating factor (GM-CSF) was incorporated into the phage genomic DNA. There was 100% survival of mice treated with the phage, while the untreated group recorded only 40% survival within the study period. Tumour growth was remarkably inhibited in the treated group. Immunological responses were also observed during treatment. Such responses include increased

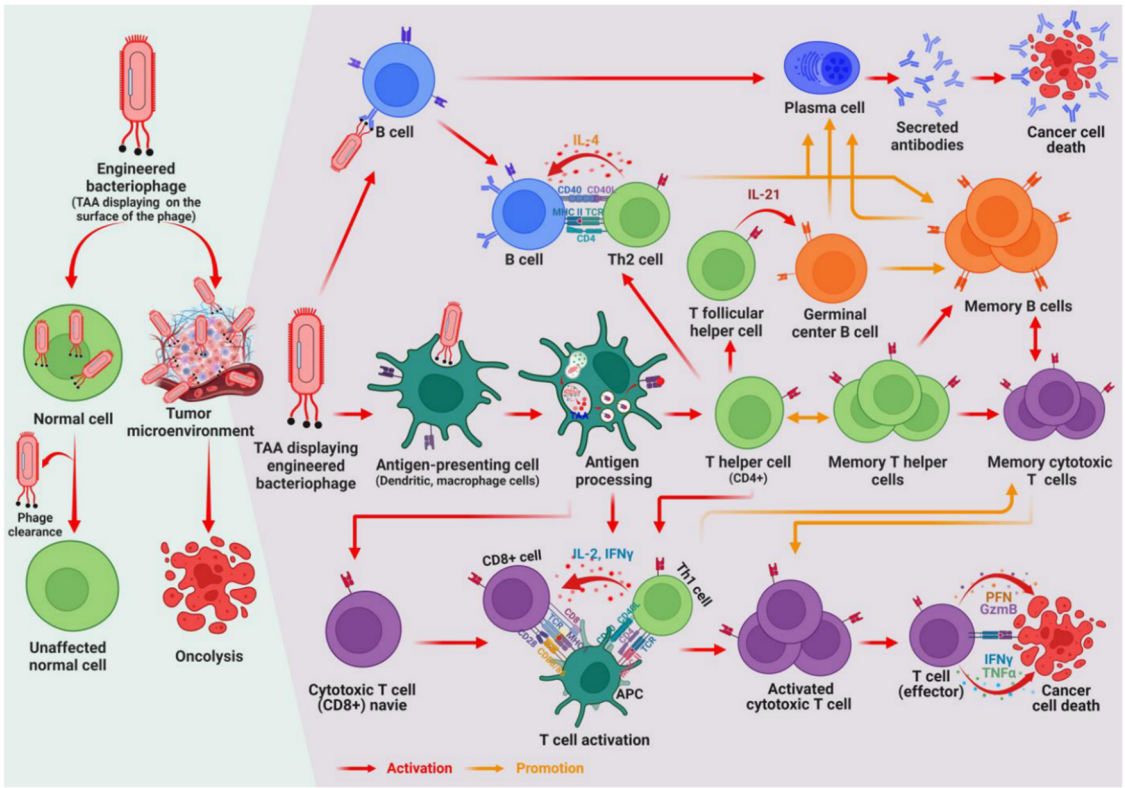

Fig. 6 Mechanism of phage-induced immunity. Reprinted from Ragothaman et al., 2023 with permission from MDPI.[150]

