

ENGINEERED PHAGE-BASED THERAPEUTIC COMPOSITIONS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims priority to U.S. Provisional Patent application serial no. 63/597,619, filed November 9, 2023, which is incorporated herein by reference in its entirety.

STATEMENT OF GOVERNMENTAL SUPPORT

[002] This invention was made with government support under GM148249 awarded by the National Institutes of Health. The government has certain rights in the invention.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[003] The instant application contains a Sequence Listing conforming the rules of WIPO Standard ST.26 which has been submitted electronically in XML format and is hereby incorporated by reference in its entirety. The XML copy, created on November 6, 2024, is named P-628519-PC_SQL_ST26_06NOV24.xml and is 48,767 bytes in size.

FIELD OF INTEREST

[004] This disclosure relates to engineered bacteriophages comprising a bioactive agent and a targeting agent to a pathogen or pathogenic cell, for treatment of infections and cancer, among other uses.

BACKGROUND

[005] Pathogens, such as infectious bacteria, and pathogenic cells, such as cancer cells, comprise vast targets of health care attention and medical interest. Bacterial diseases remain major causes of morbidity and mortality, as is cancer. Both need better means for control and treatment, in particular for antibiotic-resistant bacteria and for cancers resistant to standard of care treatment.

[006] Bacteremia, the presence of bacteria in the bloodstream, can arise from various infection sites caused by both Gram positive and Gram negative bacterial organisms. These organisms can rapidly develop into life-threatening conditions such as sepsis and septic shock.

Empiric intravenous antibiotics is the standard of care to treat bacteremia. However, antibiotic resistance, especially in Gram negative bacteria such as *Escherichia coli* with extended-spectrum β -lactamase (beta-lactamase) or resistant to multiple classes of antibiotics often leave bacteremia with significantly higher mortality rate or extended stay in the intensive care unit. More than half of all *E. coli* bacteremia cases originate from urinary tract infection (UTI) leading to bacteremia. The in-hospital 7-day case fatality risk ranges from 8.9-11%. Due to the fact that Gram negative bacteria are intrinsically more resistant to antibiotics in general, they pose a much more serious threat in the intensive care unit (ICU) due to the increasing rate of infections caused by multidrug resistance Gram-negative bacteria. *E. coli* is the most common bloodstream pathogen for both hospital acquired (hospital onset) infections (HAI) or community acquired (community onset) infections (CAI). In community onset bacteremia, it is ranked at the top and accounted for 26.6% of cases; and similarly, it is ranked second and underlying 21.3% within hospital onset bacteremia. The ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) are antibiotic-resistant priority pathogens highlighted by the World Health Organization (WHO) for which new antimicrobial developments are urgently needed.

[007] Cancer is the second most common cause of death after cardiovascular diseases. Statistics from the US showed that the number of people diagnosed with cancer was 1.7 million in 2017 with 0.6 million deaths. Ninety percent of deaths from cancer result from the development of drug resistance, which leads to the ineffectiveness of chemotherapeutic agents. Drug resistance can be defined as the ability of cancer cells to reduce the potency and efficacy of chemotherapeutic agents. In some types of cancer such as renal cancer and hepatocellular carcinoma, malignant cells start resistance without previous exposure to chemotherapeutic agents (intrinsic resistance) resulting in a poor response to initial treatment. In other cases, cancer cells exhibit initial sensitivity to chemotherapy followed by a poor response due to the development of resistance (acquired resistance).

[008] Thus, there remains an unmet need for therapeutic compositions and methods of treatment of microbial infections, for example, but not limited to, treatment of Gram negative and Gram positive bacterial infections, and fungal infections. A major gap in treatment exists, wherein there is an inability to provide local factors where most needed in the treatment of

“superbug” (i.e., super-resistant) bacterial infections, particularly with respect to the need for broad-based therapies. Likewise, cancer and in particular drug resistant cancer remain a major and serious unmet need.

[0009] Similarly, there remains an unmet need for compositions and methods of treatment with reduced side-effects. A major gap in treatment exists, wherein there is an inability to provide broad therapies against super-resistant bacterial infections, while reducing harsh side-effects commonly associated with treatment of super-resistant bacterial infections, including Gram negative and Gram positive bacterial infections. Similarly, targeted cancer therapies with reduced side effects on non-cancer cells, represents a major gap in treatment.

SUMMARY

[0010] In one aspect, a filamentous phage is provided comprising:

a binding moiety to a target cell, said binding moiety expressed on a minor capsid protein of the filamentous phage; and

a bioactive agent bound to a plurality of major capsid proteins of the filamentous phage, said bioactive agent exerting an activity on the target cell.

[0011] In some embodiments, the target cell is an infectious organism or a pathological cell. In some embodiments, the infectious organism is a bacterium, fungus or parasite.

[0012] In some embodiments, the binding moiety binds to peptidoglycan. In some embodiments, the bacterium is a Gram positive bacterium, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Clostridium difficile*, *Clostridium botulinum*, *Clostridium tetani*, *Listeria monocytogenes*, *Bacillus anthracis* or *Corynebacterium diphtheriae*. In some embodiments, the binding moiety binds to lipopolysaccharide (LPS). In some embodiments, the bacterium is a Gram negative bacterium such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Salmonella* spp. *Shigella* spp. and *Vibrio cholerae*. In some embodiments, the binding moiety binds to a yeast cell surface structure, protein and/or cell wall component. In some

embodiments, the fungus is a yeast.

[0013] In some embodiments, the pathological cell is a cancer cell, an infected cell, an immune cell or an autoreactive immune cell. In some embodiments, the cancer cell is a solid tumor cancer cell.

[0014] In some embodiments, the binding moiety is expressed on minor capsid protein pIII, pVI, pVII or pIX of the filamentous phage. In some embodiments, the binding moiety expressed on a minor capsid protein of the filamentous phage replaces the N-terminal domain of the minor capsid protein. In some embodiments, the binding moiety to the target cell comprises an antibody, binding fragment thereof, or cell-targeted receptor binding domain. In some embodiments, the binding moiety comprises a Fab, scFab, scFv or dAb. In some embodiments, the antibody or binding fragment thereof comprises a scFab domain or binding fragment thereof.

[0015] In some embodiments, the infectious organism is a Gram negative bacterium and the scFab domain comprises WN1 222-5.

[0016] In some embodiments, the filamentous phage is a Ff phage. In some embodiments, the filamentous phage is a M13 phage. In some embodiments, the minor capsid protein is pIII, pVI, pVII or pIX. In some embodiments, the minor capsid protein is pIII.

[0017] In some embodiments, the binding moiety expressed on pIII comprises SEQ ID NO:2 (HLL-pIII) or SEQ ID NO:4 (LLH-pIII).

[0018] In some embodiments, the phage does not predominantly bind to bacterial pili. In some embodiments, the filamentous phage is unable to enter bacterial cells. In some embodiments, the phage is non-replicating.

[0019] In some embodiments, the major capsid protein is pVIII.

[0020] In some embodiments, the bioactive agent is a cytotoxic agent, anticancer agent, immune checkpoint inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme. In some embodiments, the bioactive agent is an antibiotic, antimicrobial, antifungal, antiparasitic, immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent. In some embodiments, the bioactive agent is bound covalently to the

major capsid protein. In some embodiments, the bioactive agent is covalently bound via reactive groups thereon to a reactive group on the major capsid protein by means of direct conjugation or a bifunctional cross-linking agent.

[0021] In some embodiments, the antibiotic is a Gram positive bacterium targeting antibiotic, such as daptomycin, penicillin, tetracycline, erythromycin, vancomycin, or an analogue thereof. In some embodiments, the antibiotic is a fungus targeting antibiotic, such as amphotericin B, an echinocandin or an analogue thereof. In some embodiments, the antibiotic is a Gram negative bacterium targeting antibiotic, such as targeting *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Salmonella* spp. *Shigella* spp. or *Vibrio cholerae*. In some embodiments, the antibiotic is streptomycin or an analogue thereof. In some embodiments, the Gram negative antibiotic is a lipopeptide. In some embodiments, the lipopeptide is a cyclic lipopeptide. In some embodiments, the cyclic lipopeptide is a polymyxin. In some embodiments, the polymyxin is polymyxin B or polymyxin E.

[0022] In some embodiments, the major capsid protein is pVIII. In some embodiments, the pVIII is bound to polymyxin B by amide linkages. In some embodiments, the ratio of antibiotic to major coat protein is less than or equal to 3:1.

[0023] In some embodiments, the minimal inhibitory concentration of the filamentous phage is at least 2X, 5X, 10X, 20X, 50X or 100X lower than the minimal inhibitory concentration of the antibiotic alone. In some embodiments, the nephrotoxicity thereof is reduced compared to that of the antibiotic alone. In some embodiments, an infectious organism that is resistant to the antibiotic alone is sensitive to the filamentous phage.

[0024] In some embodiments, the anticancer agent is a cytotoxic agent, immune checkpoint inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme. The filamentous phage of claim 35, wherein the antibiotic is an amphotericin B, an echinocandin or an analogue thereof. In some embodiments, the cytotoxic agent is an antibiotic, antimicrobial, antifungal, antiparasitic, immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent.

[0025] In one aspect, a method is provided for treating a target cell in a subject comprising

exposing a subject in need thereof to filamentous phage comprising:

a binding moiety to the target cell, said binding moiety expressed on a minor capsid protein of the filamentous phage; and

a bioactive agent that exerts an activity on the target cell, the bioactive agent bound to a plurality of major capsid proteins of the filamentous phage.

[0026] In some embodiments, the target cell is an infectious organism or a pathological cell. In some embodiments, the pathological cell is a cancer cell, an infected cell, an immune cell or an autoreactive immune cell.

[0027] In some embodiments, the binding moiety is expressed on minor capsid protein pIII, pVI, pVII or pIX of the filamentous phage. In some embodiments, the binding moiety expressed on a minor capsid protein of the filamentous phage replaces the N-terminal domain of the minor capsid protein. In some embodiments, the binding moiety comprises a Fab, scFab, scFv or dAb. In some embodiments, the binding domain comprises a scFab domain or binding fragment thereof.

[0028] In some embodiments, the filamentous phage is a Ff phage. In some embodiments, the filamentous phage is a M13 phage. In some embodiments, the minor capsid protein is pIII, pVI, pVII or pIX. In some embodiments, the minor capsid protein is pIII. In some embodiments, the filamentous phage does not predominantly bind to bacterial pili. In some embodiments, the filamentous phage is unable to enter bacterial cells. In some embodiments, the filamentous phage is non-replicating. In some embodiments, the major capsid protein is pVIII.

[0029] In some embodiments, the bioactive agent is a cytotoxic agent, anticancer agent, immune checkpoint inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme. In some embodiments, the cytotoxic agent is an antibiotic, antimicrobial, antifungal, antiparasitic, immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent. In some embodiments, the bioactive agent is bound covalently to the major capsid protein. In some embodiments, the bioactive agent is covalently bound via reactive groups thereon to a reactive group on the major capsid protein by means of direct conjugation or a bifunctional cross-linking agent.

[0030] In one aspect, a method is provided for treating a pathogen or pathogenic cell comprising exposing a subject in need thereof to filamentous phage comprising:

a binding moiety to the pathogen or pathogenic cell, said binding moiety expressed on a minor capsid protein of the filamentous phage; and

a bioactive agent that exerts an activity against the pathogen or pathogenic cell, the bioactive agent bound to a plurality of major capsid proteins of the filamentous phage.

[0031] In some embodiments, the pathogen or pathogenic cell is an infectious organism or a pathological cell. In some embodiments, the pathogen is an infectious organism is a bacterium, fungus or parasite. In some embodiments, the binding moiety binds to peptidoglycan. In some embodiments, the bacterium is a Gram positive bacterium, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Clostridium difficile*, *Clostridium botulinum*, *Clostridium tetani*, *Listeria monocytogenes*, *Bacillus anthracis* or *Corynebacterium diphtheriae*. In some embodiments, the binding moiety binds to lipopolysaccharide (LPS). In some embodiments, the bacterium is a Gram negative bacterium, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Salmonella* spp. *Shigella* spp. or *Vibrio cholerae*. In some embodiments, the binding moiety binds to a yeast cell surface structure, protein and/or cell wall component. In some embodiments, the fungus is a yeast. In some embodiments, the pathological cell is a cancer cell, an infected cell, an immune cell or an autoreactive immune cell. In some embodiments, the cancer cell is a solid tumor cancer cell.

[0032] In some embodiments, the binding moiety is expressed on minor capsid protein pIII, pVI, pVII or pIX of the filamentous phage. In some embodiments, the binding moiety expressed on a minor capsid protein of the filamentous phage replaces the N-terminal domain of the minor capsid protein. In some embodiments, the binding moiety to the pathogen or pathogenic cell comprises an antibody, binding fragment thereof, or cell-targeted receptor binding domain. In some embodiments, the binding moiety comprises a Fab, scFab, scFv or dAb. In some embodiments, the antibody or binding fragment thereof comprises a scFab

domain or binding fragment thereof. In some embodiments, the infectious organism is a Gram negative bacterium and the scFab domain comprises WN1 222-5.

[0033] In some embodiments, the filamentous phage is a Ff phage. In some embodiments, the filamentous phage is a M13 phage. In some embodiments, the minor capsid protein is pIII, pVI, pVII or pIX. In some embodiments, the minor capsid protein is pIII. In some embodiments, the binding moiety expressed on pIII comprises SEQ ID NO:2 (HLL-pIII) or SEQ ID NO:4 (LLH-pIII). In some embodiments, the phage does not predominantly bind to bacterial pili. In some embodiments, the filamentous phage is unable to enter bacterial cells. In some embodiments, the phage is non-replicating.

[0034] In some embodiments, the major capsid protein is pVIII. In some embodiments, the bioactive agent is a cytotoxic agent, anticancer agent, immune checkpoint inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme. In some embodiments, the cytotoxic agent is an antibiotic, antimicrobial, antifungal, antiparasitic, immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent. In some embodiments, the bioactive agent is bound covalently to the major capsid protein. In some embodiments, the bioactive agent is covalently bound via reactive groups thereon to a reactive group on the major capsid protein by means of direct conjugation or a bifunctional cross-linking agent. In some embodiments, the pathogen is a Gram negative bacterium, and the bioactive agent is an antibiotic.

[0035] In one aspect, a method is provided for treating an microbial infection comprising exposing a subject in need thereof to filamentous phage comprising:

a binding moiety to an infectious organism, said binding moiety expressed on a minor capsid protein of the filamentous phage; and

an antibiotic active against the infectious organism, the antibiotic bound to a plurality of major capsid proteins of the filamentous phage.

[0036] In some embodiments, the infectious organism is a bacterium, fungus or parasite. In some embodiments, the binding moiety binds to peptidoglycan. In some embodiments, the bacterium is a Gram positive bacterium such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus*

saprophyticus, *Enterococcus faecalis*, *Clostridium difficile*, *Clostridium botulinum*, *Clostridium tetani*, *Listeria monocytogenes*, *Bacillus anthracis* or *Corynebacterium diphtheriae*. In some embodiments, the binding moiety binds to lipopolysaccharide (LPS). In some embodiments, the bacterium is a Gram negative bacterium, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Salmonella* spp. *Shigella* spp. and *Vibrio cholerae*. In some embodiments, the binding moiety binds to a yeast cell surface structure, protein and/or cell wall component. In some embodiments, the fungus is a yeast. In some embodiments, the binding moiety is expressed on minor capsid protein pIII, pVI, pVII or pIX of the filamentous phage. In some embodiments, the binding moiety expressed on a minor capsid protein of the filamentous phage replaces the N-terminal domain of the minor capsid protein. In some embodiments, the binding moiety to the target cell comprises an antibody, binding fragment thereof, or cell-targeted receptor binding domain. In some embodiments, the binding moiety comprises a Fab, scFab, scFv or dAb. In some embodiments, the antibody or binding fragment thereof comprises a scFab domain or binding fragment thereof. In some embodiments, the infectious organism is a Gram negative bacterium and the scFab domain comprises WN1 222-5.

[0037] In some embodiments, the filamentous phage is a Ff phage. In some embodiments, the filamentous phage is a M13 phage. In some embodiments, the minor capsid protein is pIII, pVI, pVII or pIX. In some embodiments, the minor capsid protein is pIII. In some embodiments, the binding moiety expressed on pIII comprises SEQ ID NO:2 (HLL-pIII) or SEQ ID NO:4 (LLH-pIII). In some embodiments, the phage does not predominantly bind to bacterial pili. In some embodiments, the filamentous phage is unable to enter bacterial cells. In some embodiments, the phage is non-replicating.

[0038] In some embodiments, the major capsid protein is pVIII. In some embodiments, the antibiotic is bound covalently to the major capsid protein. In some embodiments, the antibiotic is covalently bound via reactive groups thereon to a reactive group on the major capsid protein by means of direct conjugation or a bifunctional cross-linking agent. In some embodiments, the antibiotic is a Gram positive bacterium targeting antibiotic, such as daptomycin, penicillin, tetracycline, erythromycin, vancomycin, or an analogue thereof. In some embodiments, the antibiotic is a fungus targeting antibiotic such as amphotericin B, an echinocardin or an

analogue thereof. In some embodiments, the antibiotic is a Gram negative bacterium targeting antibiotic, such as streptomycin or an analogue thereof. In some embodiments, the Gram negative antibiotic is a lipopeptide. In some embodiments, the lipopeptide is a cyclic lipopeptide. In some embodiments, the cyclic lipopeptide is a polymyxin. In some embodiments, the polymyxin is polymyxin B or polymyxin E. In some embodiments, the pVIII is bound to polymyxin B or polymyxin E by amide linkages. In some embodiments, the ratio of antibiotic to major coat protein is less than or equal to 3:1. In some embodiments, the minimal inhibitory concentration of the filamentous phage is at least 2X, 5X, 10X, 20X, 50X or 100X lower than the minimal inhibitory concentration of the antibiotic alone. In some embodiments, the nephrotoxicity of the filamentous phage is reduced compared to that of the antibiotic alone. In some embodiments, the infectious organism that is resistant to the antibiotic alone is sensitive to the filamentous phage.

[0039] In one aspect, a method is provided for treating cancer comprising exposing a subject in need thereof to filamentous phage comprising:

a binding moiety to the cancer, said binding moiety expressed on a minor capsid protein of the filamentous phage; and

a bioactive agent that exerts activity against the cancer, the bioactive agent bound to a plurality of major capsid proteins of the filamentous phage.

[0040] In some embodiments, the cancer cell is a solid tumor cancer cell. In some embodiments, the binding moiety is expressed on minor capsid protein pIII, pVI, pVII or pIX of the filamentous phage. In some embodiments, the binding moiety expressed on a minor capsid protein of the filamentous phage replaces the N-terminal domain of the minor capsid protein. In some embodiments, the binding moiety to the cancer comprises an antibody, binding fragment thereof, or cell-targeted receptor binding domain. In some embodiments, the binding moiety comprises a Fab, scFab, scFv or dAb. In some embodiments, the antibody or binding fragment thereof comprises a scFab domain or binding fragment thereof.

[0041] In some embodiments, the filamentous phage is a Ff phage. In some embodiments, the filamentous phage is a M13 phage. In some embodiments, the minor capsid protein is pIII, pVI, pVII or pIX. In some embodiments, the minor capsid protein is pIII. In some

embodiments, the filamentous phage does not predominantly bind to bacterial pili. In some embodiments, the filamentous phage is unable to enter bacterial cells. In some embodiments, the phage is non-replicating.

[0042] In some embodiments, the major capsid protein is pVIII. In some embodiments, the bioactive agent against the cancer is a cytotoxic agent, anticancer agent, immune checkpoint inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme. In some embodiments, the bioactive agent against the cancer is an immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent. In some embodiments, the bioactive agent is bound covalently to the major capsid protein. In some embodiments, the bioactive agent is covalently bound via reactive groups thereon to a reactive group on the major capsid protein by means of direct conjugation or a bifunctional cross-linking agent. In some embodiments, the ratio of bioactive agent to major coat protein is less than or equal to 3:1. In some embodiments, the minimal effective dose of the filamentous phage is at least 2X, 5X, 10X, 20X, 50X or 100X lower than the minimal effective dose of the bioactive agent alone.

[0043] In any of the foregoing methods, the filamentous phage is administered topically, intranasally, parenterally or by inhalation. In some embodiments, parenterally is paracancerally, intramuscularly, intravenously, intradermally, subcutaneously, intraperitoneally, intraventricularly, intracranially or intratumorally.

[0044] In some embodiments, the subject is in septic shock. In some embodiments, the subject has a community acquired infection. In some embodiments, the subject has community-onset bacteremia. In some embodiments, the subject has a urogenital infection. In some embodiments, the subject has a systemic fungal infection. In some embodiments, the subject has cancer.

[0045] In one aspect, a method is provided for inhibiting microorganism growth on a surface comprising treating the surface with a filamentous phage as described herein. In some embodiments, the surface is a biological surface, implantable medical device, catheter, surgical or medical instrument, implantable prosthesis, artificial joint, or dental post.

[0046] In one aspect, a method is provided for making a filamentous phage comprising a

binding moiety to a target cell expressed on a minor capsid protein thereof, and comprising a bioactive agent bound to a plurality of major capsid proteins thereof, wherein said bioactive agent exerting an activity on the target cell, the method comprising the steps of:

producing filamentous phages that express the minor capsid protein
comprising the binding moiety to the target cell; and

binding the bioactive agent to the major capsid protein thereof.

[0047] In some embodiments, the target cell is an infectious organism or a pathological cell. In some embodiments, the infectious organism is a bacterium, fungus or parasite. In some embodiments, the binding moiety binds to peptidoglycan. In some embodiments, the bacterium is a Gram positive bacterium such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Clostridium difficile*, *Clostridium botulinum*, *Clostridium tetani*, *Listeria monocytogenes*, *Bacillus anthracis* or *Corynebacterium diphtheriae*. In some embodiments, the binding moiety binds to lipopolysaccharide (LPS). In some embodiments, the bacterium is a Gram negative bacterium such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Salmonella* spp. *Shigella* spp. and *Vibrio cholerae*. In some embodiments, the binding moiety binds to a yeast cell surface structure, protein and/or cell wall component. In some embodiments, the fungus is a yeast. In some embodiments, the pathological cell is a cancer cell, an infected cell, an immune cell or an autoreactive immune cell. In some embodiments, the cancer cell is a solid tumor cancer cell. In some embodiments, the binding moiety is expressed on minor capsid protein pIII, pVI, pVII or pIX of the filamentous phage. In some embodiments, the binding moiety expressed on a minor capsid protein of the filamentous phage replaces the N-terminal domain of the minor capsid protein. In some embodiments, the binding moiety to the target cell comprises an antibody, binding fragment thereof, or cell-targeted receptor binding domain. In some embodiments, the binding moiety comprises a Fab, scFab, scFv or dAb. In some embodiments, the antibody or binding fragment thereof comprises a scFab domain or binding fragment thereof. In some embodiments, the infectious organism is a Gram negative bacterium and the scFab domain comprises WN1 222-5.