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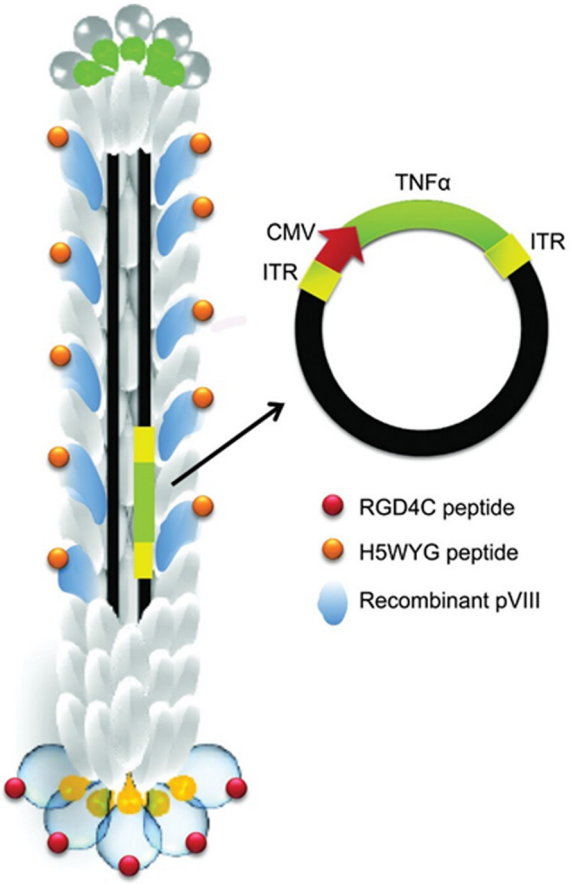

Fig. 7 Schematic representation of the RGD4C-targeted phage encoding TNFα. Modified phage capsid with RGD4C ligand and endosomal escape peptide, H5WYG, displayed on the pIII minor and pVIII major coat proteins. The incorporated transgene expression cassette was composed of tumour necrosis factor alpha (TNFα) transgene, cytomegalovirus (CMV), and inverted terminal repeats (ITRs) *cis*-elements from adeno-associated virus (AAV2). Adapted from Chongchai *et al.*, 2021 with permission from Wiley.[152]

antitumor immune cells, including IL-1α, TNF-α, and GM-CSF, and the migration of macrophages, dendritic cells (DCs), and CD8+ T cells into tumour cells.

Another strategy for cancer therapy is *via* the vascular endothelial growth factor (VEGF) signalling pathway. This pathway is essential in the angiogenesis of cancer cells. T4 phages displaying the extracellular domain of VEGF receptor 2 were investigated owing to their anticancer activity.[155] The VEGF receptor 2 phages specifically bind to VEGF, causing the inhibition of VEGF-mediated phosphorylation of the VEGF receptor and other signalling pathways, including extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK). Hence, murine Lewis lung and colon carcinoma models hinder cell proliferation and tumour growth. In addition, the survival time was prolonged in the tumour-bearing mice.

Several reports have shown the potential of microRNAs in cancer therapy. However, instability and low cellular delivery efficiency hinder their use. Bacteriophages can be used to deliver microRNAs to cells and protect them from enzymatic degradation by RNase. Sun *et al.*[156] developed PP7 bacteriophage-based virus-like particles to provide microRNAs using an *E. coli* expression system. They conjugated particles with cell-penetrating particles (transactivated transcription (TAT) peptide) to enhance the *in vivo* delivery of RNA. The authors reported that the phage virus-like particles modified with a penetrating peptide penetrated the liver cancer cells. The upregulation of RNA and the subsequent reduction of liver-intestine cadherin expression led to the suppression of hepatoma. Previous studies using phage virus-like particles conjugated to cell-penetrating peptides in liver cancer reported similar findings.[157,158]

A combination of photothermal and chemotherapy was studied using a genetically engineered T7 phage. Oh *et al.*[159] investigated the T7 phage with a prostate cancer cell-binding peptide and a gold-binding peptide for targeted photothermal therapy of prostate cancer. Self-assembling gold nanoparticles and the engineered phage formed phage-gold nanoparticle clusters. Compared to non-targeted gold nanoparticles, the targeted phage-gold nanoparticles destroyed the cancer cells within few minutes when irradiated. The results demonstrated the uptake of phage-gold nanoparticles by human prostate carcinoma cells when treated with the engineered phage.[159] This selective accumulation of nanoparticles presents a prostate cancer cell-targeted delivery.

4.2 Therapeutic effects against bacterial infections

Abuse of antibiotics in humans has led to the development and spread of antimicrobial resistance worldwide.[160,161] Common multidrug-resistant organisms are ESKAPE pathogens, which include *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa*, and *Enterobacter species*.[162] Efforts are geared toward solving this public health issue. One of the approaches investigated is the use of bacteriophages. Their specificity and role in maintaining healthy microbiota make them an exciting and promising option. Phages can engage with pattern recognition receptors to activate immune signalling pathways and to generate and recruit immune cells to the infection site. Bioengineered phages with surface peptides are also engulfed by APC and presented to MHC to generate cytotoxic T lymphocytes and T helper cells to kill the infected cells directly or to produce antigen-specific antibodies against the causative organisms.

Eradication of ESKAPE pathogens using bacteriophages alone or with antibiotics has been reported in the literature.[163,164] Diabetic foot ulcers are deadly diabetic complications characterised by infections with multidrug-resistant bacteria. Ghanaim *et al.*[165] investigated the topical use of a phage cocktail to treat diabetic foot ulcers infected by *S. aureus*, *P. aeruginosa, Klebsiella variicola, Escherichia coli*, and *Proteus mirabilis*, with the therapy showing better wound healing and anti-inflammatory parameters than those treated with ceftriaxone. Hence, phage therapy could be an effective option for treating multidrug polymicrobial infections. MMI-*Ps*1 is a newly isolated phage with a powerful lytic effect. A single dose of this phage administered immediately or 12 hours post-infection exhibited a protective effect in a mouse model of acute lung infection with *P. aeruginosa*. The phage therapy also enhanced the complement-mediated lysis and elimination of the resistant *Pseudomonas strain*.[166]

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Lytic enzymes from bacteriophages (lysins, endolysins, or enzybiotics) are potent antibacterial agents with faster killing rates than standard antibiotics. The benefits of using phage lysins as an antimicrobial agent include high specificity, activity against multidrug-resistant organisms, low toxicity, and low chances of resistance development.[167] The antimicrobial activity of three lysins- wild-type Cpl-1, the engineered Cpl-7S, and the chimaera Cpl-711 were evaluated against a pneumococcal colonised nasopharyngeal mouse model.[168] Human nasopharyngeal and lung epithelial cells were infected with three different multidrug-resistant clinical isolates of S. pneumonia, and a single dose of complementary lysin was given afterwards. The engineered Cpl-7S had no activity against the test organism, while Cpl-1 and Cpl-711 effectively killed the organism. Treatment with Cpl-711 showed superior antimicrobial activity against the multidrug-resistant organism than Cpl-1. Furthermore, a significant reduction in pneumococcal colonisation was observed in mice treated with Cpl-711, with no detection of bacterial load in 20 to 40% of the mice.[168]

In a randomised clinical trial, intravesical bacteriophage therapy exhibited efficacy similar to standard antibiotic therapy in treating urinary tract infections in patients undergoing transurethral resection of the prostate with a better safety profile.[169] Apart from the efficacy of bacteriophages in treating diseases, their safety is of utmost importance. Fabijan *et al.*[170] assessed the safety of phage therapy in patients with severe *S. aureus* infection. Patients who were given a phage cocktail of three *Myoviridae* bacteriophages (AB-SA01) intravenously twice daily for two weeks showed no sign of adverse reactions within a study period of 3 months.

4.3 Gene therapy

Gene therapy requires safe and efficient delivery systems that can target a specific cell or tissue. Viruses are natural carriers of genetic materials and can transfer the same into other biological systems.[21] The intrinsic potential for bacteriophages and phage particles to be loaded with genetic materials makes them attractive nano-vehicles for gene delivery. Phages equally display foreign proteins on their surfaces, enabling targeted gene therapy to specific cells.[140,171,172] Ghaemi *et al.*[173] conducted several experiments to study lambda-ZAP bacteriophage-mediated gene transfer and expression *in vitro*. In their study, genes encoding an enhanced green fluorescent protein (EGFP) and E7 of human papillomavirus (HPV) were conjugated to recombinant lambda-phage nanobioparticles to enhance the anti-tumour immune response against HPV. Dual modification of bacteriophages is a promising strategy for improving targeted gene delivery in combating disease conditions. In this method, the phage surface displays specific ligands, while the genes of interest are encapsulated in the phage capsid, where they are protected from enzymatic degradation.[174] Thomas *et al.*[175] established that dual modification could elicit higher immune responses. Transacting activation transduction (TAT) peptides, which serve as cell-penetrating ligands, were used in a study conducted by Tian *et al.*,[176] who conjugated this TAT peptide onto the exterior surface of a TMV phage. The TMV–TAT conjugation system successfully delivered green fluorescent protein (GFP) silencing RNA (siRNA) into GFP-expressing hepatocellular carcinoma tumours *in vivo.*