[0048] In some embodiments, the filamentous phage is a Ff phage. In some embodiments, the filamentous phage is a M13 phage. In some embodiments, the minor capsid protein is pIII, pVI, pVII or pIX. In some embodiments, the minor capsid protein is pIII. In some embodiments, the binding moiety expressed on pIII comprises SEQ ID NO:2 (HLL-pIII) or SEQ ID NO:4 (LLH-pIII).

[0049] In some embodiments, the major capsid protein is pVIII. In some embodiments, the bioactive agent is a cytotoxic agent, anticancer agent, immune checkpoint inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme. In some embodiments, the bioactive agent is an antibiotic, antimicrobial, antifungal, antiparasitic, immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent. In some embodiments, the bioactive agent is bound covalently to the major capsid protein. In some embodiments, the bioactive agent is covalently bound via reactive groups thereon to a reactive group on the major capsid protein by means of direct conjugation or a bifunctional cross-linking agent. In some embodiments, the antibiotic is a Gram positive bacterium targeting antibiotic. In some embodiments, the antibiotic is daptomycin, penicillin, tetracycline, erythromycin, vancomycin, or an analogue thereof. In some embodiments, the antibiotic is a fungus targeting antibiotic. In some embodiments, the fungus targeting antibiotic is amphotericin B, an echinocardin or an analogue thereof. In some embodiments, the antibiotic is a Gram negative bacterium targeting antibiotic. In some embodiments, the Gram negative bacterium is *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Salmonella* spp. *Shigella* spp. or *Vibrio cholerae*. In some embodiments, the antibiotic is streptomycin or an analogue thereof. In some embodiments, the Gram negative antibiotic is a lipopeptide. In some embodiments, the lipopeptide is a cyclic lipopeptide. In some embodiments, the cyclic lipopeptide is a polymyxin. In some embodiments, the polymyxin is polymyxin B or polymyxin E. In some embodiments, the major capsid protein is pVIII. In some embodiments, the pVIII is bound to polymyxin B by amide linkages. In some embodiments, the ratio of antibiotic to major coat protein is less than or equal to 3:1. In some embodiments, the minimal inhibitory concentration of the filamentous phage is at least 2X, 5X, 10X, 20X, 50X or 100X lower than the minimal inhibitory concentration of the antibiotic alone.

[0050] In some embodiments, the anticancer agent is a cytotoxic agent, immune checkpoint

inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme. In some embodiments, the cytotoxic agent is an antibiotic, antimicrobial, antifungal, antiparasitic, immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent. In some embodiments, the binding moiety binds to a cancer cell.

[0051] In one aspect, a pIII-anti-LPS fusion protein comprising SEQ ID NO:2 or SEQ ID NO:4 is provided. In one aspect, a pIII-anti-LPS fusion protein consisting of SEQ ID NO:2 or SEQ ID NO:4 is provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0052] The subject matter disclosed herein is particularly pointed out and distinctly claimed in the concluding portion of the specification. The disclosure, however, both as to organization and method of operation, together with objects, features, and advantages thereof, may best be understood by reference to the following detailed description when read with the accompanying drawings in which:

[0053] **FIGURES 1A-1B-1C** show a schematic representation of the attachment of polymyxin-recombinant M13 bacteriophage to a bacterium, the polymyxin core structure and attachment sites on the bacteriophage. **FIGURE 1A** is a schematic representation of a bacterium to which several PMB-Phage are attached. **FIGURE 1B** shows the chemical structure of two polymyxin antibiotics – polymyxin B and polymyxin E (colistin). **FIGURE 1C** is a schematic representation of a bacteriophage, showing the g3p (also called pIII; the F' attachment protein) at one end of the bacteriophage and attached to the deoxyribonucleic acid (DNA) core of the bacteriophage. The g8p (also called pVIII) in the envelope are stacked at a 20-degree (20°) angle with the N-termini exterior to the DNA core and the C-termini interior to the DNA core. The other end of the bacteriophage comprises g9p attached to the DNA core and g7p attached to the g6p in the envelope. The polymyxin may be bound to free NH₂ or COOH moieties on the g8p proteins on the surface of the bacteriophage coat.

[0054] **FIGURE 2** is a schematic illustration of wild-type M13 and two recombinant derivatives with modified receptor-binding protein constructs. Rectangles indicate separate protein domains as labeled. In the recombinants, the C-terminal domain and Linker 2 on the phagemid vector were retained, while the N2, Linker 1, and N1 domains were replaced by

single-chain fragment antigen binding regions of antibody WN1 222-5. The two constructs of LLH and HLL differ from each other by the order of the heavy chain and light chain.

[0055] **FIGURES 3A-3B-3C-3D-3E-3F** shows binding of anti-LPS phage to Gram-negative pathogen using transmission electron microscopy (TEM; negative stain). Recombinant M13 phages have filamentous morphology (3A) that is similar to bacteria pili and other structures. Therefore, anti-LPS phages were labeled using a mouse monoclonal anti-pVIII primary antibody, followed by gold nanoparticles (dark spheres) coated with donkey anti-mouse secondary antibody, allowing phages to be easily identified (red darts). Gold-labeled anti-LPS phage is shown (3B) without bacteria; or incubated with (3C) *E. coli* DH5 α ; (3D) *P. aeruginosa* strain ATCC 25102; (3E) *K. pneumoniae* strain ATCC 700603; and (3F) *A. baumannii* ATCC 19606. Note that phage width is increased by the labeling reagents. Scale bars = 500 nm.

[0056] **FIGURES 4A-4B-4C** depict binding curve showing the amount of anti-LPS phage bound to cells determined by qPCR, given an increasing incubation amount of phages with a constant amount of bacterial cells ($\sim 10^8$ colony forming units (cfu)). At 10^7 virions/mL and above, the amount of phage bound to either Gram-negative organism significantly exceeds that bound to *S. aureus* by > 1 order of magnitude. Error bars are standard deviation calculated from triplicates. FIG. 4A. Anti-LPS phage binding curve for *E. coli* DH5 α , *E. coli* ATCC BAA 1161 and negative control strain *S. aureus* ATCC 25904. Fig. 4B shows Anti-LPS phage binding curve for *P. aeruginosa* strain ATCC 25102, clinical strain A, G and J, as well as negative control strain *S. aureus* ATCC 25904. FIG. 4C shows anti-LPS phage binding curve for *K. pneumoniae* strain ATCC 700604 and clinical strain A, *A. baumannii* strain ATCC 19606, *B. cepacia* strain ATCC 25904 and negative control strain *S. aureus* ATCC 25904.

[0057] **FIGURES 5A-5B** show the amount of anti-LPS phage bound to various bacterial strains ($\sim 10^8$ colony forming units (cfu) mixed with 1×10^{11} anti-LPS phage virions) determined by qPCR, and a live cell count of viable *E. coli*. FIG. 5A. The bacterial strains are *E. coli* (E.C., navy blue) 1: DH5 α , 2: ATCC BAA 1161; *P. aeruginosa* (P.A., red) 1: ATCC 25102, 2: clinical strain A, 3: clinical strain B, 4: clinical strain C, 5: clinical strain E, 6: clinical strain F, 7: clinical strain G, 8: clinical strain J, 9: PAKpmrB6; *K. pneumoniae* (K.P., brown) 1: ATCC 700603, 2: clinical strain A; *A. baumannii* ATCC 19606 (A.B.1, sky blue); *B. cepacia* ATCC 25416 (B.C.1, pink); *S. aureus* ATCC 25904 (S.A.1). All Gram-negative strains show

substantial binding compared to *S. aureus*. FIG. 5B. Decrease of viable *E. coli* (ATCC BAA 1161) cells over time when incubating with PMB (blue) or PMB-Phage (yellow) at their respective MIC concentration. Similarly rapid cell death is observed in both cases. The y-axis value is normalized by 50,000, which is the number of cells estimated in each well at $t=0$ h, based on OD600 ($n=3$).

[0058] **FIGURES 6A-6B-6C-6D** are transmission electron microscopy (TEM; negative stain) image of anti-LPS phages labeled using a mouse monoclonal anti-pVIII primary antibody, followed by gold nanoparticles (dark spheres) coated with donkey anti-mouse secondary antibody, allowing phages to be easily identified (red darts). Gold-labeled anti-LPS phage is incubated with (6A) *E. coli* BAA 1161; (6B) *P. aeruginosa* strain PAKpmrB6; (6C) *K. pneumoniae* strain clinical strain A; and (6D) *B. cepacia* ATCC 25416. Note that phage width is increased by the labeling reagents. Scale bars = 500 nm.

[0059] **FIGURE 7** is a TEM image of PMB-Phage. The sample was incubated with anti-PMB mouse monoclonal antibodies and subsequently using gold nanoparticles (black dots) coated with Donkey-anti-Mouse secondary antibodies, demonstrating successful conjugation of PMB to the phage. Compared with anti-LPS phage lacking PMB, PMB-Phage virions show a tendency toward self-association, likely due to reduced surface charge. Scale bar = 500 nm.

[0060] **FIGURES 8A-8B** depict cytotoxicity studies of PMB-Phage. (8A) Hemolysis assay of PMB-Phage on sheep red blood cells. Less than 2% of hemolysis is considered non-hemolytic, as observed for PMB-Phage at these concentrations. (8B) HEK 293 cell viability, indicated by cell metabolic activity measured using MTT assay, when incubated in growth media containing PMB-Phage. >80% cell viability is considered acceptable.

[0061] **FIGURES 9A-9B-9C-9D** depict biodistribution of ^{89}Zr -labeled PMB-Phage (100 μCi) representative $\mu\text{PET/CT}$ images across the 72-hour time period are shown in (9A), for one animal in the group with (top row) or without (bottom row) bacterial injection. The distribution into different organs and tissues over time were measured for (9B) the whole animal, (9C) blood, and (9D) left kidney over a 72-hour time period. Mice were injected with PMB-Phage alone (blue) or were pre-injected with 1×10^7 cfu *E. coli* ATCC BAA 1161 (red). Error bars show standard deviation ($N=3$). Data were quantified from μPET images. Signals are measured as percentages of the injected dose (ID) per volume (cc).

[0062] **FIGURE 10** depicts the efficacy of PMB-Phage in the immunocompetent lung infection model with multidrug-resistant *P. aeruginosa*. Test animals were intranasally (IN) inoculated with 2.8×10^7 CFU of *P. aeruginosa* strain AR Bank #0266. 1x PBS (orange circles), anti-LPS phage (blue triangles), wild-type M13 phage (WT-M13; green triangles), PMB sulfate (yellow diamonds), or PMB-phage (pink hexagons), was administered IN at 1 h post infection. Animals were sacrificed at 1 or 25 h post infection and the lung tissues harvested. The bacterial counts (CFU/lung) of lung tissue homogenates were measured. Baseline, 1-log kill, and 2-log kill levels were defined with respect to the CFU counts at 1 h post infection, without treatment. Statistical significance of comparisons to the 1x PBS control group was determined by one-way ANOVA and Dunnett's test. (*) means $p < 0.05$; (***) means $p < 0.001$; (****) means $p < 0.0001$. The percentage number (d) indicates the percentage of animals found dead before 25 h in each group. QD: once per day (single dose). N = 5 including animals that died.

[0063] **FIGURES 11A-11B** show the efficacy of PMB-Phage in a mouse keratitis model with *P. aeruginosa* infection. Animals were subject to corneal scratch and inoculated with 5×10^4 CFU of *P. aeruginosa* strain PAO1-GFP. Anti-LPS phage, WT-M13 phage, PMB sulfate, or PMB-phage was administered topically as treatment. Animals were sacrificed at 48 h post-infection, and the corneal tissues were harvested for CFU counts. The CFU/mL of homogenized tissue were for animals in each group were plotted in panel (A) and representative brightfield and fluorescent images of the infect eyes of animal in each group were shown in panel (B). Statistical significance of comparisons to the 1x PBS control were determined by one-way ANOVA and Tukey's test. (**) means $p < 0.01$; (****) means $p < 0.0001$. v = virions.

[0064] **FIGURE 12** is a schematic illustration of wild-type and recombinant M13 receptor-binding protein domains. The wild-type M13 gp3 is composed of the N1, N2, and C terminal domains. A glycine-rich linker is responsible for connecting adjacent domains. In recombinant M13 receptor binding proteins, the N1 and N2 domains as well as the linker connecting them were replaced with the receptor binding domain engineered from a phage metagenomic library.

[0065] **FIGURES 13A-13B-13C-13D-13E-13F** show recombinant PAbinder phage, selected from the Pa-RBD library, bound to different *P. aeruginosa* strains as visualized by

transmission electron microscopy (TEM; negative stain). Filamentous phages have similar morphology to bacterial structures such as pili. To distinguish them, PAbinder phages were specifically labeled with gold nanoparticles (dark spheres) coated with antibodies against pVIII, allowing phages to be easily differentiated (yellow darts) from other structures. Gold-labeled PAbinder phages are shown (FIGURE 13A) without bacteria; or incubated with *P. aeruginosa* strain (FIGURE 13B) ATCC 25102; (FIGURE 13C) clinical strain A; (FIGURE 13D) clinical strain B; (FIGURE 13E) and clinical strain C; (FIGURE 13F) polymyxin-resistant mutant PAK *pmrB6*. Note that phage width is increased by the labeling reagents. Scale bars = 500 nm.

[0066] It will be appreciated that for simplicity and clarity of illustration, elements shown in the figures have not necessarily been drawn to scale. For example, the dimensions of some of the elements may be exaggerated relative to other elements for clarity.

DETAILED DESCRIPTION

[0067] In the following detailed description, numerous specific details are set forth in order to provide a thorough understanding of bioactive agent-bacteriophage conjugations, bacteriophage expressing antibody fragments or other moieties that target a type of cell, methods of making them, and methods of use thereof. However, it will be understood by those skilled in the art that the production of these bioactive agent-bacteriophage conjugations and bacteriophage expressing antibody fragments or other binding moieties, and uses thereof together or separately, may be practiced without these specific details. In other instances, well-known methods, procedures, and components have not been described in detail so as not to obscure their description.

[0068] The ensuing description of bioactive agent-bacteriophage conjugations include examples such as an antibiotic conjugated to a pathogenic bacterium-targeted bacteriophage, and an anticancer agent conjugated to a tumor cell-targeted bacteriophage. Such bacteriophages are referred to variously as antibiotic-recombinant phage conjugate, antibiotic-phage conjugate, anticancer agent-recombinant phage conjugate, anticancer agent-phage conjugate, antibiotic-recombinant phage, anticancer agent-phage, and more generally as phage-drug conjugate, drug-phage conjugate, bioactive agent-recombinant phage conjugate, bioactive agent-phage conjugate, and other variants that will be readily be understood to refer

to the recombinant phages and their bound bioactive agents and are not intended to be limiting. Moreover, these examples are merely exemplary of various bioactive agents that can be targeted to a desired cell type using an engineered bacteriophage as described herein, and are not intended to be limiting. As noted below, pathogenic organisms such as bacteria represent one example of a cell target by bacteriophages disclosed herein; other pathogenic organisms other than bacteria are similarly addressable by the teachings herein. Also as noted below, cancer cells represent one example of a target cell addressable by the teachings herein; other cell types associated with or able to participate in the attack on cancer cells, such as immune cells, as targets for the bacteriophages disclosed herein are also embraced by the teachings herein.

[0069] In other embodiments, described herein are bacteriophages to which a bioactive agent is bound to a major capsid protein, such as any bioactive agent disclosed herein. Such bacteriophages may not also comprise a recombinantly expressed targeting moiety on a minor capsid protein, but other comprise the “native” bacteriophage. Also embodied herein are bacteriophages expressing on a minor capsid protein a targeting moiety to a target cell such as a pathogen or pathogenic cell, but without a bioactive agent bound to a major capsid protein thereof. As noted herein, in some embodiments, the targeting moiety is expressed in place of a portion of a minor capsid protein, such as at the N-terminus of pIII (e.g., the native binding domain), and is not merely extended from the native pIII protein or a substantial portion of the native pIII protein.

[0070] Lipopolysaccharide (LPS) is a common structure on Gram negative bacteria and a critical element forming their bacterial outer membranes. Provided herein, in one embodiment, are recombinant M13 bacteriophages engineered to target and bind to LPS present on the outer membranes of a broad spectrum of Gram negative bacterial strains and species, including, but not limited to, all Gram negative members of strains of the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), six bacterial pathogens highlighted by the World Health Organization (WHO) for which new antimicrobial development is urgently needed. Also provided herein, in one embodiment, are an antibiotic-recombinant bacteriophage conjugate and methods of use thereof, e.g., using the recombinant M13 phages as drug delivery vehicles to specifically deliver the antibiotic polymyxin B as an

antibiotic-recombinant bacteriophage conjugate comprising polymyxin B and recombinant M13 bacteriophage.

[0071] Gram negative bacteria, including *E. coli*, differ from Gram positive bacteria such as *Staphylococcus aureus* by having an extra outer membrane that contributes greatly to antibiotic resistance. Lipopolysaccharide (LPS) is a common structure on the Gram negative bacteria outer membrane and a critical element forming their bacterial outer membranes. It contains three domains in the order from inner to outer domains: the lipid A, the core oligosaccharides and the O antigen (a region composed of extremely diverse surface polysaccharide). While the overall structure of LPS is conserved, variation on each of the three domains exist between bacteria species or strains. The O antigen has the greatest diversity, even across bacterial strains. The lipid A and core oligosaccharide are relatively more conserved within species. WN1 222-5 is an example of a neutralizing antibody that displays specificity for an epitope within the structurally conserved region of LPS core from a large number of pathogenic *E. coli*, *Salmonella*, *Shigella*, and *Citrobacter serovars*. Its sequence has also been published (see, e.g., US 00/5858728A).

[0072] Other medically important bacteria include *Clostridium difficile*, *Clostridium botulinum*, *Clostridium tetani*, *Listeria monocytogenes*, *Bacillus anthracis* and *Corynebacterium diphtheriae*.

[0073] A bioactive agent as used herein refers to the agent bound to a bacteriophage having a binding moiety to a target cell, as described herein, where the bioactive agent exerts one or more activities on the target cell to which the phage becomes bound. Such activities include but are not limited to killing pathogen cells (e.g., a bacterium or parasite), killing pathogenic cells (e.g., tumor cells), killing bacterial cells, killing parasite cells, killing cancer cells, killing tumor cells, suppressing growth of tumor cells, suppressing growth of cancer cells, suppressing metastasis of cancer cells, activating immune cells, activating T cells to increase anticancer activity against a tumor, suppressing regulatory T cells, etc. In some embodiments, pathogenic cell may be an autoreactive T cell or any other cell that exerts a pathology in the subject. In some embodiments, the target cell may be an immune cell involved in the development of an immune response to recognize and/or attack a pathogenic cell such as a cancer cell. In some embodiments, the target cell may be an immune cell involved in the development of an autoimmune response to recognize and/or attack a non-pathogenic cell

such as a normal cell of the body, e.g., neuronal, muscular, renal, skeletal, pancreatic, etc., or components of normal cells such as histones.

[0074] Such activities may include, but are not limited to, killing or inducing the killing of a pathogenic organism such as a bacterium, killing or inducing the killing of a cancer cell, such as a cancer cell of a solid tumor. In some embodiments, the bioactive agent exerts its activity on the target cell while bound to the phage. In some embodiments, the bioactive agent is an antibiotic. In some embodiments, the bioactive agent is an anticancer agent. In some embodiments, the bioactive agent is an immunostimulatory agent. Non-limiting examples of bioactive agents are described in ensuing sections.

[0075] Polymyxins are a family of cyclic lipopeptides that display exceptional antimicrobial activities toward Gram-negative bacteria such as *E. coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and others. Polymyxin B and polymyxin E (colistin) are the only members of this family that are currently being used clinically. After polymyxin B was introduced clinically in the late 1950s, due to its high (up to 60%) rate of associated nephrotoxicity and development of safer class of antibiotics such as quinolones, it was sidelined in the 1970s. Today, with the growing emergence of “superbug” and a dry antibiotic discovery pipeline, polymyxin B are increasingly used today as a last-line therapy to treat infections such as systemic multidrug resistant *E. coli* infections, including carbapenem-resistant *E. coli*. The use of polymyxin B is still extremely conserved and the nephrotoxicity associated is the major dose-limiting factor for polymyxin B administration. The tradeoff between its efficacy and toxicity is preventing its use in earlier lines of treatment to improve clinical outcomes. Moreover, polymyxin B has numerous side effects. Common side effects of polymyxin B, when given by injection, include kidney problems, neurological problems, fever, itchiness, and rash. Injections into muscle may result in significant pain. Other serious side effects may include fungal infections, anaphylaxis, and muscle weakness.

[0076] Anticancer drugs comprise a large and diverse array of small molecules and macromolecules including proteins and nucleic acids. By way of non-limiting examples, some of which are described in detail herein, include cytotoxic agents, immune checkpoint inhibitors, ligand or receptor inhibitors, radioligand conjugates with bound isotopes, immunostimulatory agents such as a cytokine, enzymes, cell-death inducer, etc. As noted herein, the anticancer agent may be conjugated to a bacteriophage that targets a cancer cell,

or may be targeted to immune or other cells that stimulate the immune system to attack the cancer. Thus, in some embodiments, the bioactive agent exerts its effect directly on the cancer cell to which the bacteriophage is bound. In some embodiments the bioactive agent exerts its effect on a cell that is not the cancer cell, such as an immune cell, but such cell then exerts its anticancer effect on the cancer cell.

[0077] Bacteriophages (or phages) are viruses that are specific to bacteria and harmless to humans. Due to their biocompatibility and therapeutic potential, phages are increasingly studied and engineered for various applications such as drug delivery, biosensing, cancer therapy and tissue regeneration. Phage capsids are generally composed of repetitions of a small number of capsid proteins. While they encompass a large diversity of morphologies, two major morphological classes are the tailed phages, which include an icosahedral head, and the filamentous, or rod-like, phages. One of the most well-characterized phages is M13, a filamentous phage that is about 1 μm (micrometer) in length and 6 nm in diameter. M13 has a single-stranded DNA genome packaged inside its capsid, which is composed of approximately 2700 copies of the major capsid protein pVIII (also called g8p), forming the bulk of the filamentous structure, and 3 to 5 copies of four minor capsid proteins, including the receptor-binding protein pIII (also called g3p). M13 phage is a prominent tool in molecular biology as a system for phage display technology, in which protein variants can be rapidly selected, typically for binding activity. Major applications include display of a heterologous mutant library of antibody fragments or display of a random library of peptides on the major capsid protein (pVIII) or receptor binding protein (pIII). The expression and selection of these proteins allows discovery of recombinant M13 phages engineered to bind to different targets. As will be seen in the examples herein as non-limiting ways to prepare a targeted pIII, an anti-LPS antibody Fab domain (as a single-chain Fab; scFab) replaced the N-terminal domain of pIII; in another, a specific bacterium-targeted bacterial receptor binding domain was selected for from a library of recombinant bacteriophages.

[0078] Provided herein, in one embodiment, is a recombinant M13 phage that is able to target LPS and to bind specifically to Gram negative bacteria species including *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumonia*, *Burkholderia cepacia*, and more. Such phage is also conjugated with an antibiotic. Because these recombinant anti-LPS M13 phages are only able to bind but not able to infect and replicate,

methods are provided for delivering an antibiotic such as polymyxin B, having a mechanism of action outside of the cells and thus circumventing dose limitations.

[0079] In one aspect, a high concentration of polymyxin B molecules are conjugated on recombinant phage major capsid proteins and are delivered to bacteria cell surfaces through phage receptor binding protein binding to cell surface lipopolysaccharide (LPS) (see, e.g., **FIGURE 1A**). The polymyxin B – recombinant M13 phage conjugate (referred to herein as Poly B-Phage, PMB-Phage, PMB-Phage conjugate, and other syntactic variants) can effectively lower the minimal inhibitory concentration (MIC) of polymyxin B by, in one embodiment, an order of magnitude in vitro compared to polymyxin B in its free drug formulation (see examples herein). Through achieving a higher local concentration, bacteria that were previously resistant to polymyxin B have become sensitive to the PMB-Phage. Since an order of magnitude less polymyxin B is needed to achieve bactericidal activity on the same level, the new formulation (PMB-Phage) would have lower nephrotoxicity to achieve the same therapeutic efficacy.

[0080] Such principles are applicable to target bacteriophages to other types of infectious microorganisms such as Gram positive bacteria and fungi, and to use the respective infectious microorganism sensitive antibiotic on the bacteriophages to increase MIC and decrease toxicity. Moreover, antibiotics that are too toxic for general use (and many have been withdrawn from the market for such reason) are useful for the purposes disclosed herein, as the lower amount of antibiotic and/or its conjugation to the bacteriophage increases potency and reduces toxicity.

[0081] Furthermore, similar principles are used as disclosed herein to design phage that can both target cancer cells and deliver a cytotoxic agent. Such methods enable delivery of a high concentration of anticancer agent to cancer cells, increasing efficacy and lowering toxicity. Such phage may be used as first line cancer therapy in particular for solid tumors, or in one embodiment for tumors that have failed first line therapy and developed resistance to standard of care therapy.

[0082] Thus, provided herein is a recombinant M13 phage that is able to target Gram positive bacteria and Gram negative bacteria. Because these recombinant phages are only able to bind but not able to infect and replicate, also provided herein are methods of delivering an antibiotic

having a mechanism of action outside of the cells and circumventing dose limitations.

[0083] Thus, provided herein is a recombinant M13 phage that is able to target pathogenic among other fungi and to bind specifically to such fungi. Because these recombinant M13 phages are only able to bind but not able to infect and replicate, also provided herein are methods of delivering an antibiotic having a mechanism of action outside of the cells and circumventing dose limitations.

[0084] Thus, provided herein is a recombinant M13 phage that is able to target pathologic cells such as cancer cells. Because these recombinant M13 phages are only to selectively bind target cancer cells but less or unable to bind to normal cells, also provided herein are methods of selectively delivering an anticancer agent and thus reducing or circumventing dose limitations.

[0085] Furthermore, the principles disclosed herein are applicable to filamentous bacteriophages in general, and the skilled artisan will be guided to alternate designs of the compositions disclosed herein for the purposes herein. The disclosure is thus not limiting to any particular type of bacteriophage, pathogenic organism, antibiotic, pathological cell or anticancer agent. Furthermore, in addition to the recombinant modification of the minor capsid protein as disclosed herein to comprise a targeting moiety, the major capsid protein may also be modified (e.g. mutated) recombinantly, for example, to provide one or more amino acids for conjugation to a bioactive moiety. While in some embodiments the native major capsid protein may comprise such conjugation sites (e.g., one or more amino groups, carboxylic acid groups, phenol groups and/or sulfhydryl groups), introducing a conjugation site may provide a more facile way for achieving the desired conjugation to the bioactive agent. Any such modified major capsid protein for this purpose is embraced herein. As noted elsewhere herein, certain conjugation sites may be blocked in order to obtain the desired conjugation(s).

[0086] In some embodiments, mutations may be introduced into the major capsid protein to reduce aggregation of the phages after conjugation of the bioactive agent.

Bacteria

[0087] Provided herein are an antibiotic-recombinant bacteriophage conjugate and methods of use thereof. In some embodiments, provided herein are an antibiotic-recombinant

bacteriophage conjugate and methods of use thereof for inhibiting or treating bacterial infection or disease.

[0088] “Bacteria” are ubiquitous, mostly free-living organisms, typically consisting of one biological cell. They constitute a large domain of prokaryotic microorganisms and are members of the Monista kingdom, along with the archaea. While some bacteria are harmless to most other organisms (including vertebrates, e.g., mammals and birds), other bacteria cause infection or disease.

[0089] The bacterial cell is surrounded by a cell membrane, which is made primarily of phospholipids. This membrane encloses the contents of the cell and acts as a barrier to hold nutrients, proteins and other essential components of the cytoplasm within the cell. Unlike eukaryotic cells, bacteria usually lack large membrane-bound structures in their cytoplasm such as a nucleus, mitochondria, chloroplasts and the other organelles present in eukaryotic cells. However, some bacteria have protein-bound organelles in the cytoplasm which compartmentalize aspects of bacterial metabolism, such as the carboxysome. Additionally, bacteria have a multi-component cytoskeleton to control the localization of proteins and nucleic acids within the cell, and to manage the process of cell division. Their genetic material is typically a single circular bacterial chromosome of DNA located in the cytoplasm in an irregularly shaped body called the nucleoid. The nucleoid contains the chromosome with its associated proteins and RNA. Like other organisms, bacteria contain ribosomes for the production of proteins, but the structure of the bacterial ribosome is different from that of eukaryotes and archaea.

[0090] The bacterial “cell wall” surrounds the bacterial “cell membrane.” Bacterial cell walls are made of peptidoglycan (also called murein), which is made from polysaccharide chains cross-linked by peptides containing D-amino acids. Bacterial cell walls are different from the cell walls of plants and fungi, which are made of cellulose and chitin, respectively. The cell wall of bacteria is also distinct from that of archaea, which do not contain peptidoglycan. The cell wall is essential to the survival of many bacteria.

[0091] There are broadly speaking two different types of cell wall in bacteria, that classify bacteria into “Gram positive bacteria” and “Gram negative bacteria.” The names originate from the reaction of cells to the Gram stain, a long-standing test for the classification of

bacterial species. Gram positive bacteria possess a thick cell wall containing many layers of peptidoglycan and teichoic acids. In contrast, Gram negative bacteria have a relatively thin cell wall consisting of a few layers of peptidoglycan surrounded by a second lipid membrane containing lipopolysaccharides and lipoproteins. Most bacteria have the Gram negative cell wall, and these differences in structure can produce differences in antibiotic susceptibility. Some bacteria have cell wall structures that are neither classically Gram positive nor Gram negative.

[0092] “Flagella” are rigid protein structures that are used for motility. “Fimbriae” (“attachment pili”) are fine filaments of protein. They are distributed over the surface of the cell and may resemble fine hairs when seen under the electron microscope. Fimbriae are believed to be involved in attachment to solid surfaces or to other cells and are essential for the virulence of some bacterial pathogens. “Pili” (singularly “pilus”) are cellular appendages, slightly larger than fimbriae, that can transfer genetic material between bacterial cells during bacterial conjugation.

[0093] “Gram positive bacteria” are bacteria that give a positive result in the Gram stain test, which is traditionally used to classify bacteria quickly into two broad categories according to their type of cell wall. Gram positive bacteria take up the Gram stain (crystal violet) and appear purple-colored when viewed through an optical microscope, because the thick layer of peptidoglycan in the bacterial cell wall retains the stain after it is washed away during the decolorization stage of the test. Notwithstanding their thicker peptidoglycan layer, Gram positive bacteria are more receptive to cell wall-targeting antibiotics than Gram negative bacteria, due to the absence on Gram positive bacteria of the outer membrane found on Gram negative bacteria. Representative examples of Gram positive bacteria especially pathogenic in humans include, but are not limited to, *Streptococcus* and *Staphylococcus*, which are cocci, and *Corynebacterium*, *Listeria*, *Bacillus*, and *Clostridium*, which are bacilli. Many of these now pose a medical problem, due to antibiotic resistance. Representative examples of Gram positive bacteria especially pathogenic in plants include, but are not limited to, *Rathyabacter*, *Leifsonia*, and *Clavibacter*.

[0094] Other medically important bacteria include *Clostridium difficile*, *Clostridium botulinum*, *Clostridium tetani*, *Listeria monocytogenes*, *Bacillus anthracis* and *Corynebacterium diphtheriae*. Others include *Neisseria gonorrhoeae*, *Neisseria meningitidis*,

Salmonella spp. *Shigella spp.* and *Vibrio cholerae*.

[0095] “Gram-negative bacteria” are bacteria that do not retain the Gram stain (crystal violet stain) used in the Gram staining method of bacterial differentiation. They are characterized by their cell envelopes, which are composed of a thin peptidoglycan cell wall sandwiched between an inner cytoplasmic cell membrane and a bacterial outer membrane. They pose a significant medical challenge as their outer membrane protects them from many antibiotics (including penicillin), detergents that would normally damage the inner cell membrane, and lysozyme, an antimicrobial enzyme produced by animals that forms part of the innate immune system. Additionally, the outer leaflet of this membrane comprises a complex lipopolysaccharide (LPS) whose lipid A component can cause a toxic reaction when bacteria are lysed by immune cells. This toxic reaction may lead to low blood pressure, respiratory failure, reduced oxygen delivery, and lactic acidosis – manifestations of septic shock.

[0096] “Lipopolysaccharides” (LPS) are large molecules consisting of a lipid and a polysaccharide that are bacterial toxins. They are composed of an O-antigen, an outer core, and an inner core all joined by covalent bonds, and are found in the outer membrane of Gram-negative bacteria, such as *E. coli* and *Salmonella*. Today, the term endotoxin is often used synonymously with LPS, although there are a few endotoxins (in the original sense of toxins that are inside the bacterial cell that are released when the cell disintegrates) that are not related to LPS, such as the so-called delta endotoxin proteins produced by *Bacillus thuringiensis*. Lipopolysaccharides can have substantial impacts on human health, primarily through interactions with the immune system. LPS is a potent activator of the immune system and pyrogen (agent that causes fever). In severe cases, LPS can play a role in causing septic shock. In lower amounts and over a longer time period, there is evidence LPS may play an important and harmful role in autoimmunity, obesity, depression, and cellular senescence.

[0097] “Peptidoglycan” or murein, is a unique large macromolecule, a polysaccharide, consisting of sugars and amino acids that forms a mesh-like peptidoglycan layer outside the plasma membrane, the rigid cell wall (murein sacculus) characteristic of most bacteria. The sugar component consists of alternating residues of β -(1,4) linked N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). Attached to the N-acetylmuramic acid is an oligopeptide chain made of three to five amino acids. The peptide chain can be cross-linked to the peptide chain of another strand forming the 3D mesh-like layer. Peptidoglycan serves a

structural role in the bacterial cell wall, giving structural strength, as well as counteracting the osmotic pressure of the cytoplasm. This repetitive linking results in a dense peptidoglycan layer which is critical for maintaining cell form and withstanding high osmotic pressures, and it is regularly replaced by peptidoglycan production.

[0098] The proteobacteria are a major superphylum of Gram-negative bacteria, including *Escherichia coli*, *Salmonella*, *Shigella*, and other *Enterobacteriaceae*, as well as *Pseudomonas*, *Moraxella*, *Helicobacter*, *Stenotrophomonas*, *Bdellovibrio*, acetic acid bacteria, *Legionella*, and others. Other notable groups of Gram-negative bacteria include the cyanobacteria, spirochaetes, green sulfur, and green non-sulfur bacteria. Medically relevant Gram-negative cocci include the four types that cause a sexually transmitted disease (*Neisseria gonorrhoeae*), a meningitis (*Neisseria meningitidis*), and respiratory symptoms (*Moraxella catarrhalis*, *Haemophilus influenzae*). Medically relevant Gram-negative bacilli include a multitude of species. Some of them cause primarily respiratory problems (*Klebsiella pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*), primarily urinary problems (*Escherichia coli*, *Proteus mirabilis*, *Enterobacter cloacae*, *Serratia marcescens*), and primarily gastrointestinal problems (*Helicobacter pylori*, *Salmonella enteritidis*, *Salmonella typhi*). Gram-negative bacteria associated with hospital-acquired infections include *Acinetobacter baumannii*, which cause bacteremia, secondary meningitis, and ventilator-associated pneumonia in hospital intensive-care units. Gram negative soil species include, but are not limited to, *Pseudomonas stutzeri* and *Acinetobacter baylyi*, and Gram-negative plant pathogens include, but are not limited to, *Ralstonia solanacearum* and *Xylella fastidiosa*. Other medically important bacteria include *Clostridium difficile*, *Clostridium botulinum*, *Clostridium tetani*, *Listeria monocytogenes*, *Bacillus anthracis* and *Corynebacterium diphtheriae*. Others include *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Salmonella spp.*, *Shigella spp.* and *Vibrio cholerae*.

[0099] Gram negative bacteria, including *E. coli*, differ from Gram positive bacteria such as *Staphylococcus aureus* by having an extra outer membrane that contributes greatly to antibiotic resistance. Lipopolysaccharide (LPS) is a common structure on the Gram negative bacteria outer membrane and a critical element forming their bacterial outer membranes. It contains three domains in the order from inner to outer domains: the lipid A, the core oligosaccharides and the O antigen (a region composed of extremely diverse surface

polysaccharide). While the overall structure of LPS is conserved, variation on each of the three domains exist between bacteria species or strains. The O antigen has the greatest diversity, even across bacterial strains. The lipid A and core oligosaccharide are relatively more conserved within species.

Gram negative bacteria binding moieties

[00100] WN1 222-5 is a neutralizing antibody that displays specificity for an epitope within the structurally conserved region of LPS core from a large number of pathogenic *E. coli*, *Salmonella*, *Shigella*, and *Citrobacter serovars* (DiPadova *et al.* (1993) Infect. Immun. 61(9): 3863-3872). Its sequence has also been published (see, e.g., US 00/5858728A).

[00101] As noted herein, a binding moiety provided herein is a monoclonal antibody or an antigen-binding fragment. In some embodiments, the antigen-binding fragment provided herein is a single chain Fv (scFv), a diabody, a tri(a)body, nanobody, a di-or tri-tandem scFv, a scFv-Fc bivalent molecule, an Fab, scFab, Fab', Fv, F(ab')₂ or an antigen binding scaffold (e.g., affibody, monobody, anticalin, DARPIn, Knottin, etc.). "Affibodies" are small proteins engineered to bind to a large number of target proteins or peptides with high affinity, often imitating monoclonal antibodies, and are antibody mimetics. As noted herein, an engineered phage comprising an antibody or antibody fragment binding moiety is expressed on a minor capsid protein and retains its binding activity towards its ligand.

Gram positive bacteria binding moieties

[00102] Antibodies to peptidoglycan useful for the purposes disclosed herein include, by way of non-limiting examples, monoclonal antibody 3F6B3 (10H6) from Invitrogen (catalog no. MA5-16509), and monoclonal antibody BDI380 from Santa Cruz Biotechnology, Inc. (catalog no. sc-57752). In other embodiments, antibodies may be used that are identified and isolated using phage display.

[00103] As noted herein, a binding moiety provided herein is a monoclonal antibody or an antigen-binding fragment. In some embodiments, the antigen-binding fragment provided herein is a single chain Fv (scFv), a diabody, a tri(a)body, a di-or tri-tandem scFv, a scFv-Fc bivalent molecule, an Fab, Fab', Fv, F(ab')₂ or an antigen binding scaffold (e.g., affibody, monobody, anticalin, DARPIn, Knottin, etc.). "Affibodies" are small proteins engineered to

bind to a large number of target proteins or peptides with high affinity, often imitating monoclonal antibodies, and are antibody mimetics.

Bacterial binding moieties

[00104] In addition to the antibodies and fragments thereof as binding moieties to bacteria or other types of cells (e.g., cancer cells) as described herein, other methods for designing a targeting moiety to a specific or preselected target cell are fully embraced by the present disclosure, and encompassing such target cell binding moieties is nonlimiting. By way of example, and as described in the examples herein, in one embodiment, a library of different bacterial receptor binding domains may be expressed on bacteriophage, and bacterial specificity of certain bacteriophages identified using binding studies, to identify bacteriophages useful for specific target bacteria. Such specific bacterial targeted bacteriophages may be bound to an antibiotic and thus embodied within the disclosure. In other embodiments, such library method for creating a large variety of bacteriophages then screening for desired subsets that bind target cells, similar approaches may be used to identify bacteriophages for targeting specific types of cancer cells, for cancer cells in general, and for disease-associated cells that a bioactive agent may exert an activity that treats an infection, a cancer, or other pathogen or pathogenic cell as described herein. As such, the same approach is applicable to any type of cell, a pathogen, pathogenic cell such as a cancer cell, or cell associated with a pathogenic cell such as an immune cell; such approach providing a receptor binding domain for binding to any desired cell type.

Pathogenic Fungi

[00105] “Pathogenic fungi” are fungi that cause disease in humans or other organisms. Although fungi are eukaryotic, many pathogenic fungi are microorganisms. Approximately 300 fungi are known to be pathogenic to humans. Fungal infections kill more people than either tuberculosis or malaria—about 2 million people per year. In 2022 the World Health Organization (WHO) published a list of fungal pathogens which should be a priority for public health action. Deemed of critical importance were *Candida albicans*, *Aspergillus fumigatus*, *C. auris* and *Cryptococcus neoformans*, followed by *Candida glabrata*, *H. capsulatum* and the several fungi causing mucormycosis or mycetoma. *Fusarium* spp., *Candida tropicalis* and *Candida parapsilosis* were deemed to be of high importance. Medium

priority fungi are *Scedosporium* spp., *Lomentospora prolificans*, *Coccidioides* spp., *Pichia kudriavzevii* (*Candida krusei*), *Cryptococcus gattii*, *Talaromyces marneffeii*, *Pneumocystis jirovecii*, *Paracoccidioides* spp.

Pathogenic fungi binding moieties

[00106] Pathogenic fungi binding moieties may be genera binding moieties to fungal cell wall components, or may be specific to a particular species of fungus. By way of non-limiting examples, binding moieties to fungi may be selected from a phage display library.

[00107] As noted herein, a binding moiety provided herein is a monoclonal antibody or an antigen-binding fragment. In some embodiments, the antigen-binding fragment provided herein is a single chain Fv (scFv), a diabody, a tri(a)body, a di- or tri-tandem scFv, a scFv-Fc bivalent molecule, an Fab, Fab', Fv, F(ab')₂ or an antigen binding scaffold (e.g., affibody, monobody, anticalin, DARPIn, Knottin, etc.). "Affibodies" are small proteins engineered to bind to a large number of target proteins or peptides with high affinity, often imitating monoclonal antibodies, and are antibody mimetics.

Other pathogen binding moieties

[00108] In other embodiments, binding moieties targeting parasites are embraced herein. Non-limiting examples include malaria, babesia, etc., scabies, Chagas, *Hypoderma tarandi*, amoebae, roundworm, *Trichomonas vaginalis*, *Giardia lamblia* and *Toxoplasma gondii*.

Cancers

[00109] In some embodiments, the tumor is a solid tumor. In some embodiments, the tumor, suspected tumor, or resected tumor comprises a cancerous, pre-cancerous, or non-cancerous tumor. In some embodiments, the tumor comprises a sarcoma or a carcinoma, a fibrosarcoma, a myxosarcoma, a liposarcoma, a chondrosarcoma, an osteogenic sarcoma, a chordoma, an angiosarcoma, an endotheliosarcoma, a lymphangiosarcoma, a lymphangioendotheliosarcoma, a synovioma, a mesothelioma, an Ewing's tumor, a leiomyosarcoma, a rhabdomyosarcoma, a colon carcinoma, a pancreatic cancer or tumor, a breast cancer or tumor, an ovarian cancer or tumor, a prostate cancer or tumor, a squamous cell carcinoma, a basal cell carcinoma, an adenocarcinoma, a sweat gland carcinoma, a

sebaceous gland carcinoma, a papillary carcinoma, a papillary adenocarcinomas, a cystadenocarcinoma, a medullary carcinoma, a bronchogenic carcinoma, a renal cell carcinoma, a hepatoma, a bile duct carcinoma, a choriocarcinoma, a seminoma, an embryonal carcinoma, a Wilm's tumor, a cervical cancer or tumor, a uterine cancer or tumor, a testicular cancer or tumor, a lung carcinoma, a small cell lung carcinoma, a bladder carcinoma, an epithelial carcinoma, a glioma, an astrocytoma, a medulloblastoma, a craniopharyngioma, an ependymoma, a pinealoma, a hemangioblastoma, an acoustic neuroma, an oligodendroglioma, a schwannoma, a meningioma, a melanoma, a neuroblastoma, or a retinoblastoma, esophageal cancer, pancreatic cancer, metastatic pancreatic cancer, metastatic adenocarcinoma of the pancreas, bladder cancer, stomach cancer, fibrotic cancer, glioma, malignant glioma, diffuse intrinsic pontine glioma, recurrent childhood brain neoplasm renal cell carcinoma, clear-cell metastatic renal cell carcinoma, kidney cancer, prostate cancer, metastatic castration resistant prostate cancer, stage IV prostate cancer, metastatic melanoma, melanoma, malignant melanoma, recurrent melanoma of the skin, melanoma brain metastases, stage IIIA skin melanoma; stage IIIB skin melanoma, stage IIIC skin melanoma; stage IV skin melanoma, malignant melanoma of head and neck, lung cancer, non-small cell lung cancer (NSCLC), squamous cell non-small cell lung cancer, breast cancer, recurrent metastatic breast cancer, hepatocellular carcinoma, Hodgkin's lymphoma, follicular lymphoma, non-Hodgkin's lymphoma, advanced B-cell NHL, HL including diffuse large B-cell lymphoma (DLBCL), multiple myeloma, chronic myeloid leukemia, adult acute myeloid leukemia in remission; adult acute myeloid leukemia with Inv(16)(p13.1q22); CBFβ-MYH11; adult acute myeloid leukemia with t(16;16)(p13.1;q22); CBFβ-MYH11; adult acute myeloid leukemia with t(8;21)(q22;q22); RUNX1-RUNX1T1; adult acute myeloid leukemia with t(9;11)(p22;q23); MLLT3-MLL; adult acute promyelocytic leukemia with t(15;17)(q22;q12); PML-RARA; alkylating agent-related acute myeloid leukemia, chronic lymphocytic leukemia, Richter's syndrome; Waldenstrom's macroglobulinemia, adult glioblastoma; adult gliosarcoma, recurrent glioblastoma, recurrent childhood rhabdomyosarcoma, recurrent Ewing sarcoma/ peripheral primitive neuroectodermal tumor, recurrent neuroblastoma; recurrent osteosarcoma, colorectal cancer, MSI positive colorectal cancer; MSI negative colorectal cancer, nasopharyngeal nonkeratinizing carcinoma; recurrent nasopharyngeal undifferentiated carcinoma, cervical adenocarcinoma; cervical adenosquamous carcinoma; cervical squamous cell carcinoma; recurrent cervical carcinoma;

stage IVA cervical cancer; stage IVB cervical cancer, anal canal squamous cell carcinoma; metastatic anal canal carcinoma; recurrent anal canal carcinoma, recurrent head and neck cancer; carcinoma, squamous cell of head and neck, head and neck squamous cell carcinoma (HNSCC), ovarian carcinoma, colon cancer, gastric cancer, advanced GI cancer, gastric adenocarcinoma; gastroesophageal junction adenocarcinoma, bone neoplasms, soft tissue sarcoma; bone sarcoma, thymic carcinoma, urothelial carcinoma, recurrent Merkel cell carcinoma; stage III Merkel cell carcinoma; stage IV Merkel cell carcinoma, myelodysplastic syndrome and recurrent mycosis fungoides and Sezary syndrome. In some embodiments, at the site, T cells are stimulated to target the tumor, suspected tumor, or resected tumor, and the induction of Tregs is suppressed.