4.4 Diagnosis, disease detection, and monitoring

Traditional molecular imaging methods using one biomarker have resulted in inaccurate conclusions. Their use is also limited by the high cost of fabrication and the need for sophisticated handling. Therefore, multi-modification of phages could be used in the accurate and precise diagnosis of diseases, which would ultimately enable personalized therapy.[177,178] Viruses, such as bacteriophages, can carry a wide range of targeting agents, immune-fluorescent labels and various markers to target particular cells and tissues. Viruses bearing imaging labels have the advantage of short retention times, making them easily removed from the system compared to other non-viral biomaterials. Virus-based nanocarriers have, therefore, been used in imaging processes, such as fluorescence imaging, magnetic resonance imaging (MRI), positron emission tomography (PET), computed tomography (CT), and others.[179,180] The use of single-walled carbon nanotubes (SWNTs) in medical diagnosis has been flawed because of their lack of stability in biological environments. To proffer a solution, Yi *et al.*[181] bound SWNTs to the pVIII of M13 phage peptides specific to prostate cancer. The aim was to generate a deep-tissue diagnostic imaging agent. From the M13-SWCNT phage, cell targeting was achieved with highly visible fluorescent images of the tumour, and more biologically stable SWNTs were obtained in the biological environment. It was, therefore, concluded that phage-assembled SWNTs may be used as reliable imaging agents. There have been reports of *in vivo* optical imaging with fluorescent dyes for the detection of bacterial infections using bacteriophage M13.[182] They demonstrated that by conjugating these dyes to bacteriophage M13, *F*-pili expressing and *F*-negative strains of *E. coli* could be identified. The phage coat was equally modified by adding biotin acceptor peptides to enable the detection of other bacterial strains as well. It was reported that the biotinylated M13 phage specifically detected and targeted *S. aureus* infections. Phages engineered with fluorescent or radiolabelled peptides on their protein coats are usually employed in molecular imaging to distinguish between various cancer or metastatic cells. For example, in these studies, molecular imaging was carried out *in vivo* using green fluorescent protein (GFP) conjugated to phages.[183,184] Using recombinant DNA technology, Choi *et al.*[185] co-engineered a bacteriophage that expressed cyclic RGD (cRGD) peptides on the pVIII major coat protein. They proposed that upon modification of this cRGD peptide, engineered phages with therapeutic or imaging agents could find use in therapy or diagnosis. Bacteriophages can also be used in theranostics when loaded simultaneously with imaging biomarkers and therapeutics, as described by Dymova *et al.*[186] using a tumor-targeting bacteriophage technology, diagnostic imaging and therapeutic agents were delivered to malignant triple negative MDA-MB-231 breast cancer cells. They proposed that modification of phages using modifying agents, such as FAM–NHS dye, enhanced

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internalization of the phages into cancer cells, specifically MDA-MB-231 cells.

Autoantibodies for disease conditions were obtained from serum. This indicates that serum can be used to screen, diagnose, or monitor diseases.[187] In the diagnosis of serological biomarkers, large volumes of serum and serum samples are typically used, which is very costly. The T7 phage display system can detect serological biomarkers more accurately and cheaply. This was demonstrated by Talwar *et al.*[188] in detecting antibodies reactive to *Mycobacterium tuberculosis* (MTB) antigens in bodily fluids. In this study, sarcoidosis, which has clinical and pathological characteristics similar to MTB, was used. Four T7 phage display cDNA libraries were prepared and screened using sera from healthy controls, sarcoidosis and culture-positive MTB patients. The results showed highly sensitive and specific biomarkers for MTB in the sera of subjects with culture-positive MTB.[188] In a similar study by this group, T7 phages were immune screened with sera of smear-positive TB patients. This was done to identify specific diagnostic biomarkers from the sera of TB-positive patients. From the results, highly specific TB clones, which can be used to differentiate TB patients from healthy controls and sarcoidosis patients, were clearly detected.[189] The pIII protein of filamentous phage nanocarriers can be used to display a targeting sequence for the biodetection of specific antigens from sera, while diagnostic peptides can be engineered to the pVIII protein coat.[190] Lee *et al.*[190] showed that pVIII modification with plasmon-resonant gold nanoparticles could rapidly detect antigens initially recognized by peptides displayed on the pIII protein.

4.5 Detection of bacteria in food

To avoid developing and disseminating food-borne diseases, a strategy for food safety assessments could be achieved using genetically engineered bacteriophages. Wisuthiphaet *et al.*[191] demonstrated this using phage T7-ALP, which expresses the enzyme alkaline phosphatase. By infecting the phage with *E. coli*, there was overexpression of alkaline phosphatase. This serves as a signal indicating bacterial presence in the model beverage samples. This process was followed by fluorescence imaging and image analysis for the more sensitive detection of single-cell bacteria. Another advantage of this method is the relatively short completion time of less than 6 h, which makes it ideal for use in major industrial food processing facilities with up to 8 h operational times. Another T7 phage conjugated to iron oxide magnetic beads was developed to rapidly detect and separate *E. coli* in drinking water.[192] The T7 phages lysis *E. coli* cells and endogenous β-galactosidase were then detected using chlorophenol red-β-D-galactopyranoside, a colourimetric substrate, which changes from yellow to red in the presence of β-galactosidase. This strategy showed high sensitivity and specificity against a background of competing bacteria.

4.6 Phage-based vaccination

The application of phage-based nanocarriers in vaccination has emerged as an intriguing area for researchers. Immunity can be achieved with bacteriophages by direct vaccination with antigen-loaded phage particles or by delivering DNA vaccines expressed in the phage genome. Antigens of interest can be displayed on phages, which trigger an antibody response upon administration, thereby generating immunity.[193,194] Deng *et al.*[195] fused an extracellular domain of matrix protein 2 to the N terminus (pVIII) of M13 phages. The recombinant phages were assessed *in vivo* for immunogenic potentials. A very significant protein 2 immune response was elicited, and the protein-phage was able to confer protection in mice infected by human and avian type A influenza viruses. Ghaemi *et al.*[196] conducted a study on phage vaccination against cervical cancer. The gene coding for human papillomavirus 16 (HPV16) proteins E7 was incorporated into the Lambda ZAP CMV vector to give a ZAP HPV16 E7 bacteriophage complex. The immunogenicity of this complex was tested *ex vivo* on cells from immunized mice. There was an increased release of granzyme B, which is actively implicated in cytotoxicity and antiviral activities. Phage-based vaccination has also been applied to fungal infections. In *Candida albicans*, the secretory aspartyl proteinase (SAP) family proteins are known to possess virulence attributes. Filamentous fd phages displaying the epitope (the peptide Val-Lys-Tyr-Thr-Ser) from SAP2 were developed and established to produce a strong immune response *in vivo*. Mice infected with *C. albicans* and vaccinated using the fd phage-peptide dramatically increased survival compared to non-vaccinated mice.[197] It was concluded that this phage-based vaccine showed promising potential and could be studied further in clinical trials.

Interestingly, it has been noted that phage-based vaccines play roles in disease prevention. Additionally, they have been studied for hormonal control. An example was cited where fertility could be controlled with gonadotropin-releasing hormone-based antigenic vaccines. It was proposed that this vaccine could inactivate endogenous gonadotropin-releasing hormones, thereby regulating their release and, by extension, fertility.[198] Bacteriophage-particle vaccines have been developed for viral infections, such as Zika, influenza A, and Norwalk, and bacterial diseases, such as brucellosis, anthrax, and bubonic plague.[104] Phage virus-like particles (VLPs) have also presented the human papillomavirus (HPV) L2 epitope, leading to the development of HPV-neutralizing antibodies.[199] By conjugating a tandem HPV31/16L2 peptide (amino acid 17–31) onto the surface of bacteriophage MS2 virus-like particles (VLPs), immunized mice models released high antibodies against individual L2 epitopes and were protected from multiple HPV types.[199]

Some of the studies on the application of bacteriophages are summarized in Table 3.