[00110] In some embodiments, cancers include lung cancer, breast cancer, ovarian cancer, prostate cancer, bladder cancer, melanoma, among other solid tumors.

Cancer cell binding moieties

[00111] In some embodiments, a binding moiety to a cancer or tumor cell is directed to a neoantigen or other tumor cell surface marker that is not expressed or differentially expressed in normal cells of the cancer patient, such that specific targeting of the tumor cells by the engineered phages disclosed here may be achieved with targeted delivery of an anticancer drug, in particular one with a low or lower therapeutic index than the unbound anticancer agent. In other embodiments, the anticancer bioactive agent may be active on an immune or other cell that then exerts its activity against a cancer cell.

[00112] In some embodiments, cancer cell binding moieties are antibodies or antigen-binding fragments such as a single chain Fv (scFv), a diabody, a tri(a)body, a di- or tri-tandem scFv, a scFv-Fc bivalent molecule, an Fab, Fab', Fv, F(ab')₂ or an antigen binding scaffold (e.g., affibody, monobody, anticalin, DARPin, Knottin, etc.). Non-limiting examples of tumor cell targets to which a targeting moiety may be used includes B-cell maturation antigen, CD38, CD46, CD74, CD138, CD56, CD52, VEGFR, EGFR, RANKL, GD2, SLAMF7, HER2, CCR4, CD20, PDGFR α , CD19, CD30, nectin-4, CD33, CD22, folate receptor, CD79b, TROP-2, MET, gp100 and CD3. Non-limiting examples of tumor-binding antibodies include those in the following drugs: alemtuzumab, bevacizumab, blinatumomab, brentuximab vedotin, catumaxomab, cetuximab, elotuzumab, daratumumab, denosumab,

dinutuximab, ibritumomab tiuxetan, ipilimumab, isatuximab, margetuximab, mogamulizumab, naxitamab, necitumumab, obinutuzumab, ofatumumab, olaratumumab, panitumumab, pertuzumab, ramucirumab, rituximab, siltuximab, tafasitamab, trastuzumab and trastuzumab emtansine. Any of the foregoing antibodies or antibody components thereof, or antigen-binding fragments such as described herein, may be used in the bacteriophages disclosed herein.

[00113] In some embodiments, the antibodies used in the bacteriophages disclosed herein target immune checkpoint inhibitors, such as ipilimumab, pembrolizumab, nivolumab and atezolizumab. Other examples are provided herein below.

Other target cells

[00114] In some embodiments, the bacteriophage delivery of a bioactive agent by a target cell specific bacteriophage may be used to target cells associated with an autoimmune disease, such that the bioactive agent suppresses the disease. By way of non-limiting example, a bacteriophage embodied herein may target pancreatic islet cells and deliver an immunosuppressive bioactive agent, or may enhance the formation of regulatory T cells that suppress an immune response. In some embodiments, the target cell may be an autoreactive immune cell.

Binding moiety expressed on minor capsid protein

[00115] Numerous non-limiting examples of binding moieties to infectious agents and cancer cells are described herein. As noted herein, in some embodiments, the binding moiety is expressed as a N-terminal fusion with the anchoring, C-terminal domain of a phage minor capsid protein, such as the C-terminal domain of pIII, wherein in one embodiment, a binding moiety to a target cell is expressed as the N-terminal portion of the engineered pIII protein. In some embodiments, the C-terminal domain of pIII comprises only from the glycine-rich linker between the N2 domain and the C-terminal domain (for example, amino acids 236 to 274 of M13 gp3p), wherein the pIII has its N-terminal domain replaced with the binding moiety disclosed herein. As noted for example in Figure 2, the Fab binding domain is located at the N-terminus, fused to the glycine-rich linker and the C-terminal domain. As noted herein, pIII possesses two domains connected by a flexible glycine rich linker region: the C terminus

domain responsible for anchoring the pIII protein to the phage virions and the N terminus domain responsible for binding and infecting, e.g., *E. coli* cells bearing F sex pili. Thus, a recombinant pIII herein, in one embodiment, has its N terminus domain responsible for binding replaced with a binding moiety selected from among those described herein, to target the engineered bacteriophage to the desired target cell. In one embodiment, the selected binding domain is not fused onto or near the N-terminus of native pIII. Such strategy on modifying any minor capsid protein of a bacteriophage to achieve the targeting function by replacing the native binding domain on the minor capsid protein with a selected binding domain, and retaining the C-terminal anchoring domain, is embraced herein.

[00116] In some embodiments, a single chain binding moiety may be fused to the pIII C-terminal domain, retaining the target cell binding activity of the binding moiety. As noted herein in the examples, the orientation of components of the binding moiety may be optimized in selecting the phage composition: for a Fab sequence, both orientations were made and tested: light chain - linker - heavy chain (LLH; SEQ ID NO:1)), and heavy chain - linker - light chain (HLL; SEQ ID NO:4), with the linker sequence being (GGGGS)₃. Any construction of the minor capsid protein that expressed the binding moiety capable of binding the phage to the target cell is embraced by the disclosure, and one of skill by following the guidance herein facility construct a binding moiety expressed on a minor capsid protein embraced herein.

Bacteriophage

[00117] In some embodiments, disclosed herein is a recombinant bacteriophage comprising a binding moiety to an infectious microorganism. In some embodiments, the recombinant bacteriophage comprises a recombinant M13 bacteriophage.

[00118] A “bacteriophage” or “phage” is a virus that infects and replicates within bacteria and archaea. Bacteriophages are composed of proteins that encapsulate a DNA or RNA genome, and may have structures that are either simple or elaborate. Their genomes may encode as few as four genes (e.g., MS2) and as many as hundreds of genes. Phages replicate within the bacterium following the injection of their genome into its cytoplasm.

[00119] Filamentous phages are a family of viruses (Inoviridae) that infect bacteria. They

are named for their filamentous shape, a worm-like chain (long, thin and flexible, reminiscent of a length of cooked spaghetti), in some embodiments about 6 nm in diameter and about 1000-2000 nm long. This distinctive shape reflects their method of replication: the coat of the virion comprises in some embodiments five types of viral protein, which are located in the inner membrane of the host bacterium during phage assembly, and these proteins are added to the nascent virion's DNA as it is extruded through the membrane. The simplicity of filamentous phages makes them an appealing model organism for research in molecular biology, and they have also shown promise as tools in nanotechnology and immunology.

[00120] The family of filamentous phages contains 29 defined species, divided among 23 genera. Three filamentous bacteriophages, fd, f1 and M13, were isolated and characterized by three different research groups in the early 1960s, but they are so similar that they are sometimes grouped under the common name "Ff", which are members of genus Inovirus, as acknowledged by the International Committee on Taxonomy of Viruses (ICTV). The single-stranded Ff phage DNA runs down the central core of the phage, and is protected by a cylindrical protein coat built from thousands of identical α -helical major coat protein subunits coded by phage gene 8 (pVIII or g8p). The two ends of the phage are capped by a few copies of proteins that are important for infection of the host bacteria, and also for assembly of nascent phage particles. These proteins are the products of phage genes 3 and 6 at one end of the phage, and phage genes 7 and 9 at the other end. The fiber diffraction studies identified two structural classes of phage, differing in the details of the arrangement of the gene 8 protein. Class I has a rotation axis relating the gene 8 coat proteins, whereas for Class II this rotation axis is replaced by a helix axis. This technical difference has little noticeable effect on the overall phage structure, but the extent of independent diffraction data is greater for symmetry Class II than for Class I.

[00121] Structural Class I includes strains fd, f1, M13 of genus Inovirus as well as If1 (of ICTV's species *Escherichia virus If1*, genus *Infulavirus*) and Ike (of ICTV's species *Salmonella virus Ike*, genus *Lineavirus*), whereas Class II includes strains Pf1 (of ICTV's species *Pseudomonas virus Pf1* of genus *Primolicivirus*), and perhaps also Pf3 (of ICTV's species *Pseudomonas virus Pf3* of genus *Tertilicivirus*), Pf4 and PH75 (of NCBI's proposed species *Thermus phage PH75*, *incertae sedis* within *Inoviridae*).

[00122] The "M13 bacteriophage" (M13 phage, M13) is a well-characterized and studied

viral scaffold for phage display (Wang *et al.* Anal. Bioanal. Chem. 415(18): 3927-3944). Within its filamentous capsid, its 10 genes are encoded on a single stranded circular DNA that can be used to clone in recombinant genes using common molecular biology tools such as polymerase chain reaction (PCR) and restriction enzymes. The receptor binding protein pIII possesses two domains connected by a flexible glycine rich linker region: the C terminus domain responsible for anchoring the pIII protein to the phage virions and the N terminus domain responsible for binding and infecting, e.g., *E. coli* cells bearing F sex pili.

[00123] The term “operably linked” encompasses components to which the term is applied are in a relationship that allows them to carry out their inherent functions under suitable conditions. For example, a transcription control sequence “operably linked” to a protein coding sequence is ligated thereto so that expression of the protein coding sequence is achieved under conditions compatible with the transcriptional activity of the control sequences.

[00124] The term “control sequence” as used herein encompasses polynucleotide sequences that can affect expression or processing of coding sequences to which they are ligated or operably linked. The nature of such control sequences may depend upon the elements included in the cell-free transcription and translation system. In particular embodiments, transcription control sequences may include a promoter, ribosomal binding site, and transcription termination sequence. In some embodiments, transcription control sequences may include promoters comprising one or a plurality of recognition sites for transcription factors, transcription enhancer sequences, transcription termination sequences and polyadenylation sequences. In certain embodiments, “control sequences” can include leader sequences and/or fusion partner sequences.

[00125] The term “polynucleotide” as used herein encompasses single-stranded or double-stranded nucleic acid polymers. In certain embodiments, the nucleotides comprising the polynucleotide can be ribonucleotides or deoxyribonucleotides or a modified form of either type of nucleotide. The term “polynucleotide” specifically includes single and double stranded forms of DNA.

[00126] In some embodiments, at least a portion of the recombinant bacteriophage is produced via a plasmid. In some embodiments, the plasmid comprises a nucleic acid

comprising control sequences operably linked to the nucleic acid sequence encoding the LLH or HLL bound to receptor binding proteins of the bacteriophage (see **FIGURE 2**). In some embodiments, the plasmid comprises an expression vector.

[00127] In some embodiments, the expression vector may contain a variety of elements for controlling expression, including without limitation, promoter sequences, transcription initiation sequences, enhancer sequences, selectable markers, and signal sequences. These elements may be selected as appropriate by a person of ordinary skill in the art. For example, the promoter sequences may be selected to promote the transcription of the polynucleotide in the vector. Suitable promoter sequences include, without limitation, T7 promoter, T3 promoter, SP6 promoter, beta-actin promoter, EF1a promoter, CMV promoter, and SV40 promoter, or any promoter sequence known in the art. Enhancer sequences may be selected to enhance the transcription of the polynucleotide.

[00128] Transcription and translation of an encoded peptide or protein by the cell-free machinery are ATP-dependent processes. Therefore, availability of ATP, may in certain embodiments, regulate expression of a peptide or protein encoded by a plasmid vector. Caged ATP molecules may be used to regulate ATP release rapidly at sites of biological interest, at a desired time. A skilled artisan would appreciate and know how to select a caged ATP molecule, wherein they are well known in the art. In some embodiments, a caged ATP molecule may encompass a photoactivatable ATP molecule, for example but not limited to DMNPE-caged ATP (Adenosine 5'-Triphosphate, P3-(1-(4,5-Dimethoxy-2-Nitrophenyl)ethyl) Ester, Disodium Salt; THERMO FISHER SCIENTIFIC™ Cat No. A1049) or NPE-caged ATP (Adenosine 5'-Triphosphate, P3-(1-(2-Nitrophenyl)Ethyl) Ester, Disodium Salt; THERMO FISHER SCIENTIFIC™ Cat No. A1048; Jena Science, Germany Cat. No. NU-301S).

[00129] As used herein, a “targeting agent,” “binding moiety” or “affinity reagent,” is a molecule that binds to an antigen or receptor or other molecule. In some embodiments, a “targeting agent” is a molecule that specifically binds to an antigen or receptor or other molecule. In certain embodiments, some or all of a targeting agent is composed of amino acids (including natural, non-natural, and modified amino acids), nucleic acids, or saccharides. In certain embodiments, a “targeting agent” is a small molecule. In some embodiments, the binding moiety is an antibody fragment.

[00130] As used herein, the term “antibody” encompasses the structure that constitutes the natural biological form of an antibody. In most mammals, including humans, and mice, this form is a tetramer and consists of two identical pairs of two immunoglobulin chains, each pair having one light and one heavy chain, each light chain comprising immunoglobulin domains VL and CL, and each heavy chain comprising immunoglobulin domains VH, C-gamma-1 (C γ 1), C-gamma-2 (C γ 2), and C-gamma-3 (C γ 3). In each pair, the light and heavy chain variable regions (VL and VH) are together responsible for binding to an antigen, and the constant regions (CL, C γ 1, C γ 2, and C γ 3, particularly C γ 2, and C γ 3) are responsible for antibody effector functions. In some mammals, for example in camels and llamas, full-length antibodies may consist of only two heavy chains, each heavy chain comprising immunoglobulin domains VH, C γ 2, and C γ 3. By “immunoglobulin (Ig)” herein is meant a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. Immunoglobulins include but are not limited to antibodies. Immunoglobulins may have a number of structural forms, including but not limited to full-length antibodies, antibody fragments, and individual immunoglobulin domains including but not limited to VH, C γ 1, C γ 2, C γ 3, VL, and CL.

[00131] Depending on the amino acid sequence of the constant domain of their heavy chains, intact antibodies can be assigned to different “classes”. There are five-major classes (isotypes) of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into “subclasses”, e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called alpha, delta, epsilon, gamma, and mu, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known to one skilled in the art.

[00132] As used herein, the term “immunoglobulin G” or “IgG” refers to a polypeptide belonging to the class of antibodies that are substantially encoded by a recognized immunoglobulin gamma gene. In humans this class comprises IgG1, IgG2, IgG3, and IgG4. In mice this class comprises IgG1, IgG2a, IgG2b, IgG3. As used herein, the term “modified immunoglobulin G” refers to a molecule that is derived from an antibody of the “G” class. As used herein, the term “antibody” refers to a protein consisting of one or more polypeptides substantially encoded by all or part of the recognized immunoglobulin genes. The recognized immunoglobulin genes, for example in humans, include the kappa (κ), lambda (λ), and heavy

chain genetic loci, which together comprise the myriad variable region genes, and the constant region genes mu (μ), delta (δ), gamma (γ), sigma (σ), and alpha (α) which encode the IgM, IgD, IgG, IgE, and IgA isotypes or classes, respectively.

[00133] The term “antibody” is meant to include full-length antibodies, and may refer to a natural antibody from any organism, an engineered antibody, or an antibody or antibody fragment generated recombinantly for experimental, therapeutic, or other purposes as further defined below. Furthermore, full-length antibodies comprise conjugates as described and exemplified herein. As used herein, the term “antibody” comprises monoclonal and polyclonal antibodies. Antibodies can be antagonists, agonists, neutralizing, inhibitory, or stimulatory.

[00134] The “variable region” of an antibody contains the antigen binding determinants of the molecule, and thus determines the specificity of an antibody for its target antigen. The variable region is so named because it is the most distinct in sequence from other antibodies within the same isotype. The majority of sequence variability occurs in the complementarity determining regions (CDRs). There are 6 CDRs total, three each per heavy and light chain, designated VH CDR1, VH CDR2, VH CDR3, VL CDR1, VL CDR2, and VL CDR3. The variable region outside of the CDRs is referred to as the framework (FR) region. Although not as diverse as the CDRs, sequence variability does occur in the FR region between different antibodies. Overall, this characteristic architecture of antibodies provides a stable scaffold (the FR region) upon which substantial antigen binding diversity (the CDRs) can be explored by the immune system to obtain specificity for a broad array of antigens.

[00135] Furthermore, antibodies may exist in a variety of other forms including, for example, Fv, Fab, and (Fab')₂, as well as bi-functional (i.e., bi-specific) hybrid antibodies (see, e.g., Lanzavecchia et al., *Eur. J. Immunol.* 17, 105 (1987)) and in single chains (see, e.g., Huston et al., *Proc. Natl. Acad. Sci. U.S.A.*, 85, 5879-5883 (1988) and Bird et al., *Science*, 242, 423-426 (1988)). (See, generally, Hood et al., “Immunology”, Benjamin, N.Y., 2nd ed. (1984), and Hunkapiller and Hood, *Nature*, 323, 15-16 (1986)).

[00136] The term “epitope” as used herein refers to a region of the antigen that binds to the antibody or antigen-binding fragment. It is the region of an antigen recognized by a first antibody wherein the binding of the first antibody to the region prevents binding of a second

antibody or other bivalent molecule to the region. The region encompasses a particular core sequence or sequences selectively recognized by a class of antibodies. In general, epitopes are comprised by local surface structures that can be formed by contiguous or noncontiguous amino acid sequences.

[00137] As used herein, the terms “selectively recognizes”, “selectively bind” or “selectively recognized” mean that binding of the antibody, antigen-binding fragment or other bivalent molecule to an epitope is at least 2-fold greater, preferably 2-5 fold greater, and most preferably more than 5-fold greater than the binding of the molecule to an unrelated epitope or than the binding of an antibody, antigen-binding fragment or other bivalent molecule to the epitope, as determined by techniques known in the art and described herein, such as, for example, ELISA or cold displacement assays.

[00138] As used herein, the term “Fc domain” encompasses the constant region of an immunoglobulin molecule. The Fc region of an antibody interacts with a number of Fc receptors and ligands, imparting an array of important functional capabilities referred to as effector functions, as described herein. For IgG, the Fc region comprises Ig domains CH2 and CH3. An important family of Fc receptors for the IgG isotype are the Fc gamma receptors (FcγRs). These receptors mediate communication between antibodies and the cellular arm of the immune system.

[00139] As used herein, the term “Fab domain” encompasses the region of an antibody that binds to antigens. The Fab region is composed of one constant and one variable domain of each of the heavy and the light chains.

[00140] In one embodiment, the term “antibody” or “antigen-binding fragment” respectively refer to intact molecules as well as functional fragments thereof, such as Fab, a scFv-Fc bivalent molecule, F(ab')₂, and Fv that are capable of specifically interacting with a desired target. In some embodiments, the antigen-binding fragments comprise:

[00141] (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, which can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain;

[00142] (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

[00143] (3) (Fab')₂, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')₂ is a dimer of two Fab' fragments held together by two disulfide bonds;

[00144] (4) Fv, a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

[00145] (5) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule. In some embodiments, the SCA has the light chain portion at the N terminus and the heavy chain portion at the C terminus; in other embodiments, the heavy chain portion is at the N terminus and the light chain portion at the C terminus. In some embodiments, the two domains are connected through a linker such as a glycine-rich linker, e.g., (GGGGS)₄.

[00146] (6) scFv-Fc, is produced in one embodiment, by fusing single-chain Fv (scFv) with a hinge region from an immunoglobulin (Ig) such as an IgG, and Fc regions.

[00147] In some embodiments, an antibody provided herein or source of the antigen-binding fragment is a monoclonal antibody. In some embodiments, the antigen-binding fragment provided herein is a single chain Fv (scFv), a diabody, a tri(a)body, a di- or tri-tandem scFv, a scFv-Fc bivalent molecule, an Fab, Fab', Fv, F(ab')₂ or an antigen binding scaffold (e.g., affibody, monobody, anticalin, DARPIn, Knottin, etc.). "Affibodies" are small proteins engineered to bind to a large number of target proteins or peptides with high affinity, often imitating monoclonal antibodies, and are antibody mimetics.

[00148] As used herein, the terms "bivalent molecule" or "BV" refer to a molecule capable of binding to two separate targets at the same time. The bivalent molecule is not limited to having two and only two binding domains and can be a polyvalent molecule or a molecule comprised of linked monovalent molecules. The binding domains of the bivalent molecule can selectively recognize the same epitope or different epitopes located on the same target or

located on a target that originates from different species. The binding domains can be linked in any of a number of ways including, but not limited to, disulfide bonds, peptide bridging, amide bonds, and other natural or synthetic linkages known in the art (see, e.g., Spatola et al., "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Morley, J. S., "Trends Pharm Sci." (1980) pp. 463-468; Hudson et al., Int. J. Pept. Prot. Res. (1979) 14, 177-185; Spatola et al., Life Sci. (1986) 38, 1243-1249; Hann, M. M., J. Chem. Soc. Perkin Trans. I (1982) 307-314; Almquist et al., J. Med. Chem. (1980) 23, 1392-1398; Jennings-White et al., Tetrahedron Lett. (1982) 23, 2533; Szelke et al., European Application EP 45665; Chemical Abstracts 97, 39405 (1982); Holladay, et al., Tetrahedron Lett. (1983) 24, 4401-4404; and Hruby, V. J., Life Sci. (1982) 31, 189-199).

[00149] As used herein, the terms "binds" or "binding" or grammatical equivalents, refer to compositions having affinity for each other. "Specific binding" is where the binding is selective between two molecules. A particular example of specific binding is that which occurs between an antibody and an antigen. Typically, specific binding can be distinguished from non-specific when the dissociation constant (KD) is less than about 1×10^{-5} M or less than about 1×10^{-6} M or 1×10^{-7} M. Specific binding can be detected, for example, by ELISA, immunoprecipitation, coprecipitation, with or without chemical crosslinking, two-hybrid assays and the like. Appropriate controls can be used to distinguish between "specific" and "non-specific" binding.

[00150] "Binding" or "bound" as used herein also refers to the bioactive agent bound to the major capsid protein of the bacteriophage. Description of such binding, such as achieved by a cross-linking agent, is described elsewhere herein.