5. Challenges

Though inherently considered safe, there are often issues with safety considerations, depending on the phage type, preparation, and route of administration.[200] These include the potential ability to trigger immune responses, neutralization and clearance, and chances of toxicity attributable to the phage component or loaded

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Table 3 Summary of some studies on the applications of bacteriophages

Disease	Type of therapy	Bacteriophage	Cargo	Phage displays protein or targeting ligand	Ref.
Hepatocellular carcinoma	Immunotherapy	Lambda (λ) phage	—	Aspartate β-hydroxylase (ASPH)	148
Breast cancer	Immunotherapy	M13 bacteriophages	—	Extracellular and transmembrane domains of human HER2 or its Δ16HER2 splice variant	149
Chondrosarcoma	Gene therapy	M13 phage	Tumour necrosis factor-alpha (TNFα) transgene cassette	RGD4C	152
Glioblastoma multiforme	Gene and drug therapy	M13 phage	Adeno-associated virus genome, Grp 78, temozolomide)	RGD4C	153
Melanoma	Gene therapy	T7 bacteriophage	Granulocyte macrophage-colony stimulating factor (GM-CSF	Pep42 peptide	154
Lung carcinoma and colon carcinoma	Targeted therapy	T4 phages	—	Vascular endothelial growth factor (VEGF)	155
Hepatoma	Gene therapy	PP7 bacteriophage-based virus-like particles	MicroRNAs	Transactivated transcription (TAT) peptide	156
Human papilloma virus (HPV)	Gene therapy (gene transfer and expression)	Lambda-ZAP bacteriophage	Recombinant lambda-phage nanobioparticles	Enhanced green fluorescent protein and E7 of HPV	173
Hepatocellular carcinoma tumours	Silencing RNA (siRNA)	TMV phage	TMV–TAT conjugation system	Transacting activation transduction (TAT)	176
Prostate cancer	Deep-tissue diagnostic imaging	M13 phages	M13-SWCNT phage	Single-walled carbon nanotubes (SWNTs)	181
E. coli and *S. aureus* bacterial infections	*In vivo* optical imaging	M13 phages coat	Biotinylated M13 phage	Fluorescent dyes, biotin acceptor peptides	182
Cancerous and metastatic cells	Molecular imaging	Engineered phage protein coat	Engineered phage protein coat	Green fluorescent protein (GFP)	183 and 184
—	Diagnosis	—	pVIII major coat protein	Cyclic RGD (cRGD)	185
Malignant triple negative MDA-MB-231 breast cancer cells	Therapy and diagnosis	Tumor-targeting bacteriophage	—	FAM–NHS dye	186
Mycobacterium tuberculosis	Diagnosis	T7 phage	—	Sarcoidosis antigens	189
E. coli infection	Detection of *E. coli in* Food-borne infection	T7 phage	Phage T7-ALP	Alkaline phosphatase	191
E. coli infection	Detection and separation of *E. coli* in drinking water	T7 phage	T7 phage-conjugated to iron oxide magnetic beads	—	192
Human and avian type A influenza viruses	Immunogenicity	Recombinant M13 phages	N terminus (pVIII) of M13 phages	Protein 2	195
Cervical cancer	Vaccination	Lambda ZAP CMV	ZAP HPV16 E7 bacteriophage complex	Gene coding for human papillomavirus 16 (HPV16) proteins E7	196
Candida albicans infection	Immune response	Filamentous fd phages	—	Val-Lys-Tyr-Thr-Ser epitope from secretory aspartyl proteinase (SAP)	197
Fertility control	Hormonal control	—	—	—	198
Human papillomaviruses (HPVs)	Immunization	MS2 phage-like particles	Phage virus-like particles (VLPs)	Human papillomaviruses (HPVs) L2 epitope (HPV31/16L2 peptide)	199

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cargoes (*e.g.* phage-associated conversion of Tox− *Streptococcus pyogenes* into Tox+ bacteria *in vivo*, as described by Broudy *et al.*,[201] especially in temperate phages[202]), unintended accumulation in non-target tissues, conferment of resistance genes to pathogenic bacterial strains, poor storage and stability profile, and unintended potential consequences of genetic engineering. Thus, safety evaluations are essential to harness their therapeutic effects safely and effectively in targeted drug delivery.

The regulatory challenges of using bacteriophages as nanocarriers in targeted drug delivery and therapeutic applications are complex and multifaceted but are centred on specific considerations, such as classification, economic viability and manufacturing standards, safety assessments, quality control, clinical trials, intellectual property, regulatory guidance, environmental impact, global harmonization, and post-approval monitoring. Long-term monitoring of safety and efficacy post-approval is essential, especially given the evolving nature of bacteriophage interactions and potential unforeseen effects. The regulation of phage therapy is growing, and addressing these regulatory concerns would require close collaboration among researchers, regulatory agencies, and other stakeholders.[203]

The other apparent challenges include the high selectivity and specificity of bacteriophages to limited species of host bacteria, thus limiting their applicability against a broad range of pathogens and diseases; rapid and over-amplification of therapeutic phages *in vivo* in the presence of their host bacteria, leading to unintended consequences; the potential development of resistance of target pathogen to bacteriophage formulations and undermining the long-term efficacy of the phage-based treatments; complexities associated with the delivery of phage formulations to target sites within the body owing to certain factors, including the pharmacokinetics and bioavailability; ethical concerns and public acceptance of genetically modified bacteriophages; limited data and clinical trials to validate research claims, effectiveness, long-term effects, and optimal usage; and the inadequate standardization of large scale phage formulation/preparations and production.[204–207]

6. Future perspectives and research directions

Surprisingly, low phage cytotoxicity studies have been published; thus, there is a need to advance this area.[208] As Pirisi[209] rightly summed: ''What we think we know about phages has to be verified and then deemed reproducible, safe and effective''. Currently, most phage products are administered as suspensions or dressings (for skin infections), which are usually unsuitable for targeting specific target sites, as phages are rapidly cleared around these application areas.[208] Pharmaceutical formulations must encapsulate and protect phages from the environment while preserving their biological activity, especially their lytic activity. Moreover, the excipients selected must be biocompatible and can release phages at the desired site of infection.[208] The treatment outcomes of self-replicating pharmaceutical agents depend critically on various density-dependent thresholds, often with apparent paradoxical consequences; thus, the ability to predict these thresholds and associated critical time points becomes necessary for the clinical use of phage therapy.[210] The exact modalities for using each phage product must be ascertained, including treatment regimens, route of administration, concentration and quantity, and methods.[208] Finally, in the interim, the potential of phage therapy may be fulfilled using individual preparations on a named-patient basis, with extensive monitoring and multidisciplinary team input.[202]

7. Conclusion

The efficient delivery of therapeutic agents to target sites is a crucial problem and area in managing disease conditions. Nanotechnological advances utilising various nano-sized particles present promising platforms for this course. VLPs, specifically bacteriophages, with their desirable properties, including having potential therapeutic effects and serving as alternatives to antibiotics, also present the opportunity for use as effective nanocarriers in the delivery of drugs. Their use in drug delivery has many advantages, including biocompatibility, large loading capabilities, stability, evasion of immune responses, and easy modifiability. The various forms of the use of bacteriophages in this regard include their use in the capsid-associated display of peptides and antibodies, chemical conjugation of molecules, polymer coating of capsids, capsid manipulations and encapsulation, attachment of multi-targeting ligands and moieties, and image tagging. The bacteriophage-mediated drug delivery has many applications, such as in cancer therapies, bacterial infections and gene therapies. Although there are currently certain safety and regulatory challenges, the continuous research effects promise to open more doors increasingly.

Author contributions

S. C. E.: conceptualization, writing – original draft, writing – review & editing; A. L. O.: conceptualization, writing – original draft, writing – review & editing; C. F. K.: writing – original draft; P. N. E.: writing – original draft; O. C. E.: writing – original draft; M. M. D. C. V.: supervision, validation; V. M. B.: supervision, validation; A. A. A.: supervision, validation; E. B. O.: supervision, validation.

Conflicts of interest

There are no conflicts to declare.

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