[00151] In addition to antibody sequences, an antibody according to the present disclosure may comprise other amino acids, e.g., forming a peptide or polypeptide, such as a folded domain, or to impart to the molecule another functional characteristic in addition to ability to bind antigen. For example, antibodies of the disclosure may carry a detectable label, such as fluorescent or radioactive label, or may be conjugated to a toxin (such as a holotoxin or a hemitoxin) or an enzyme, such as beta-galactosidase or alkaline phosphatase (e.g., via a peptidyl bond or linker).

[00152] In one embodiment, an antibody of the disclosure comprises a stabilized hinge region. The term “stabilized hinge region” will be understood to mean a hinge region that has been modified to reduce Fab arm exchange or the propensity to undergo Fab arm exchange or formation of a half-antibody or a propensity to form a half-antibody. “Fab arm exchange” refers to a type of protein modification for human immunoglobulin, in which a human immunoglobulin heavy chain and attached light chain (half-molecule) is swapped for a heavy-light chain pair from another human immunoglobulin molecule. Thus, human immunoglobulin molecules may acquire two distinct Fab arms recognizing two distinct antigens (resulting in bispecific molecules). Fab arm exchange occurs naturally in vivo and can be induced in vitro by purified blood cells or reducing agents such as reduced glutathione. A “half-antibody” forms when a human immunoglobulin antibody dissociates to form two molecules, each containing a single heavy chain and a single light chain. In one embodiment, the stabilized hinge region of human immunoglobulin comprises a substitution in the hinge region.

[00153] In one embodiment, the term “hinge region” as used herein refers to a proline-rich portion of an immunoglobulin heavy chain between the Fc and Fab regions that confers mobility on the two Fab arms of the antibody molecule. It is located between the first and second constant domains of the heavy chain. The hinge region includes cysteine residues which are involved in inter-heavy chain disulfide bonds. In one embodiment, the hinge region includes cysteine residues which are involved in inter-heavy chain disulfide bonds.

[00154] In one embodiment, the antibody or antigen-binding fragment binds its target with a KD of 0.1 nM – 10 mM. In one embodiment, the antibody or antigen-binding fragment binds its target with a KD of 0.1 nM – 1 mM. In one embodiment, the antibody or antigen-binding fragment binds its target with a KD within the 0.1 nM range. In one embodiment, the antibody or antigen-binding fragment binds its target with a KD of 0.1-2 nM. In another embodiment, the antibody or antigen-binding fragment binds its target with a KD of 0.1-1 nM. In another embodiment, the antibody or antigen-binding fragment binds its target with a KD of 0.05-1 nM. In another embodiment, the antibody or antigen-binding fragment binds its target with a KD of 0.1-0.5 nM. In another embodiment, the antibody or antigen-binding fragment binds its target with a KD of 0.1-0.2 nM.

[00155] In some embodiments, the antibody or antigen-binding fragment thereof provided herein comprises a modification. In another embodiment, the modification minimizes conformational changes during the shift from displayed to secreted forms of the antibody or antigen-binding fragment. It is to be understood by a skilled artisan that the modification can be a modification known in the art to impart a functional property that would not otherwise be present if it were not for the presence of the modification. Encompassed are antibodies which are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited, to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, etc.

[00156] In some embodiments, the modification is one as further defined herein below. In some embodiments, the modification is a N-terminus modification. In some embodiments, the modification is a C-terminal modification. In some embodiments, the modification is an N-terminus biotinylation. In some embodiments, the modification is an C-terminus biotinylation. In some embodiments, the secretable form of the antibody or antigen-binding fragment comprises an N-terminal modification that allows binding to an Immunoglobulin (Ig) hinge region. In some embodiments, the Ig hinge region is from but is not limited to, an IgA hinge region. In some embodiments, the secretable form of the antibody or antigen-binding fragment comprises an N-terminal modification that allows binding to an enzymatically biotinylatable site. In some embodiments, the secretable form of the antibody or antigen-binding fragment comprises an C-terminal modification that allows binding to an enzymatically biotinylatable site. In some embodiments, biotinylation of said site functionalizes the site to bind to any surface coated with streptavidin, avidin, avidin-derived moieties, or a secondary reagent.

[00157] It will be appreciated that the term “modification” can encompass an amino acid modification such as an amino acid substitution, insertion, and/or deletion in a polypeptide sequence.

[00158] In a further embodiment, the amino acid sequences of the disclosure may be homologues, variants, isoforms, or fragments of the sequences presented. The term

“homolog” as used herein refers to a polypeptide having a sequence homology of a certain amount, namely of at least 70%, e.g., at least 80%, 90%, 95%, 96%, 97%, 98%, 99% of the amino acid sequence it is referred to. Homology refers to the magnitude of identity between two sequences. Homolog sequences have the same or similar characteristics, in particular, have the same or similar property of the sequence as identified. The term ‘variant’ as used herein refers to a polypeptide wherein the amino acid sequence exhibits substantially 70, 80, 95, or 99% homology with the amino acid sequence as set forth in the sequence listing. It should be appreciated that the variant may result from a modification of the native amino acid sequences, or by modifications including insertion, substitution or deletion of one or more amino acids. The term “isoform” as used herein refers to variants of a polypeptide that are encoded by the same gene, but that differ in their isoelectric point (pI) or molecular weight (MW), or both. Such isoforms can differ in their amino acid composition (e.g., as a result of alternative splicing or limited proteolysis) and in addition, or in the alternative, may arise from differential post-translational modification (e.g., glycosylation, acylation, phosphorylation deamidation, or sulphation). As used herein, the term “isoform” also refers to a protein that exists in only a single form, i.e., it is not expressed as several variants. The term “fragment” as used herein refers to any portion of the full-length amino acid sequence of protein of a polypeptide of the disclosure which has less amino acids than the full-length amino acid sequence of a polypeptide of the disclosure. The fragment may or may not possess a functional activity of such polypeptides.

Expression of binding moiety on minor capsid protein

[00159] As disclosed herein, bacteriophages comprise a binding moiety to a target cell expressed on a minor capsid protein. In some embodiments of M13 phage, the minor capsid proteins are pIII, pVI, pVII or pIX. In some embodiments, the minor capsid protein is pIII.

[00160] As seen in FIG. 2, the binding moiety is expressed on the N-terminus of pIII, as replacements of the wild-type pIII N-terminal domains (N1 and N2). Such replacement provides an surface-exposed binding moiety that can bind the target cell. The examples herein describe on such method for expressing a binding moiety (in this example, a Fab fragment targeting LPS) to the C-terminus of M13 pIII. Various ways of expressing the binding moiety fused to the minor capsid protein are embraced herein. Analogous capsid proteins of other filamentous and other phages may be similarly modified for such binding moiety expression

following the teachings herein.

Cross-linking agents

[00161] A cross-linking agent may be used to conjugate the antibiotic to the capsid protein, via a reactive groups on each, such as but not limited to independently free amino groups and/or carboxyl groups and/or phenol groups and/or sulfhydryl groups. The reactive moiety on the antibiotic and the capsid protein may be the same or different. By way of non-limiting examples, chemistry for such conjugation may comprise the direct conjugation of the antibiotic to the capsid protein (e.g., by an amide bond between an amino group on one and a carboxyl group on the other), or a bifunctional cross-linking agent may be used, that conjugates the same reactive groups on both moieties (e.g., a homobifunctional crosslinking agent) such as amine to amine or carboxyl to carboxyl, or may comprise different reactive groups on the two moieties (e.g., a heterobifunctional crosslinking agent) such as an amine (on one or the other) to a carboxylic acid (on the other). Non-limiting examples of direct conjugation include the use of a carbodiimide such as EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) to form an amide bond. Non-limiting examples of other carbodiimides include 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide (CMC) and 1-ethyl-3-(4-azonia-44-dimethylpentyl) carbodiimide. Other cross-linking reagents include cyanogen bromide, glutaraldehyde and succinic anhydride. Non-limiting examples of homobifunctional agents include including a homo-bifunctional aldehyde, a homo-bifunctional epoxide, a homo-bifunctional imido-ester, a homo-bifunctional N-hydroxysuccinimide ester, a homo-bifunctional maleimide, a homo-bifunctional alkyl halide, a homo-bifunctional pyridyl disulfide, a homo-bifunctional aryl halide, a homo-bifunctional hydrazide, a homo-bifunctional diazonium derivative or a homo-bifunctional photoreactive compound. Non-limiting examples of heterobifunctional agents include compounds having an amine-reactive and a sulfhydryl-reactive group, compounds with an amine-reactive and a photoreactive group and compounds with a carbonyl-reactive and a sulfhydryl-reactive group.

[00162] In other examples, the homo-bifunctional cross-linking agents include the bifunctional N-hydroxysuccinimide esters dithiobis(succinimidylpropionate), disuccinimidyl suberate, and disuccinimidyl tartarate; the bifunctional imido-esters dimethyl adipimidate, dimethyl pimelimidate, and dimethyl suberimidate; the bifunctional sulfhydryl-reactive crosslinkers 1,4-di-[3'-(2'-pyridyl)dithio]propionamido]butane, bismaleimido-hexane, and bis-

N-maleimido-1,8-octane; the bifunctional aryl halides 1,5-difluoro-2,4-dinitrobenzene and 4,4'-difluoro-3,3'-dinitrophenylsulfone; bifunctional photoreactive agents such as bis-[b-(4-azidosalicylamido)ethyl]disulfide; the bifunctional aldehydes formaldehyde, malondialdehyde, succinaldehyde, glutaraldehyde, and adipaldehyde; a bifunctional epoxide such as 1,4-butanediol diglycidyl ether; the bifunctional hydrazides adipic acid dihydrazide, carbonyldihydrazide, and succinic acid dihydrazide; the bifunctional diazoniums o-tolidine, diazotized and bis-diazotized benzidine; the bifunctional alkylhalides N,N'-ethylene-bis(iodoacetamide), N,N'-hexamethylene-bis(iodoacetamide), N,N'-undecamethylene-bis(iodoacetamide), as well as benzylhalides and halomustards, such as a,a'-diiodo-p-xylene sulfonic acid and tri(2-chloroethyl)amine, respectively.

[00163] In other embodiments, hetero-bifunctional cross-linking agents used to link the antibiotic to phage capsid proteins include, but are not limited to, SMCC (succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate), MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester), SIAB (N-succinimidyl(4-iodoacetate)aminobenzoate), SMPB (succinimidyl-4-(p-maleimidophenyl)butyrate), GMBS (N-(gamma-maleimidobutyryloxy)succinimide ester), MPBH (4-(4-N-maleimidophenyl) butyric acid hydrazide), M2C2H (4-(N-maleimidomethyl) cyclohexane-1-carboxyl-hydrazide), SMPT (succinimidyl-oxycarbonyl-a-methyl-a-(2-pyridyldithio)toluene), and SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate).

[00164] Other methods for binding the bioactive agent to the major capsid protein are embraced herein, such as using a disulfide bond. In other embodiments, click chemistry may be used (see, e.g., H. C. Kolb; M. G. Finn; K. B. Sharpless (2001). "Click Chemistry: Diverse Chemical Function from a Few Good Reactions". *Angewandte Chemie International Edition*. 40 (11): 2004–2021; R. A. Evans (2007). "The Rise of Azide–Alkyne 1,3-Dipolar 'Click' Cycloaddition and its Application to Polymer Science and Surface Modification". *Australian Journal of Chemistry*. 60 (6): 384–395). In other embodiments, cyclodextrin-adamantine or other cyclodextrin binding moieties may be used to bind a bioactive moiety to a phage.

Bioactive agent

[00165] As used herein, a bioactive agent is any agent bound to a phage having a binding moiety to a target cell, as described herein, where the bioactive agent exerts one or more activities on the target cell to which the phage becomes bound. Such activities may include, but are not limited to, killing or inducing the killing of a pathogenic organism such as a bacterium, killing or inducing the killing of a cancer cell, such as a cancer cell of a solid tumor. In some embodiments, the bioactive agent exerts its activity on the target cell while remaining bound to the phage. In some embodiments, the bioactive agent exerts its activity after being cleaved or dissociated from the phage. In some embodiments, the bioactive agent exerts its activity on another bioactive agent such as a prodrug, wherein the result of such exerted activity (e.g., converting the prodrug into the active drug) then acts on the target cell. In some embodiments, the bioactive agent is an antibiotic. In some embodiments, the bioactive agent is an anticancer agent. In some embodiments, the bioactive agent is a peptide. In some embodiments, the bioactive agent is a stapled peptide. In some embodiments, the bioactive agent is an enzyme. Non-limiting examples of bioactive agents are described in the ensuing sections. As described herein, a bioactive agent comprises at least two domains, one domain responsible for exerting the activity on the target cell (e.g., a bacterial cell or a cancer cell), referred to herein as the activity domain, and another domain comprising a moiety that can be bound to a major capsid protein of a bacteriophage, as described herein, referred to herein as the linking domain. In some embodiments, the activity domain is responsible for cytotoxic activity, cytosuppressive activity, immunostimulatory activity, etc., and the linking domain having a reactive group such as an amino, carboxylic acid, or sulfhydryl group, that can be linked to a reactive group on the major capsid protein. The binding of the bioactive agent via the linking domain to the bacteriophage does not adversely affect the activity of the activity domain, e.g., an antibiotic bound to a bacteriophage retains antibacterial activity, or an anticancer agent bound to a bacteriophage retains anticancer activity. In some embodiments, a reduction in the bioactivity of the bioactive agent may be observed but adequate bioactivity is retained for the bacteriophage to be active for their intended uses.

[00166] In some embodiments, up to about 99% of the conjugated bioactive agent is active; in other embodiments, up to about 95%, up to about 90%, up to about 99%, up to about 85%, up to about 80%, up to about 75%, up to about 70%, up to about 65%, up to about 60%, up to about 55%, or up to about 50% of the conjugate bioactive agent is active. In some embodiments, a lower percent active bound bioactive agent is provided. In some

embodiments, up to about 99% of the conjugated antibiotic molecules are active; in other embodiments, up to about 95%, up to about 90%, up to about 99%, up to about 85%, up to about 80%, up to about 75%, up to about 70%, up to about 65%, up to about 60%, up to about 55%, or up to about 50% of the conjugate antibiotic molecules are active. In some embodiments, a lower percent active bound antibiotic is provided. In some embodiments, up to about 99% of the conjugated anticancer agents are active; in other embodiments, up to about 95%, up to about 90%, up to about 99%, up to about 85%, up to about 80%, up to about 75%, up to about 70%, up to about 65%, up to about 60%, up to about 55%, or up to about 50% of the conjugate anticancer molecules are active. In some embodiments, a lower percent active bound anticancer agent is provided.

[00167] In any embodiment herein, the bioactive agent bound to the phage may comprise one bioactive agent, or may comprise two or more different bioactive agents, such as two different antibiotics or two different anticancer agents, or two different types of bioactive agents. In some embodiments, the two or more different bioactive agents may be bound to the phage at the same time as a mixture, or in other embodiments, each bioactive bound to the major coat protein using different conjugation chemistries and/or at different times. Such successive conjugation may be performed to load phages with desired ratios of the two (or more) different bioactive agents, or separately conjugated using different conjugation chemistries. As noted here, reactive sites on the major coat protein may be blocked or reversibly blocked in order to conjugate different bioactive moieties each with different reactive groups for conjugation.

Antibiotics

[00168] In some embodiments, a therapeutic moiety is conjugated to the bacteriophage. A “therapeutic moiety” comprises a substance for inhibiting viability, growth, maintenance, or proliferation of, e.g., a bacterium or other microorganism. In some embodiments, the therapeutic moiety is an antibiotic. As conjugated to the bacteriophage, the antibiotic is active, e.g., it retains its microbicidal activity against the infectious microorganism to which it is sensitive. As noted herein, in some embodiments where an infectious microorganism is resistant to a certain antibiotic, that antibiotic conjugated to a bacteriophage as disclosed herein overcomes the resistance thereto.

[00169] In some embodiments, the therapeutic moiety acts on the surface of the microorganism, such as but not limited to surface-active antibiotics, cationic antibiotics, cationic antimicrobial peptides, and other moieties that when covalent bound to a bacteriophage major capsid protein, are capable of killing bacteria when in contact therewith. Such contact may be, as described herein, the targeting of the antibiotic-bacteriophage to particular types of microorganisms (e.g., Gram negative, Gram positive, fungi) by means of a targeting moiety recombinantly expressed on a minor capsid protein thereof.

[00170] Thus, in some embodiments, the therapeutic moiety is a microorganism surface active agent. In some embodiments the therapeutic moiety is microorganism cell surface active. In some embodiments the therapeutic moiety is cell wall active. In some embodiments the therapeutic moiety is cell membrane active. In some embodiments the therapeutic moiety is a surface active antibiotic. One of skill in the art will be directed following the disclosure herein and the type of infectious microorganism to be targeted to select an appropriate therapeutic moiety that when conjugated to the phage will kill the intended target microorganism. The terms “therapeutic moiety”, “antibiotic” and other variants are used herein to refer to the compound conjugated to the major capsid protein of the phage that targets infectious microorganisms, and the various aforementioned terms used to describe variations thereon as disclosed herein.

[00171] In some embodiments, the antibiotic is a peptide such as a gramicidin, tyrocidin, alamethicin or polymyxin, or an analogue thereof. In some embodiments the antibiotic is a cationic peptide. Other antibiotics and types of antibiotics include magainin, pardaxin, cecropin, dermaseptin, brevinine, protegrin I, tachyplesin I, β -defensin-1, and analogues thereof.

[00172] In some embodiments, the antibiotic is a lytic peptide.

[00173] In some embodiments, the antibiotic comprises a polymyxin. In some embodiments, the antibiotic comprises polymyxin B (PolyB), polymyxin E (PolyE), or a combination thereof. Other polymyxins and analogues thereof are embraced within the disclosure.

[00174] In some embodiments, the antibiotic comprises polymyxin B (PolyB; N-[(2S)-4-

amino-1-[[[(2S,3R)-1-[[[(2S)-4-amino-1-oxo-1-[[[(3S,6S,9S,12S,15R,18R,21S)-6,9,18-tris(2-aminoethyl)-15-benzyl-3-[(1R)-1-hydroxyethyl]-12-(2-methylpropyl)-2,5,8,11,14,17,20-hepta-oxo-1,4,7,10,13,16,19-heptazacyclotricos-21-yl]amino]butan-2-yl]amino]-3-hydroxy-1-oxobutan-2-yl]amino]-1-oxobutan-2-yl]-6-methyloctanamide). The structure of PolyB is shown in **FIGURE 1B**.

[00175] In some embodiments, the antibiotic comprises polymyxin E (PolyE, colistin; N-[(2S)-4-amino-1-[[[(2S,3R)-1-[[[(2S)-4-amino-1-oxo-1-[[[(3S,6S,9S,12S,15R,18S,21S)-6,9,18-tris(2-aminoethyl)-3-[(1R)-1-hydroxyethyl]-12,15-bis(2-methylpropyl)-2,5,8,11,14,17,20-hepta-oxo-1,4,7,10,13,16,19-heptazacyclotricos-21-yl]amino]butan-2-yl]amino]-3-hydroxy-1-oxobutan-2-yl]amino]-1-oxobutan-2-yl]-5-methylheptanamide). The structure of PolyE is shown in **FIGURE 1B**.

[00176] While the disclosure is directed in certain embodiments to surface active therapeutic moieties (e.g., antibiotics), the use of other types of antibiotics is equally embraced herein. Drugs commonly used to treat Gram negative infections include amino, carboxy and ureido penicillins (ampicillin, amoxicillin, piperacillin, ticarcillin) these drugs may be combined with beta-lactamase inhibitors to combat the presence of enzymes that can digest these drugs (known as beta-lactamases) in the peri-plasmic space. Other classes of drugs that have Gram negative spectrum include cephalosporins, monobactams (aztreonam), aminoglycosides, quinolones, macrolides, chloramphenicol, folate antagonists, and carbapenems.

[00177] In some embodiments, the antibiotic comprises an amino penicillin, a carboxy penicillin, or a ureido penicillin. In some embodiments, the antibiotic comprises an ampicillin, an amoxicillin, a piperacillin, or a ticarcillin. In some embodiments, the antibiotic further comprises a beta-lactamase inhibitor. In some embodiments, the antibiotic comprises a cephalosporin, a monobactam, an amino glycoside, a quinolone, a macrolide, a chloramphenicol, a folate antagonist, or a carbapenem.

[00178] Drugs commonly used to treat Gram positive infections include daptomycin, penicillin, cloxacillin, tetracycline, erythromycin, vancomycin, or an analogue thereof.

[00179] Drugs commonly used to treat fungal infections include amphotericin B, an echinocandins or an analogue thereof. Some examples of echinocandins are anidulafungin, rezafungin, asposfungin, caspofungin, cilofungin or micafungin, or an analogue thereof.

[00180] In one embodiment, antibiotics or analogues thereof comprises a reactive or otherwise pendant moiety that can be used, or chemically modified then used, to link the antibiotic to the major capsid protein of a phage, and wherein the so-linked antibiotic retains its activity against its target microorganism or microorganisms. In some embodiments, an antibiotic comprises such a reactive or linkable moiety that can be used for such linking and the antibiotic retains activity. In some embodiments, an antibiotic is modified to comprise such a reactive or pendant moiety that upon linking to the phage, retains its antibiotic activity. In some embodiments, a known (e.g., tested, marketed) antibiotic does not comprise such a reactive or pendant moiety, but an analogue of the antibiotic does comprise the reactive or pendant moiety, and the antibiotic analogue retains activity after linking to the phage protein. Analogues of any of the aforementioned antibiotics are known in the art and are fully embraced herein.

[00181] Among antibiotics active against bacteria or fungi, in certain embodiments, those that have proven too toxic for use or have other undesirable properties for development as a drug (e.g., never developed through clinical testing; approved but taken off the market; having poor pharmacokinetics or other properties unsuitable for use as a drug) are suitable antibiotic candidates for the phages disclosed here. In some embodiments, a toxic antibiotic when bound to a phage provides enhanced efficacy and reduced toxicity. In some embodiments, an antibiotic with poor pharmacokinetic or other properties, when bound to a phage, provides desired efficacy.

Anticancer agents

[00182] The present disclosure is not limited to any particular anticancer agent or other bioactive agent that is salutary in treating cancer. In one embodiment, an anticancer agent with a low or small therapeutic index, e.g., having toxicity to the patient near the effective dose to treat the tumor, are particularly amenable to the methods disclosed herein. The therapeutic index of a toxic anticancer agent can be expanded manifold by following the teachings herein.

[00183] An anticancer bioactive agent conjugated to the phage may be a toxin such as a toxin that is used in antibody-drug conjugates. Non-limiting examples of such toxins include a dolastatin such as monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF), a maytansanoid such as maytansine, a taxoid such as taxol or taxotere, a calicheamicin, a CC-1065 analog, a duocarmycin, or an amatoxin such as alpha-amanitin. In other embodiments, the anticancer bioactive agent may be an alkylating agent (e.g., mechlorethamine, cyclophosphamide, cisplatin, carmustine, busulfan, and dacarbazine), an antimetabolite (e.g., methotrexate, 5-fluorouracil, thioguanine, and 6-mercaptopurine), thalidomide and its analogs, e.g., lenalidomide, pomalidomide, hydroxyurea, tretinoin, or estramustine.

[00184] An anticancer bioactive agent conjugated to the phage may be an immune checkpoint inhibitor that inhibits a checkpoint molecule such as but not limited to PD-1, PD-L1, CTLA-4, CTLA-4 receptor, PD1-L2, 4-1BB, OX40, LAG-3 and TIM-3. Nonlimiting examples of immune checkpoint inhibitors include ipilimumab, tremelimumab, ticilimumab; CP-675-206, nivolumab, pembrolizumab; pidilizumab, MEDI0680 (AMP-514), AMP-224, AUNP-12, BMS-936559, atezolizumab, Durvalumab, avelumab, BMS935559 (MDX-1105), rHlgM12B7, BMS-986016, GSK2831781, IMP321, lirilumab (BMS-986015), IPH2101 (1-7F9), urelumab (BMS-663513) and MEDI6469.

[00185] An anticancer bioactive agent conjugated to the phage may be a ligand or receptor inhibitor, that blocks or inhibits interaction of a cell surface ligand or a cell surface receptor (e.g., VEGFR-2 or EGFR). Non-limiting examples of bioactive agents include necitumumab and ramucirumab.

[00186] An anticancer bioactive agent conjugated to the phage may be a radioligand conjugate that delivers radiation to a cancer cell upon binding thereto. Non-limiting examples of such radioligand conjugates to which a radioactive isotope can be bound include ibritumomab tiuxetan and tositumomab.

[00187] An anticancer bioactive agent conjugated to the phage may be an immunocytokine or immunostimulant, that stimulate the immune response in the tumor microenvironment. Non-limiting examples of immunocytokines and immunostimulants include interleukin-2 (IL-2; aldesleukin) and granulocyte-macrophage colony-stimulating factor (GM-CSF;

sargramostim). Other examples include interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-10 (IL-10), interleukin-12 (IL-12), interleukin-15 (IL-15), IL-2 superkine, chemokine (C-C motif) ligand 21 (CCL21), anti-CD3 and anti-CD28.

[00188] In other embodiments, the bioactive agent may be an immunomodulatory agent, that in one embodiment may be immunostimulatory and in another embodiment may be immunosuppressive. In some embodiments, an immunomodulatory compound may be a cytokine, chemokine, or complement component that enhances expression of immune system accessory or adhesion molecules, their receptors, or combinations thereof. In some embodiments, the immunomodulating agent include interleukins, for example interleukins 1 to 15, interferons alpha, beta or gamma, tumour necrosis factor, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), chemokines such as neutrophil activating protein (NAP), macrophage chemoattractant and activating factor (MCAF), RANTES, macrophage inflammatory peptides MIP-Ia and MIP-Ib, complement components, or combinations thereof. In other embodiments, the immunomodulating agent is an agent that stimulates expression, or enhances expression of OX40, OX40L (gp34), lymphotactin, CD40, CD40L, B7.1, B7.2, TRAP, ICAM-1, 2 or 3, cytokine receptors, or combination thereof.

[00189] As noted herein, when any bioactive compound is used for treating a cancer or tumor, whether a radionuclide, toxin, immunostimulatory or any other agent related to cancer therapy, it may be referred to as an anticancer bioactive agent.

[00190] An anticancer bioactive agent conjugated to the phage may be a tumor-targeting enzyme such as one that depletes amino acids required by the cancer for survival. A non-limiting example of a tumor-targeting enzyme includes L-asparaginase. In another embodiment, the phage-bound enzyme may catalyze the release of an anticancer agent from a prodrug (e.g., a glucuronide prodrug) or other toxic precursor administered to the patient, thus concentrating the anticancer activity at the tumor cell.

[00191] An anticancer agent conjugated to the phage may be a radiolabeled conjugate to an alpha-particle emitters, wherein binding of the phage delivers radiation to the tumor. Non-limiting examples of an alpha-particle emitter includes actinium-225-labeled trastuzumab and lutetium-177-labeled PSMA.

[00192] An anticancer bioactive agent conjugate to the phage may be a selective cell-death inducer that can impair cellular functions. Non-limiting examples of selective cell-death inducers include bortezomib and carfilzomib.

[00193] An anticancer bioactive agent may be a compound that acts through immune-mediated cytotoxicity or antibody-dependent cell-mediated cytotoxicity (ADCC) when bound to cell surfaces, such as a CD38 targeting antibody, or a SLAMF7 targeting antibody. Non-limiting examples of such agents include daratumumab and elotuzumab.

[00194] An anticancer bioactive agent may be a ligand or growth factor trap, such as a trap for VEGF or HGF. Non-limiting examples include aflibercept and rilotumumab.

[00195] The foregoing examples of bioactive anticancer agents are exemplary and are not intending to be limiting.

Bioactive agent-phage conjugates

[00196] As described herein, a bioactive agent, such as an antibiotic, is covalently conjugated to the major capsid protein of a phage. Such conjugation, examples of which are described below, preserve the activity of the bioactive agent (e.g., the antibiotic activity of the antibiotic or the anticancer activity of the anticancer agent) after such conjugation, and thus may be referred to herein as a bioactive active agent (e.g., antibiotic). In some embodiments, up to about 99% of the conjugated bioactive agent is active; in other embodiments, up to about 95%, up to about 90%, up to about 99%, up to about 85%, up to about 80%, up to about 75%, up to about 70%, up to about 65%, up to about 60%, up to about 55%, or up to about 50% of the conjugate bioactive agent is active. In some embodiments, a lower percent active bound bioactive agent is provided. In some embodiments, up to about 99% of the conjugated antibiotic molecules are active; in other embodiments, up to about 95%, up to about 90%, up to about 99%, up to about 85%, up to about 80%, up to about 75%, up to about 70%, up to about 65%, up to about 60%, up to about 55%, or up to about 50% of the conjugate antibiotic molecules are active. In some embodiments, a lower percent active bound antibiotic is provided.

[00197] In some embodiments, the bioactive agent to be bound to the phage has a reactive group for such binding, e.g., amino, carboxyl, sulfhydryl). In some embodiments the bioactive

agent does not have such a reactive group but may be chemically modified to create one. Any such binding, modification, etc. allows the bioactive agent to be bound to the phage but allows the bound bioactive agent to retain activity. By way of non-limiting example, bacteriophage M13 has amino groups, carboxylic acid groups and phenol groups of tyrosine available for crosslinking to a bioactive agent. Sites on M13 for conjugation may be found in Allen et al., Expanding the chemical diversity of M13 bacteriophage, *Front. Microbiol* 07 August 2022, vol. 13. As noted herein, any of various methods may be used to conjugate a bioactive agent to a bacteriophage major coat protein, by direct conjugation, use of a linker or spacer, or modifying the bioactive agent and/or the phage to facilitate conjugation. In some embodiments, certain reactive amino acids may be blocked in order to achieve the bioactive agent conjugation to the desired amino acid or types of similar amino acids (e.g., amino groups, carboxylic acids). In some embodiments, such conjugation may utilize a spacer such as a polyethylene oxide (e.g., polyethylene glycol) polymer to provide distance between the bioactive agent and the bacteriophage. In other embodiments, the bacteriophage major capsid protein may be mutated to comprise an amino acid to facilitate cross-linking to the bioactive agent.

[00198] Conjugation of the bioactive agent may be achieved by any of known methods in the art. The conjugation to the capsid protein is via a reactive group on the capsid protein, such as a free amino and/or carboxyl groups. In one embodiment, a reactive moiety on the bioactive agent is conjugated to the capsid protein. Chemistry for such conjugation may comprise the direct conjugation of the bioactive agent to the capsid protein (e.g., by an amide bond or disulfide bond), or a bifunctional cross-linking agent may be used, that conjugates the same reactive groups on both moieties (e.g., a homobifunctional crosslinking agent) such as amine to amine or carboxyl to carboxyl, or may comprise different reactive groups on the two moieties (e.g., a heterobifunctional crosslinking agent) such as an amine (on one or the other) to a carboxylic acid (on the other). Non-limiting examples of direct conjugation include the use of a carbodiimide such as EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) to form an amide bond. Non-limiting examples of other carbodiimides, include 1-cyclohexyl-3-(2-morpholinyl)-(4-ethyl) carbodiimide (CMC) and 1-ethyl-3-(4-azonia-44-dimethylpentyl) carbodiimide. Other cross-linking reagents include cyanogen bromide, glutaraldehyde and succinic anhydride. Non-limiting examples of homobifunctional agents include including a homo-bifunctional aldehyde, a homo-bifunctional epoxide, a homo-bifunctional imido-ester,

a homo-bifunctional N-hydroxysuccinimide ester, a homo-bifunctional maleimide, a homo-bifunctional alkyl halide, a homo-bifunctional pyridyl disulfide, a homo-bifunctional aryl halide, a homo-bifunctional hydrazide, a homo-bifunctional diazonium derivative or a homo-bifunctional photoreactive compound. Non-limiting examples of heterobifunctional agents include compounds having an amine-reactive and a sulfhydryl-reactive group, compounds with an amine-reactive and a photoreactive group and compounds with a carbonyl-reactive and a sulfhydryl-reactive group.

[00199] In other examples, the homo-bifunctional cross-linking agents include the bifunctional N-hydroxysuccinimide esters dithiobis(succinimidylpropionate), disuccinimidyl suberate, and disuccinimidyl tartarate; the bifunctional imido-esters dimethyl adipimidate, dimethyl pimelimidate, and dimethyl suberimidate; the bifunctional sulfhydryl-reactive crosslinkers 1,4-di-[3'-(2'-pyridyldithio)propionamido]butane, bismaleimido-hexane, and bis-N-maleimido-1,8-octane; the bifunctional aryl halides 1,5-difluoro-2,4-dinitrobenzene and 4,4'-difluoro-3,3'-dinitrophenylsulfone; bifunctional photoreactive agents such as bis-[b-(4-azidosalicylamido)ethyl]disulfide; the bifunctional aldehydes formaldehyde, malondialdehyde, succinaldehyde, glutaraldehyde, and adipaldehyde; a bifunctional epoxide such as 1,4-butanediol diglycidyl ether; the bifunctional hydrazides adipic acid dihydrazide, carbohydrazide, and succinic acid dihydrazide; the bifunctional diazoniums o-tolidine, diazotized and bis-diazotized benzidine; the bifunctional alkylhalides N,N'-ethylene-bis(iodoacetamide), N,N'-hexamethylene-bis(iodoacetamide), N,N'-undecamethylene-bis(iodoacetamide), as well as benzylhalides and halomustards, such as a,a'-diiodo-p-xylene sulfonic acid and tri(2-chloroethyl)amine, respectively.

[00200] In other embodiments, hetero-bifunctional cross-linking agents used to link the bioactive agent to phage capsid proteins include, but are not limited to, SMCC (succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate), MBS (m-maleimido-benzoyl-N-hydroxysuccinimide ester), SIAB (N-succinimidyl(4-iodoacetate)aminobenzoate), SMPB (succinimidyl-4-(p-maleimidophenyl)butyrate), GMBS (N-(gamma-maleimidobutyryloxy)succinimide ester), MPBH (4-(4-N-maleimidophenyl) butyric acid hydrazide), M2C2H (4-(N-maleimidomethyl) cyclohexane-1-carboxyl-hydrazide), SMPT (succinimidyl-oxycarbonyl-a-methyl-a-(2-pyridyldithio)toluene), and SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate).

[00201] Other methods for binding the bioactive agent to the major capsid protein are embraced herein, such as using a disulfide bond.

[00202] As noted herein, phages may comprise more than one bioactive agent, each which may be simultaneously bound to phages, or bound separately, for example to load different amounts onto the phages, and/or conjugate to different reactive groups on the phage and bioactive agent.

Methods for Preparing Bioactive Agent Recombinant Phage Conjugates

[00203] The methods for preparing the targeted cell bioactive agent-conjugated bacteriophage and the recombinant expression of a binding moiety to the targeted cell are each described herein and some embodiments thereof described in the examples herein, which are merely illustrative and non-limiting.

[00204] **Recombinant phage.** As described herein throughout, methods are provided for producing recombinant phage comprising a minor capsid protein expressing a binding moiety to a target cell. In some embodiments, the binding moiety is selected from an antigen binding domain of an antibody, expressed to replace the N-terminal domain of a minor capsid protein such as M13 pIII. In other embodiments, a bacteriophage library may be prepared and target cell binding used to identify phages useful for the purposes herein. Methods for preparing such recombinant phages are ascertainable from the teaching herein and general methods known in the art. In some embodiments, the phagemid and helper phage vectors contain ampicillin and kanamycin selection markers, respectively. In some embodiments, phages may be prepared using a single phage vector containing an auxotrophic selection marker such as glyA, encoding serine hydroxymethyl transferase (SHMT) to replace antibiotic selection markers. Thus, in some embodiments, no antibiotic selection gene or genes are used the preparing the recombinant bacteriophages disclosed herein. In some embodiments, an auxotrophic selection gene is used. In some embodiments, a gene that expresses serine hydroxymethyltransferase is used as a selection marker.

[00205] **Conjugating the bioactive moiety.** The disclosure provides guidance on conjugating the one or more bioactive agents to the major capsid protein of the recombinant phage. Guidance on direct conjugation (e.g., forming a peptide bond between an amino group

on the phage or bioactive moiety with a carboxyl group on the other) is provided as well as non-limiting examples of homobifunctional and heterobifunctional agents that may be used. As noted herein, certain active groups on the phage may be blocked to allow the desired cross-linking chemistry to occur on unblocked groups; other strategies for forming the conjugates are fully embraced herein.

[00206] Methods for purification, quantification, and other production and process controls for the bioactive agent-recombinant phage conjugates will be evident from the teaching herein, as well as means to assess safety, stability and other features of a product intended for human use.

Methods of Use

[00207] The methods for preparing the targeted cell bioactive agent-conjugated bacteriophage and the recombinant expression of a binding moiety to the targeted cell are each described above in detail. The conjugated antibiotic-recombinant bacteriophage described above in detail, and exemplified in the Examples, may in certain embodiments be used for therapeutic treatments, for example, but not limited to treatment of microbial infections. The conjugated anticancer agent-recombinant bacteriophage as disclosed herein may be used to treat cancer. Other uses of such drug-conjugated targeted phages are described herein as non-limiting examples.

[00208] The conjugated antibiotic-bacteriophage may be used to treat microbial infections, such as Gram negative, Gram positive or fungal infections, with enhanced properties such as but not limited to reduced toxicity compared to the antibiotic alone.

[00209] The conjugated anticancer agent-bacteriophage may be used to treat cancer in a patient, such as a solid tumor. Such anticancer agents delivered specifically to a tumor by a bacteriophage as described herein reduces the toxicity of the anticancer agent if it were delivered as a free agent, such as systemically. The conjugated anticancer agent-bacteriophage reduced off-target effects of the agent and reduces adverse effects.

[00210] The infectious microorganism targeted recombinant bacteriophage may be used to detect the presence of microorganisms or their cell wall components, such as Gram negative bacteria, Gram positive bacteria, fungi, and their components such as LPS, peptidoglycan or

fungal cell wall components. Such pathogenic microorganism-targeted bacteriophages may be used as a diagnostic agent (e.g., as conjugated to an imaging agent, a radionuclide, etc.) or used in combination with further reagents that, for example, bind to the bacteriophage (e.g., to the major capsid protein) for imaging or other purposes.

[00211] In some embodiments, disclosed herein is a method of treating a microbial such as a bacterial or fungal infection in a subject in need, said method comprising administering a phage comprising both a binding moiety to an infectious microorganism expressed on a minor capsid protein thereof, and an antibiotic that is active against the infectious microorganism bound to a major capsid protein thereof.

[00212] The cancer cell targeted recombinant bacteriophage may be used to detect the presence of tumors including metastatic or secondary tumors in a patient. Such cancer cell-targeted bacteriophages may be used as a diagnostic agent (e.g., as conjugated to an imaging agent, a radionuclide, etc.) or used in combination with further reagents that, for example, bind to the bacteriophage (e.g., to the major capsid protein) for imaging or other purposes.

[00213] In some embodiments, “treating” comprises therapeutic treatment including prophylactic or preventive measures, wherein the objective is to prevent or lessen the targeted pathologic condition or disorder, for example to treat, suppress, inhibit, prevent, reduce the incidence or severity of, delay the onset of, or reduce symptoms associated with a microbial, e.g., a bacterial or fungal, infection, or of cancer. Thus, in some embodiments, treating may include directly affecting or curing, suppressing, inhibiting, preventing, reducing the severity of, delaying the onset of, reducing symptoms associated with a microbial infection or cancer, or a combination thereof. Thus, in other embodiments, treating may include directly affecting or curing, suppressing, inhibiting, preventing, reducing the severity of, delaying the onset of, reducing symptoms associated with a microbial infection or cancer, or a combination thereof. Thus, in some embodiments, “treating,” “ameliorating,” and “alleviating” refer *inter alia* to delaying progression, expediting remission, inducing remission, augmenting remission, speeding recovery, increasing efficacy of or decreasing resistance to alternative therapeutics, or a combination thereof. In some embodiments, “preventing” refers, *inter alia*, to delaying the onset of symptoms, preventing relapse to a disease, decreasing the number or frequency of relapse episodes, increasing latency between symptomatic episodes, or a combination thereof. In some embodiments, “suppressing” or “inhibiting”, refers *inter alia* to reducing the

severity of symptoms, reducing the severity of an acute episode, reducing the number of symptoms, reducing the incidence of disease-related symptoms, reducing the latency of symptoms, ameliorating symptoms, reducing secondary symptoms, reducing secondary infections, reducing metastasis, reducing secondary tumors, prolonging patient survival, or a combination thereof.

[00214] In some embodiments, the antibiotic-recombinant bacteriophage conjugate may be utilized as a means of “purifying” a biological sample. It may be utilized as a means of “preventing,” “inhibiting,” or “reducing the incidence of” infection of one or more cells, tissues, organs, or organisms brought into contact with the biological sample or to which the biological sample is administered. Applications of these embodiments include, but are not limited to, exposure of the antibiotic-recombinant bacteriophage conjugate to a pharmaceutical prior to its use; administration of the conjugate to a biopharmaceutical prior to its use; administration of the conjugate to a food or a beverage prior to its use; administration of the conjugate to an organism to be used for a food or a beverage; administration of the conjugate to an organism, wherein a portion or product thereof is to be used for a food or a beverage; administration of the conjugate for decontamination agents for cleaning medical equipment, surfaces etc.; or administration of the conjugate as an additive to handwashing products such as solid or liquid soaps; administration of the conjugate as an additive for aesthetic products such as cosmetics; or administration of the conjugate as an additive in mouthwash or other oral products for killing microorganisms in the mouth or on teeth. In other embodiments, the conjugate may be a component provided on the surface of surgical or medical equipment, catheters, implantable prostheses, and other products to prevent or reduce microorganism growth or contamination. These uses are merely exemplary of the variety of applications of the conjugates described herein. Moreover, for any of the foregoing uses, the antibiotic-bacteriophage conjugate (without the infectious microorganism targeting recombinant minor capsid protein) may be similarly used.

[00215] In some embodiments, the bacterial infection comprises a systemic bacterial infection (e.g., throughout an organism) or disease, including but not limited to sepsis. In some embodiments, the bacterial infection comprises a localized bacterial infection or bacterial disease, e.g., primarily confined or limited to a particular region of the organism, e.g., to a particular organ (e.g., a bladder, an eye, a heart, etc.), to a particular system (e.g., the urogenital

system, the central nervous system, the gastrointestinal system, etc.), or to a particular section of the organism (e.g., a finger, a toe, a hand, a foot, etc.). In some embodiments, the bacterial infection was previously a localized bacterial infection or bacterial disease, which has subsequently spread beyond the initial localized infected or diseased area. In some embodiments, the bacterial infection comprises a systemic bacterial infection or bacterial disease having systemic symptoms. In some embodiments, the bacterial infection comprises a systemic bacterial infection or bacterial disease having localized symptoms.

[00216] In some embodiments, the fungal infection comprises a systemic or disseminated fungal infection (e.g., throughout an organism) or disease, including but not limited to sepsis. In some embodiments, the fungal infection comprises a localized fungal infection or fungal disease, e.g., primarily confined or limited to a particular region of the organism, e.g., to a particular organ (e.g., a lung, etc.), to a particular system (e.g., the respiratory system, etc.), or to a particular section of the organism (e.g., a finger, a toe, a hand, a foot, etc.). In some embodiments, the fungal infection was previously a localized fungal infection or fungal disease, which has subsequently spread (disseminated) beyond the initial localized infected or diseased area. In some embodiments, the fungal infection comprises a systemic fungal infection or fungal disease having systemic symptoms. In some embodiments, the fungal infection comprises a systemic fungal infection or fungal disease having localized symptoms.

[00217] Treating cancer comprises administering a bacteriophage as described herein to a patient having cancer, such as but not limited to a solid tumor as disclosed herein. In some embodiments, at the site, T cells are stimulated to target the tumor, suspected tumor, or resected tumor, and the induction of Tregs is suppressed. In some embodiments, T cells are the targets of the bacteriophages disclosed herein, for example, targeting an immune checkpoint inhibitor to T cells to suppress their activity. In other embodiments, bacteriophages as described herein may be used to suppress regulatory T cells, that normally suppress an immune response to a tumor.

[00218] In some embodiments, said treating reduces the size of the tumor, eliminates the tumor, slows the growth or regrowth of the tumor, slows the growth or regrowth of a secondary tumor, or prolongs survival of said subject or any combination thereof.

[00219] In some embodiments, administration comprises injection and/or infusion directly

into an organism. In some embodiments, administration comprises injection and/or infusion directly into an area of localized infection in an organism. In some embodiments, administration comprises injection and/or infusion into an area adjacent to an area of localized infection in an organism. A bioactive agent-recombinant bacteriophage conjugate for injection may be in the form of a pharmaceutical composition formulated as a sterile injectable solution. In some embodiments, the bioactive agent-recombinant bacteriophage conjugate may be provided in the form of a topical ointment, mouthwash, dentifrice, pill, tablet, capsule, wafer, dry powder taken orally, nebulized and inhaled, or administered topically to surfaces of invasive medical devices.

[00220] In some embodiments, injection comprises subcutaneous injection. In some embodiments, the bioactive agent-recombinant bacteriophage conjugate is administered via a guided catheter, e.g., comprising a UV or IR light source.

[00221] In any of the foregoing examples as well as other embodiments herein, the conjugate may be the antibiotic-bacteriophage conjugate without the microorganism targeting moiety recombinantly expressed on a minor capsid protein. In any of the foregoing embodiments, the conjugate may be the targeted bacteriophage without the bioactive agent bound thereto.

[00222] In some embodiments, “administering” comprises bringing a cell, a tissue, an organ, or an organism into contact with the bioactive agent-recombinant bacteriophage conjugate or with a composition (e.g., a pharmaceutical composition) comprising the bioactive agent-recombinant bacteriophage conjugate. In some embodiments, a cell, a tissue, an organ, or an organism is perfused with a composition comprising the conjugate. In some embodiments, a cell, a tissue, an organ, or an organism is grown in media comprising the conjugate. Other methods will occur to those of ordinary skill in the art.

[00223] A “focus of interest” a “localized environment,” or a “localized site” comprises a site in which the disease, reaction, infection, injury, or other medical condition is specific to one part or area of the body; in which a symptom or condition of the medical condition is specific to one part or area of the body; or in which treatment is desired for one part or area of the body (even if the disease, reaction, infection, injury, or other medical condition affects other parts or areas of the body or the body as a whole).

[00224] In some embodiments, methods disclosed herein treat an infection or infectious disease. In some embodiments, provided herein is a method of treating a bacterial or fungal infection or bacterial or fungal disease, in a subject in need thereof, comprising the step of administering to said subject an antibiotic-recombinant bacteriophage conjugate, as described above. In some embodiments, provided herein is a method of treating a bacterial or fungal infection or bacterial or fungal disease, in a cell, tissue, organ, or organism in need thereof, comprising the step of administering to said cell, tissue, organ, or organism an antibiotic-recombinant bacteriophage conjugate, as described above.

[00225] In some embodiments, methods disclosed herein treat cancer or another dysproliferative disease. In some embodiments, provided herein is a method of treating a cancer or other dysproliferative disease in a subject in need thereof, comprising the step of administering to said subject an anticancer agent-recombinant bacteriophage conjugate, as described above. In some embodiments, provided herein is a method of treating a cancer or dysproliferative disease in a subject in need thereof, comprising the step of administering to said cell, tissue, organ, or organism an anticancer agent-recombinant bacteriophage conjugate, as described herein.

[00226] In some embodiments, methods disclosed herein treat a systemic infection or infectious disease. In some embodiments, provided herein is a method of treating a systemic bacterial or fungal infection or bacterial or fungal disease, in a subject in need thereof, comprising the step of administering to said subject an antibiotic-recombinant bacteriophage conjugate, as described above. In some embodiments, provided herein is a method of treating a systemic bacterial or fungal infection or bacterial or fungal disease, in a cell, tissue, organ, or organism in need thereof, comprising the step of administering to said cell, tissue, organ, or organism an antibiotic-recombinant bacteriophage conjugate, as described above.

[00227] In some embodiments, methods disclosed herein treat a focus of interest of a localized site of a bacterial or fungal infection or bacterial or fungal disease, a localized site of an injury or other damage, a transplant or other surgical site potentially exposed to an infection or infectious disease, or a symptom of any of these, or a combination of any of these. In some embodiments, disclosed herein is a method of treating a focus of interest of a localized infection or an infectious disease, an injury or other damage, a transplant or other surgical site, or a symptom of any of these, or a combination of any of these, in a subject in need thereof,

comprising the step of administering to said subject an antibiotic-recombinant bacteriophage conjugate, as described above, having a localized infection or an infectious disease, an injury or other damage, a transplant or other surgical site. In some embodiments, disclosed herein is a method of treating a focus of interest of a localized infection or an infectious disease, an injury or other damage, a transplant or other surgical site, or a symptom of any of these, or a combination of any of these, in a cell, tissue, organ, or organism in need thereof, comprising the step of administering to said cell, tissue, organ, or organism an antibiotic-recombinant bacteriophage conjugate, as described above, having a localized infection or an infectious disease, an injury or other damage, a transplant or other surgical site, or a symptom of any of these, or a combination of any of these. In some embodiments, the step of administering comprises administering into or adjacent to a site of a localized infection or an infectious disease, an injury or other damage, or a transplant or other surgical site, or symptom of any of these, or combination of any of these.

[00228] In some embodiments, a systemic bacterial or fungal infection or bacterial or fungal disease, a localized bacterial or fungal infection or bacterial or fungal disease, an injury or other damage, a transplant or other surgical site, or a symptom of any of these, or a combination of any of these, comprises a localized site of an infection or infectious disease, a localized site of injury or damage, a transplant or other surgical site, or another site comprising one or more localized symptoms of any of these, or a combination of any of these.

[00229] In some embodiments, an infection or an infectious disease includes, for example, but is not limited to, a fungal infection (e.g., aspergillus, coccidioidomycosis), a bacterial infection (e.g., methicillin-resistant *Staphylococcus aureus*, localized skin infections, abscesses, necrotizing fasciitis, pulmonary bacterial infections [e.g., pneumonia], bacterial meningitis, bacterial sinus infections), a viral infection (e.g., varicella-zoster/herpes zoster [shingles], *Herpes simplex I* [e.g., cold sores/fever blisters], *Herpes simplex II* [genital herpes], human papilloma virus [e.g., cervical cancer, throat cancer, esophageal cancer, mouse cancer], Epstein-Barr virus [e.g., nasopharyngeal cancer], encephalitis viruses [e.g., brain inflammation], or hepatitis viruses [e.g., liver disease; hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatitis E, hepatitis F, hepatitis G]), or a parasitic infection (e.g., a bloodborne infection of malaria, babesia, etc., or an area infected by scabies, Chagas, *Hypoderma tarandi*, amoebae, roundworm, or *Toxoplasma gondii*).

[00230] In some embodiments, the injury or other damage includes, for example, but is not limited to traumatic injury (e.g., resulting from an accident or violence, surgical injury, acute peritonitis), or chronic injury (e.g., ulcers, chronic wounds, lung conditions, chronic bowel inflammatory diseases), such as with the possibility of bacterial or fungal infection. In some embodiments, the localized site of injury comprises a muscular-skeletal injury, a neurological injury, a respiratory injury, a gastrointestinal injury, an eye or ear injury, an internal or external wound, a localized abscess, an area of mucosa that is affected (e.g., conjunctiva, sinuses, esophagus), or an area of skin that is affected (e.g., infection). In some embodiments, the transplant or other surgical site includes, for example, but is not limited to, the site and/or its local environment or surroundings of an organ, corneal, skin, limb, face, or other transplant, or a surgical site and/or its local environment or surroundings, for, e.g., but not limited to, treatment of surgical trauma, treatment of a condition related to the transplant or surgery, or prevention of infection.

[00231] In some embodiments, administration comprises injection and/or infusion directly into a cell, tissue, organ, or organism. In some embodiments, administration comprises injection and/or infusion directly into a localized site of a localized infection or an infectious disease, an injury or other damage, a transplant or other surgical site, or a combination thereof. In some embodiments, administration comprises injection and/or infusion adjacent to a localized site of a localized infection or an infectious disease, an injury or other damage, a transplant or other surgical site, or a combination thereof. The antibiotic-recombinant bacteriophage conjugate may be administered in the form of a pharmaceutical composition formulated as a sterile injectable solution. In some embodiments, injection comprises subcutaneous injection.

[00232] In some embodiments, the antibiotic-recombinant bacteriophage conjugate is administered in a composition comprising one or more of a buffer, excipient, carrier, vehicle. In some embodiments, preservatives or other components may be included. In some embodiments, a liquid formulation comprising the antibiotic-recombinant bacteriophage conjugate is provided, which may be stored frozen, refrigerated, or is stable at room temperature. In some embodiments the antibiotic-recombinant bacteriophage conjugate is provided as a lyophilized powder for reconstitution before administration, e.g., with sterile water or saline, or addition to an intravenous fluid bag or drip.

Cancer

[00233] In some embodiments described herein are uses of bacteriophages for treating cancer or other tumors. In some embodiments, the bacteriophages are systemically administered and are targeted in vivo to the cancer by means of the binding moiety. In some embodiments, the bacteriophages may be infused at the site of or near the tumor.

[00234] The conjugated anticancer agent-recombinant bacteriophage may be used to treat cancers with properties such as but not limited to reduced toxicity compared to the anticancer agent alone. As noted herein, a cytotoxic or immunomodulatory agent may be targeted to the tumor or other cells in the tumor microenvironment by the bacteriophages. In other embodiments, an enzyme bound to the bacteriophage targeted to the tumor acts on a prodrug anticancer agent administered systemically or locally to the patient, where the enzyme cleaves the prodrug at the tumor to the cytotoxic agent.

Other uses

[00235] The infectious microorganism targeted recombinant bacteriophage may be used to detect the presence of microorganisms or their cell wall components, such as Gram negative bacteria, Gram positive bacteria, fungi, and their components such as LPS, peptidoglycan or fungal cell wall components. Such pathogenic microorganism-targeted bacteriophages may be used as a diagnostic agent (e.g., as conjugated to an imaging agent, a radionuclide, etc.) or used in combination with further reagents that, for example, bind to the bacteriophage (e.g., to the major capsid protein) for imaging or other purposes.

[00236] In some embodiments, disclosed herein is a method of treating a microbial such as a bacterial or fungal infection in a subject in need, said method comprising administering a phage comprising both a binding moiety to an infectious microorganism expressed on a minor capsid protein thereof, and an antibiotic that is active against the infectious microorganism bound to a major capsid protein thereof.

[00237] In some embodiments, a parasitic infection is e.g., a bloodborne infection of malaria, babesia, etc., or an area infected by scabies, Chagas, *Hypoderma tarandi*, amoebae, roundworm, or *Toxoplasma gondii*.

[00238] In some embodiments, administration comprises injection and/or infusion directly into an organism. In some embodiments, administration comprises injection and/or infusion directly into an area of localized infection in an organism. In some embodiments, administration comprises injection and/or infusion into an area adjacent to an area of localized infection in an organism. An antibiotic-recombinant bacteriophage conjugate for injection may be in the form of a pharmaceutical composition formulated as a sterile injectable solution. In some embodiments, the antibiotic-recombinant bacteriophage conjugate may be provided in the form of a topical ointment, mouthwash, dentifrice, pill, tablet, capsule, wafer, dry powder taken orally, nebulized and inhaled, or administered topically to surfaces of invasive medical devices.

[00239] In some embodiments, injection comprises subcutaneous injection. In some embodiments, the antibiotic-recombinant bacteriophage conjugate is administered via a guided catheter.

[00240] In any of the foregoing examples as well as other embodiments herein, the conjugate may be the antibiotic-bacteriophage conjugate without the microorganism targeting moiety recombinantly expressed on a minor capsid protein.

[00241] In some embodiments, “administering” comprises bringing a cell, a tissue, an organ, or an organism into contact with the antibiotic-recombinant bacteriophage conjugate or with a composition (e.g., a pharmaceutical composition) comprising the bioactive agent-recombinant bacteriophage conjugate. In some embodiments, a cell, a tissue, an organ, or an organism is perfused with a composition comprising the conjugate. In some embodiments, a cell, a tissue, an organ, or an organism is grown in media comprising the conjugate. Other methods will occur to those of ordinary skill in the art.

[00242] A “focus of interest” a “localized environment,” or a “localized site” comprises a site in which the disease, reaction, infection, injury, or other medical condition is specific to one part or area of the body; in which a symptom or condition of the medical condition is specific to one part or area of the body; or in which treatment is desired for one part or area of the body (even if the disease, reaction, infection, injury, or other medical condition affects other parts or areas of the body or the body as a whole).

[00243] In some embodiments, methods disclosed herein treat an infection or infectious disease. In some embodiments, provided herein is a method of treating a bacterial or fungal infection or bacterial or fungal disease, in a subject in need thereof, comprising the step of administering to said subject an antibiotic-recombinant bacteriophage conjugate, as described above. In some embodiments, provided herein is a method of treating a bacterial or fungal infection or bacterial or fungal disease, in a cell, tissue, organ, or organism in need thereof, comprising the step of administering to said cell, tissue, organ, or organism an antibiotic-recombinant bacteriophage conjugate, as described above.

[00244] In some embodiments, methods disclosed herein treat a systemic infection or infectious disease. In some embodiments, provided herein is a method of treating a systemic bacterial or fungal infection or bacterial or fungal disease, in a subject in need thereof, comprising the step of administering to said subject an antibiotic-recombinant bacteriophage conjugate, as described above. In some embodiments, provided herein is a method of treating a systemic bacterial or fungal infection or bacterial or fungal disease, in a cell, tissue, organ, or organism in need thereof, comprising the step of administering to said cell, tissue, organ, or organism an antibiotic-recombinant bacteriophage conjugate, as described above.

[00245] In some embodiments, methods disclosed herein treat a focus of interest of a localized site of a bacterial or fungal infection or bacterial or fungal disease, a localized site of an injury or other damage, a transplant or other surgical site potentially exposed to an infection or infectious disease, or a symptom of any of these, or a combination of any of these. In some embodiments, disclosed herein is a method of treating a focus of interest of a localized infection or an infectious disease, an injury or other damage, a transplant or other surgical site, or a symptom of any of these, or a combination of any of these, in a subject in need thereof, comprising the step of administering to said subject an antibiotic-recombinant bacteriophage conjugate, as described above, having a localized infection or an infectious disease, an injury or other damage, a transplant or other surgical site. In some embodiments, disclosed herein is a method of treating a focus of interest of a localized infection or an infectious disease, an injury or other damage, a transplant or other surgical site, or a symptom of any of these, or a combination of any of these, in a cell, tissue, organ, or organism in need thereof, comprising the step of administering to said cell, tissue, organ, or organism an antibiotic-recombinant bacteriophage conjugate, as described above, having a localized infection or an infectious

disease, an injury or other damage, a transplant or other surgical site, or a symptom of any of these, or a combination of any of these. In some embodiments, the step of administering comprises administering into or adjacent to a site of a localized infection or an infectious disease, an injury or other damage, or a transplant or other surgical site, or symptom of any of these, or combination of any of these.

[00246] In some embodiments, a systemic bacterial or fungal infection or bacterial or fungal disease, a localized bacterial or fungal infection or bacterial or fungal disease, an injury or other damage, a transplant or other surgical site, or a symptom of any of these, or a combination of any of these, comprises a localized site of an infection or infectious disease, a localized site of injury or damage, a transplant or other surgical site, or another site comprising one or more localized symptoms of any of these, or a combination of any of these.

[00247] In some embodiments, an infection or an infectious disease includes, for example, but is not limited to, a fungal infection (e.g., aspergillus, coccidioidomycosis), a bacterial infection (e.g., methicillin-resistant *Staphylococcus aureus*, localized skin infections, abscesses, necrotizing fasciitis, pulmonary bacterial infections [e.g., pneumonia], bacterial meningitis, bacterial sinus infections), a viral infection (e.g., varicella-zoster/herpes zoster [shingles], *Herpes simplex I* [e.g., cold sores/fever blisters], *Herpes simplex II* [genital herpes], human papilloma virus [e.g., cervical cancer, throat cancer, esophageal cancer, mouse cancer], Epstein-Barr virus [e.g., nasopharyngeal cancer], encephalitis viruses [e.g., brain inflammation], or hepatitis viruses [e.g., liver disease; hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatitis E, hepatitis F, hepatitis G]), or a parasitic infection (e.g., a bloodborne infection of malaria, babesia, etc., or an area infected by scabies, Chagas, *Hypoderma tarandi*, amoebae, roundworm, or *Toxoplasma gondii*).

[00248] In some embodiments, the injury or other damage includes, for example, but is not limited to traumatic injury (e.g., resulting from an accident or violence, surgical injury, acute peritonitis), or chronic injury (e.g., ulcers, chronic wounds, lung conditions, chronic bowel inflammatory diseases), such as with the possibility of bacterial or fungal infection. In some embodiments, the localized site of injury comprises a muscular-skeletal injury, a neurological injury, a respiratory injury, a gastrointestinal injury, an eye or ear injury, an internal or external wound, a localized abscess, an area of mucosa that is affected (e.g., conjunctiva, sinuses, esophagus), or an area of skin that is affected (e.g., infection). In some embodiments, the

transplant or other surgical site includes, for example, but is not limited to, the site and/or its local environment or surroundings of an organ, corneal, skin, limb, face, or other transplant, or a surgical site and/or its local environment or surroundings, for, e.g., but not limited to, treatment of surgical trauma, treatment of a condition related to the transplant or surgery, or prevention of infection.

[00249] In some embodiments, administration comprises injection and/or infusion directly into a cell, tissue, organ, or organism. In some embodiments, administration comprises injection and/or infusion directly into a localized site of a localized infection or an infectious disease, an injury or other damage, a transplant or other surgical site, or a combination thereof. In some embodiments, administration comprises injection and/or infusion adjacent to a localized site of a localized infection or an infectious disease, an injury or other damage, a transplant or other surgical site, or a combination thereof. The antibiotic-recombinant bacteriophage conjugate may be administered in the form of a pharmaceutical composition formulated as a sterile injectable solution. In some embodiments, injection comprises subcutaneous injection.

[00250] In some embodiments, the antibiotic-recombinant bacteriophage conjugate is administered in a composition comprising one or more of a buffer, excipient, carrier, vehicle. In some embodiments, preservatives or other components may be included. In some embodiments, a liquid formulation comprising the antibiotic-recombinant bacteriophage conjugate is provided, which may be stored frozen, refrigerated, or is stable at room temperature. In some embodiments the antibiotic-recombinant bacteriophage conjugate is provided as a lyophilized powder for reconstitution before administration, e.g., with sterile water or saline, or addition to an intravenous fluid bag or drip.

[00251] Thus, in some embodiments, phages can be engineered to specifically target tumor cells or the tumor microenvironment. Conjugating cytotoxic drugs, such as chemotherapy agents, to these phages could enable targeted delivery directly to cancer cells, reducing systemic toxicity.

[00252] In some embodiments, phages can be used to deliver immune-modulating drugs or proteins directly to tumors, potentially enhancing the efficacy of existing immunotherapies such as but not limited to immune checkpoint inhibitors.

[00253] In some embodiments, phages could be engineered to target specific immune cells, such as T cells or B cells, that are involved in autoimmune diseases such as but not limited to lupus, rheumatoid arthritis, and multiple sclerosis. Conjugating these phages with immunosuppressive drugs could enable more precise modulation of the immune system.

[00254] In some embodiments, phages could be engineered to target specific immune cells, such as T cells, B cells or NK cells, that are involved in the immune response to an infectious agent or pathological cell such as a cancer cell. Conjugating these phages with immunostimulatory drugs could enable more precise enhancement of the immune response.

[00255] In some embodiments, phage-drug conjugates could be applied topically to target specific skin conditions, such as bacterial infections, inflammatory skin diseases, or even skin cancers. This targeted approach could reduce side effects and improve efficacy.

[00256] In some embodiments, phage-drug conjugates could target and break down biofilms on medical devices like catheters, implants, and stents, which are often resistant to traditional antibiotics.

[00257] In some embodiments, phage-drug conjugates could be used to treat chronic wounds where biofilms play a major role, improving healing rates and reducing infection.

[00258] In some embodiments, phage-drug conjugates could be designed to treat bacterial infections or inflammatory conditions of the eye, delivering drugs specifically to the affected tissues with minimal systemic exposure.

[00259] In some embodiments, phage-drug conjugates could be used to selectively target harmful bacteria in the gut without disrupting the overall microbiome, potentially treating conditions like inflammatory bowel disease (IBD) or *Clostridium difficile* infections.

[00260] In some embodiments, phage-drug conjugates could target drug-resistant bacterial infections in the lungs of cystic fibrosis patients. The conjugate could deliver antibiotics directly to the bacteria in the thick mucus, improving outcomes where traditional treatments struggle.

[00261] In some embodiments, phage-drug conjugates could target bacterial infections or inflammation in COPD patients, reducing exacerbations and improving lung function.

[00262] In some embodiments, phage-drug conjugates could be used to treat bone infections, particularly those caused by drug-resistant bacteria. Targeted delivery could reduce the need for long-term antibiotic therapy and lower the risk of side effects.

[00263] In some embodiments, in the case of prosthetic joint infections, phage-drug conjugates could target the bacteria directly on the implant surface, helping to avoid or minimize the need for revision surgery.

[00264] In some embodiments, phage-drug conjugates could be used to target drug-resistant bacteria in the urinary tract, offering a targeted approach to treating recurrent or chronic UTIs, especially in patients with indwelling catheters.

[00265] In some embodiments, for chronic bacterial prostatitis, phage-drug conjugates could target the infection site within the prostate, potentially providing a more effective treatment than systemic antibiotics.

[00266] In some embodiments, phage-drug conjugates could be designed to target bacteria or viruses responsible for sexually transmitted infections (STIs), delivering antiviral or antibacterial drugs directly to infected tissues.

[00267] In some embodiments, phage-drug conjugates could target inflammatory cells or pathogens contributing to these conditions, providing a more localized and effective treatment.

[00268] In some embodiments, phage-drug conjugates could be engineered to target specific leukemia cells, delivering chemotherapeutic agents or other drugs directly to the cancerous cells while sparing healthy cells, reducing side effects.

[00269] In some embodiments, phage-drug conjugates could be developed to target pathogens in the bloodstream, offering a targeted approach to treating septicemia or other blood-borne infections.

[00270] In some embodiments, phage-drug conjugates could be particularly useful in treating pediatric patients with bacterial infections that are resistant to standard antibiotics, ensuring that treatments are both effective and safe for young patients.

[00271] In some embodiments, phage-drug conjugates could target bacterial biofilms in the sinuses, which are often resistant to traditional antibiotics, providing a more effective treatment for chronic sinusitis.

[00272] In some embodiments, phage-drug conjugates could be used to treat recurrent or chronic ear infections, especially those caused by antibiotic-resistant bacteria.

[00273] In some embodiments, phage-drug conjugates could target pathogenic bacteria in the gums, offering a more effective treatment for periodontitis and potentially preventing tooth loss.

[00274] In some embodiments, phage-drug conjugates could treat bacterial infections in the oral cavity, particularly those resistant to standard antibiotic treatments, helping to manage conditions like oral thrush or bacterial endocarditis risk in at-risk patients.

[00275] In some embodiments, phage-drug conjugates could be used to treat bacterial infections in livestock, reducing the need for broad-spectrum antibiotics and helping to combat antibiotic resistance in agricultural settings.

[00276] In some embodiments, phage-drug conjugates could offer targeted treatments for infections in pets, such as skin infections, ear infections, or gastrointestinal infections, improving outcomes and reducing side effects.

[00277] In some embodiments, phage-drug conjugates could be developed to target specific biothreat agents, such as anthrax or plague, offering a targeted treatment option in the event of a bioterrorism attack.

[00278] In some embodiments, phage-drug conjugates could be rapidly developed to target emerging infectious diseases, providing a new tool for responding to pandemics with novel pathogens.

[00279] In some embodiments, phage-drug conjugates could be formulated to target and kill *Propionibacterium acnes*, the bacterium responsible for acne, delivering drugs directly to affected areas and reducing side effects associated with systemic treatments.

[00280] In some embodiments, phage-drug conjugates could be used to treat chronic wounds, such as diabetic ulcers or pressure sores, by targeting and eliminating bacterial infections that impede healing while also delivering growth factors or anti-inflammatory agents to promote tissue regeneration.

[00281] In some embodiments, phage-drug conjugates could be applied to burns to prevent infection and promote faster healing by targeting and eliminating opportunistic pathogens, such as *Pseudomonas aeruginosa*, which are common in burn wounds.

[00282] In some embodiments, phage-drug conjugates could be designed to target specific receptors on colorectal cancer cells, delivering chemotherapeutic agents directly to the tumor and minimizing damage to surrounding healthy tissues.

[00283] In some embodiments, phages could be engineered to target gastric cancer cells, with the conjugated drug inducing cell death or enhancing the immune response against the tumor.

[00284] In some embodiments, phage-drug conjugates could be developed to target and treat infections in the respiratory tract, such as those caused by drug-resistant bacteria in cases of pneumonia or bronchitis, delivering antibiotics or immune-stimulating agents directly to the mucosal surfaces.

[00285] In some embodiments, phage-drug conjugates could target pathogens or aberrant immune cells within the GALT, which is associated with conditions like inflammatory bowel disease (IBD) or certain gut infections.

[00286] In some embodiments, phage-drug conjugates could be applied to the surface of implantable medical devices, such as pacemakers, joint replacements, or dental implants, to prevent biofilm formation by delivering antimicrobial agents directly to any colonizing bacteria.

[00287] In some embodiments, phage-drug conjugates could be used as a preventive measure during or after surgery to target and kill bacteria that may cause infections around implants, such as in orthopedic or cardiac surgery.

[00288] In some embodiments, phage-drug conjugates could be designed to treat bacterial infections of the cornea, such as those caused by *Staphylococcus aureus* or *Pseudomonas aeruginosa*, delivering antibiotics directly to the site of infection to prevent vision loss.

[00289] In some embodiments, phage-drug conjugates could be designed to target and kill *Plasmodium* parasites within the bloodstream, delivering antimalarial drugs directly to infected red blood cells.

[00290] In some embodiments, phage-drug conjugates could target *Borrelia burgdorferi*, the bacteria responsible for Lyme disease, delivering antibiotics directly to infected tissues.

[00291] In some embodiments, phage-drug conjugates could be used to treat severe bacterial infections in newborns, particularly those caused by multidrug-resistant organisms, delivering antibiotics directly to the infection site and reducing the risk of systemic toxicity.

[00292] In some embodiments, phage-drug conjugates could be designed to target and treat bacterial infections in the lungs of children, especially in conditions like cystic fibrosis or bronchopulmonary dysplasia.

[00293] In some embodiments, phage-drug conjugates could be used to target and break down environmental pollutants, such as oil spills or chemical contaminants, by delivering enzymes or other bioactive agents directly to the site of contamination.

[00294] In some embodiments, phage-drug conjugates could target plant pathogens or pests, delivering biopesticides or antimicrobial agents directly to infected crops, reducing the need for chemical pesticides and improving crop yields.

[00295] In some embodiments, the disclosure is directed to a bioactive agent-bound bacteriophage, as described throughout herein, with the bioactive agent bound to a major capsid protein, but without the target cell specific minor capsid protein as described herein. Such bioactive-bound bacteriophages have various utilities as described herein, wherein targeting to specific pathogen or pathogenic cell is not required.

[00296] In some embodiments, the disclosure is directed to a bacteriophage with a recombinant minor capsid protein comprising a target cell binding moiety as described herein, wherein the bacteriophage does not have a bioactive agent bound to a major capsid protein as

described herein. Such target cell binding bacteriophages has various utilities herein, wherein targeted bacteriophages not carrying a bound bioactive agent is useful.

[00297] Thus, in some embodiments, the bioactive agent-conjugated recombinant bacteriophage comprises an anti-LPS scFab fused to the C-terminal domain of M13 g3p and polymyxin B conjugated to g8p. In some embodiments, anti-LPS scFab is fused to the C-terminal domain of M13 g3p and polymyxin E conjugated to g8p. In some embodiments, a pathogenic bacterium targeted receptor binding domain is fused to M13 g3p, and polymyxin B conjugated to g8p. In some embodiments, a pathogenic bacterium targeted receptor binding domain is fused to M13 g3p, and polymyxin E conjugated to g8p.

Pharmaceutical compositions

[00298] “Pharmaceutically acceptable carriers” include any excipient which is nontoxic to the cell, tissue, organ, organism or subject being exposed thereto at the dosages and concentrations employed. The pharmaceutical composition may include one or additional therapeutic agents.

[00299] Pharmaceutically acceptable carriers include solvents, dispersion media, buffers, coatings, antibacterial and antifungal agents, wetting agents, preservatives, buffers, chelating agents, antioxidants, isotonic agents and absorption delaying agents.

[00300] Pharmaceutically acceptable carriers include water; saline; phosphate buffered saline; dextrose; glycerol; alcohols such as ethanol and isopropanol; phosphate, citrate and other organic acids; ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; EDTA; salt forming counterions such as sodium; and/or nonionic surfactants such as TWEEN, polyethylene glycol (PEG), and PLURONICS; isotonic agents such as sugars, polyalcohols such as mannitol and sorbitol, and sodium chloride; as well as combinations thereof. Antibacterial and antifungal agents include parabens, chlorobutanol, phenol, ascorbic acid, and thimerosal.

[00301] This disclosure further embraces pharmaceutical compositions and pharmaceutical products of the bioactive agent-recombinant phage conjugates disclosed herein. The terms “pharmaceutical composition” and “pharmaceutical product” means a composition suitable for pharmaceutical use (pharmaceutical composition), as defined herein. In some embodiments, as used herein, “pharmaceutical composition” also refers to therapeutically effective amounts of the disclosed bioactive agent-recombinant phage conjugates together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvant and/or carriers.

[00302] In practice, the bioactive agent-recombinant phage conjugates can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration. E.g., oral or parenteral (including intravenous). Thus, the pharmaceutical compositions of the present invention can be presented as discrete units suitable for oral administration such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient. Further, the compositions can be presented as a powder, as granules, as a solution, as a suspension in an aqueous liquid, as a non-aqueous liquid, as an oil in water emulsion, or as a water in oil liquid emulsion.

[00303] In addition to the common dosage forms set out above, bioactive agent-recombinant phage conjugates may also be administered by controlled release means and/or delivery devices. The compositions may be prepared by any of the methods of pharmacy. In general, such methods include a step of bringing into association the active ingredient with the carrier that constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the active ingredient with liquid carriers or finely divided solid carriers or both. The product can then be conveniently shaped into the desired presentation.

[00304] Solid medicinal forms, including lyophilized formulations, can comprise inert components and carrier substances, such as calcium carbonate, calcium phosphate, sodium phosphate, lactose, starch, mannitol, alginates, gelatine, guar gum, magnesium stearate, aluminum stearate, methyl cellulose, talc, highly dispersed silicic acids, silicone oil, higher molecular weight fatty acids, (such as stearic acid), gelatine, agar agar or vegetable or animal fats and oils, or solid high molecular weight polymers (such as polyethylene glycol);

preparations which are suitable for oral administration can comprise additional flavorings and/or sweetening agents, if desired.

[00305] Liquid medicinal forms can be sterilized and/or, where appropriate, comprise auxiliary substances, such as preservatives, stabilizers, wetting agents, penetrating agents, emulsifiers, spreading agents, solubilizers, salts, sugars or sugar alcohols for regulating the osmotic pressure or for buffering, and/or viscosity regulators. Examples of such additives are tartrate and citrate buffers, ethanol and sequestering agents (such as ethylenediaminetetraacetic acid and its nontoxic salts). High molecular weight polymers, such as liquid polyethylene oxides, microcrystalline celluloses, carboxymethyl celluloses, polyvinylpyrrolidones, dextrans or gelatine, are suitable for regulating the viscosity. Examples of solid carrier substances are starch, lactose, mannitol, methyl cellulose, talc, highly dispersed silicic acids, high molecular weight fatty acids (such as stearic acid), gelatine, agar agar, calcium phosphate, magnesium stearate, animal and vegetable fats, and solid high molecular weight polymers, such as polyethylene glycol.

[00306] Oily suspensions for parenteral or topical applications can be vegetable synthetic or semisynthetic oils, such as liquid fatty acid esters having in each case from 8 to 22 C atoms in the fatty acid chains, for example palmitic acid, lauric acid, tridecanoic acid, margaric acid, stearic acid, arachidic acid, myristic acid, behenic acid, pentadecanoic acid, linoleic acid, elaidic acid, brasicid acid, erucic acid or oleic acid, which are esterified with monohydric to trihydric alcohols having from 1 to 6 C atoms, such as methanol, ethanol, propanol, butanol, pentanol or their isomers, glycol or glycerol. Examples of such fatty acid esters are commercially available miglyols, isopropyl myristate, isopropyl palmitate, isopropyl stearate, PEG 6-capric acid, caprylic/capric acid esters of saturated fatty alcohols, polyoxyethylene glycerol trioleates, ethyl oleate, waxy fatty acid esters, such as artificial ducktail gland fat, coconut fatty acid isopropyl ester, oleyl oleate, decyl oleate, ethyl lactate, dibutyl phthalate, diisopropyl adipate, polyol fatty acid esters, inter alia. Silicone oils of differing viscosity, or fatty alcohols, such as isotridecyl alcohol, 2-octyldodecanol, cetylstearyl alcohol or oleyl alcohol, or fatty acids, such as oleic acid, are also suitable. It is furthermore possible to use vegetable oils, such as castor oil, almond oil, olive oil, sesame oil, cotton seed oil, groundnut oil or soybean oil.

[00307] Suitable solvents, gelatinizing agents and solubilizers are water or water miscible solvents. Examples of suitable substances are alcohols, such as ethanol or isopropyl alcohol, benzyl alcohol, 2-octyldodecanol, polyethylene glycols, phthalates, adipates, propylene glycol, glycerol, di- or tripropylene glycol, waxes, methyl cellosolve, cellosolve, esters, morpholines, dioxane, dimethyl sulphoxide, dimethylformamide, tetrahydrofuran, cyclohexanone, etc.

[00308] Mixtures of gelatinizing agents and film-forming agents are also perfectly possible. In this case, use is made, in particular, of ionic macromolecules such as sodium carboxymethyl cellulose, polyacrylic acid, polymethacrylic acid and their salts, sodium amylopectin semiglycolate, alginic acid or propylene glycol alginate as the sodium salt, gum arabic, xanthan gum, guar gum or carrageenan. The following can be used as additional formulation aids: glycerol, paraffin of differing viscosity, triethanolamine, collagen, allantoin and novantisolic acid. Use of surfactants, emulsifiers or wetting agents, for example of Na lauryl sulphate, fatty alcohol ether sulphates, di-Na-N-lauryl- β -iminodipropionate, polyethoxylated castor oil or sorbitan monooleate, sorbitan monostearate, polysorbates (e.g. Tween), cetyl alcohol, lecithin, glycerol monostearate, polyoxyethylene stearate, alkylphenol polyglycol ethers, cetyltrimethylammonium chloride or mono-/dialkylpolyglycol ether orthophosphoric acid monoethanolamine salts can also be required for the formulation. Stabilizers, such as montmorillonites or colloidal silicic acids, for stabilizing emulsions or preventing the breakdown of active substances such as antioxidants, for example tocopherols or butylhydroxyanisole, or preservatives, such as p-hydroxybenzoic acid esters, can likewise be used for preparing the desired formulations.

[00309] Preparations for parenteral administration can be present in separate dose unit forms, such as ampoules or vials. Use is preferably made of solutions of the active compound, preferably aqueous solution and, in particular, isotonic solutions and also suspensions. These injection forms can be made available as ready-to-use preparations or only be prepared directly before use, by mixing the active compound, for example the lyophilisate, where appropriate containing other solid carrier substances, with the desired solvent or suspending agent.

[00310] Intranasal preparations can be present as aqueous or oily solutions or as aqueous or oily suspensions. They can also be present as lyophilisates which are prepared before use using the suitable solvent or suspending agent.

[00311] Inhalable preparations can be provided such as powders, solutions or suspensions. Preferably, inhalable preparations are in the form of powders, e.g., as a mixture of the active ingredient with a suitable formulation aid such as lactose.

[00312] The preparations are produced, aliquoted and sealed under the customary antimicrobial and aseptic conditions.

[00313] Thus, the present disclosure further provides a pharmaceutical composition comprising an antibiotic-recombinant phage conjugates as described herein, and a pharmaceutically acceptable carrier.

[00314] The term “carrier” refers to any chemical entity that can be incorporated into a composition containing an active agent (e.g., a compound of formula (I)) without interfering with the stability and/or activity of the agent. In some embodiments, the term “carrier” refers to a pharmaceutically acceptable carrier. An exemplary carrier herein is water.

[00315] Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines). Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral. In some embodiments, the pharmaceutical composition is administered parenterally, paracancerally, transmucosally, transdermally, intramuscularly, intravenously, intradermally, subcutaneously, intraperitoneally, intraventricularly, intravaginally, intracranially and intratumorally.

[00316] As used herein, the terms “composition” and “pharmaceutical composition” may in some embodiments, be used interchangeably having all the same qualities and meanings. In some embodiments, disclosed herein is a pharmaceutical composition for the treatment of a cancer or tumor as described herein. In some embodiments, disclosed herein is a pharmaceutical composition for the treatment of a microbial infection or cancer. In some embodiments, disclosed herein are pharmaceutical compositions for the treatment of a

systemic bacterial or fungal infection or bacterial or fungal disease, a localized bacterial or fungal infection or bacterial or fungal disease, an injury or other damage, a transplant or other surgical site, or a symptom of any of these, or a combination of any of these. In some embodiments, disclosed herein are pharmaceutical compositions for the treatment of a cancer, a tumor, a primary tumor, a secondary tumor, a metastasis, or a combination of any of these.

[00317] In some embodiments, the antibiotic-recombinant bacteriophage conjugate is administered to an animal, a plant, a fungus, or a protist or to a cell, tissue, or organ derived from an animal, a plant, or a fungus. In some embodiments, the antibiotic-recombinant bacteriophage conjugate is administered to an animal. In some embodiments, the animal is a vertebrate. In some embodiments, the vertebrate is a homeothermic vertebrate (e.g., a mammal or a bird). In some embodiments, the mammal is a human mammal. In some embodiments, the mammal is a non-human mammal.

[00318] In some embodiments, the anticancer agent-recombinant bacteriophage conjugate is administered to an animal. In some embodiments, the animal is a vertebrate. In some embodiments, the vertebrate is a homeothermic vertebrate (e.g., a mammal or a bird). In some embodiments, the mammal is a human mammal. In some embodiments, the mammal is a non-human mammal.

[00319] As noted herein, the antibiotic-phage conjugates disclosed herein, without the microorganism targeting moiety on a minor capsid protein, may be used for many if not all of the embodiments described herein, where targeting to a specific type of microorganism is not necessarily required. As noted herein, such antibiotic-phage conjugates may be used on surfaces, in medical products, and other uses where the antibiotic properties of the conjugate is desired. In some embodiments, the conjugate is of lower toxicity to a eukaryotic cell, skin, oral mucosa, gums, gastrointestinal tract, or other living organism's surfaces or patient than the antibiotic alone, but the antibiotic-phage conjugate is toxic to the target microorganism.

[00320] As noted herein, the anticancer-phage conjugates disclosed herein, without the microorganism targeting moiety on a minor capsid protein, may be used for many if not all of the embodiments described herein, where targeting to a specific type of cancer is not necessarily required.

[00321] In some embodiments, a “cancer” is one of a group of diseases characterized by uncontrollable growth and having the ability to invade normal tissues and to metastasize to other parts of the body. Cancers have many causes, including, but not limited to, diet, alcohol consumption, tobacco use, environmental toxins, heredity, and viral infections. In most instances, multiple genetic changes are required for the development of a cancer cell. Progression from normal to cancerous cells involves a number of steps to produce typical characteristics of cancer including, e.g., cell growth and division in the absence of normal signals and/or continuous growth and division due to failure to respond to inhibitors thereof; loss of programmed cell death (apoptosis); unlimited numbers of cell divisions (in contrast to a finite number of divisions in normal cells); aberrant promotion of angiogenesis; and invasion of tissue and metastasis.

[00322] Unless otherwise indicated, all numbers expressing quantities, ratios, and numerical properties of ingredients, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about”. All parts, percentages, ratios, etc. herein are by weight unless indicated otherwise.

[00323] As used herein, the singular forms “a” or “an” or “the” are used interchangeably and intended to include the plural forms as well and fall within each meaning, unless expressly stated otherwise or unless the context clearly dictates otherwise. For example, the term “a compound” or “at least one compound” may include a plurality of compounds, including mixtures thereof.

[00324] Also as used herein, “at least one” is intended to mean “one or more” of the listed elements. Singular word forms are intended to include plural word forms and are likewise used herein interchangeably where appropriate and fall within each meaning, unless expressly stated otherwise. Except where noted otherwise, capitalized and non-capitalized forms of all terms fall within each meaning.

[00325] “Consisting of” shall thus mean excluding more than traces of other elements. The skilled artisan would appreciate that while, in some embodiments the term “comprising” is used, such a term may be replaced by the term “consisting of”, wherein such a replacement would narrow the scope of inclusion of elements not specifically recited. The terms

“comprises”, “comprising”, “includes”, “including”, “having” and their conjugates encompass “including but not limited to”.

[00326] The term “about” or “approximately” means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined. In some embodiments, the term “about” refers to a deviance of between 0.0001-5% from the indicated number or range of numbers. In some embodiments, the term “about” refers to a deviance of between 1-10% from the indicated number or range of numbers. In some embodiments, the term “about” refers to a deviance of up to 25% from the indicated number or range of numbers. In some embodiments, the term “about” refers to $\pm 10\%$.

[00327] Throughout this application, various embodiments may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of certain embodiments. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[00328] Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases “ranging/ranges between” a first indicate number and a second indicate number and “ranging/ranges from” a first indicate number “to” a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

[00329] Any patent, patent application publication, or scientific publication, cited herein, is incorporated by reference herein in its entirety.

[00330] The following examples are presented in order to more fully illustrate some embodiments disclosed herein. They should, in no way be construed, however, as limiting the

broad scope of the disclosure. One skilled in the art can readily devise many variations and modifications of the principles disclosed herein without departing from the scope of the disclosure.

EXAMPLES

Example 1: Production of Recombinant Bacteriophage and Methods of Use

[00331] A recombinant bacteriophage is designed to target and bind to lipopolysaccharide (LPS) on bacterial outer membranes. A sequence encoding a protein (e.g., an antigen binding region that targets and binds to LPS on an outer membrane of a bacterium) is cloned into a bacteriophage.

Example 2: Production of Recombinant M13 Bacteriophage Using a Single Vector and Methods of Use

[00332] A recombinant bacteriophage is designed to target and bind to lipopolysaccharide (LPS) on bacterial outer membranes. A sequence encoding a protein (e.g., an antigen binding region that targets and binds to LPS on an outer membrane of a bacterium) is cloned in recombinant protein into a phage vector, a large single vector containing some or all of the 10 genes of M13 phage with size around 8 kbp including antibiotic resistance selection markers.

Example 3: Production of Recombinant M13 Bacteriophage Using a Phagemid Vector and a Helper Phage and Methods of Use

[00333] A recombinant bacteriophage is designed to target and bind to lipopolysaccharide (LPS) on bacterial outer membranes. A phagemid vector is used in combination with helper phages to construct the recombinant bacteriophage. A phagemid vector is a smaller vector with size around 5 kbp that only encodes genes for receptor binding protein pIII and other elements of a vector, which is useful for cloning large fragments of proteins, such as an antibody Fab region (e.g., capable of targeting the LPS on the bacterial outer membrane). After cloning in gene of interest, the other gene is provided by a helper phage that contains all other gene but the pIII gene. Thus, *E. coli* host cells have all the genes to make new virions but only with the recombinant pIII genes.

Example 4: Production of Recombinant M13 Bacteriophage Comprising a Therapeutic Moiety and Methods of Use

[00334] A recombinant M13 bacteriophage is designed to target and bind to lipopolysaccharide (LPS), a common structure on Gram negative bacteria and a critical element forming their bacterial outer membranes of bacteria species including, but not limited to *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumonia*, *Burkholderia cepacia*, and other Gram negative bacteria. The recombinant protein is constructed using one of the methods described above.

[00335] Because these recombinant anti-LPS M13 phages are able to bind, but not infect or replicate, a therapeutic moiety (a substance for inhibiting viability, growth, maintenance, or proliferation of the bacteria) having a mechanism of action outside the bacterial cells and in some embodiments one that is facing challenges of dose limitation is selected. The therapeutic moiety is conjugated at a high concentration on the recombinant M13 phage major capsid proteins and is delivered to the bacteria cell surface via the protein binding of the phage receptor to the cell surface LPS. This method facilitates delivery of a high concentration of the therapeutic moiety to the cell surface of a bacterium.

Example 5: Production of Antibiotic-Recombinant Bacteriophage Conjugates and Methods of Use

[00336] A recombinant bacteriophage is designed to target and bind to lipopolysaccharide (LPS) on bacterial outer membranes. Because these recombinant anti-LPS phages are able to bind, but not infect or replicate, an antibiotic having a mechanism of action outside the bacterial cells and one that is facing challenges of dose limitation is selected. The antibiotic molecules are conjugated at a high concentration on the recombinant phage major capsid proteins and are delivered to the bacteria cell surface via the protein binding of the phage receptor to the cell surface LPS.

[00337] The antibiotic-recombinant phage conjugate can effectively lower the minimal inhibitory concentration of the antibiotic by an order of magnitude *in vitro* compared with the antibiotic in its free drug formulation. Through achieving a higher local concentration, bacteria that were previously resistant to the antibiotic become sensitive to the antibiotic-recombinant bacteriophage conjugate. Due to the order of magnitude change in the minimal inhibitory concentration of the antibiotic needed to achieve bactericidal activity on the same level, it is expected that the antibiotic-recombinant bacteriophage conjugate would have lower nephrotoxicity while achieving the same therapeutic efficacy. Moreover, antibiotics too toxic

or having other undesirable or undevelopable features may be used in this phage delivery system to therapeutic advantage.

Example 6: Production of a Gram Negative Antibiotic-Recombinant M13 Bacteriophage Conjugate and Methods of Use

[00338] A recombinant M13 bacteriophage is designed to target and bind to lipopolysaccharide (LPS), a common structure on Gram negative bacteria and a critical element forming their bacterial outer membranes of bacteria species including, but not limited to *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumonia*, *Burkholderia cepacia*, and more. Because these recombinant anti-LPS M13 phages are able to bind, but not infect or replicate, an antibiotic having a mechanism of action outside the bacterial cells and one that is facing challenges of dose limitation (e.g., polymyxin B or polymyxin E) is selected. The antibiotic molecules are conjugated at a high concentration on the recombinant M13 phage major capsid proteins and are delivered to the bacteria cell surface via the protein binding of the phage receptor to the cell surface LPS.

[00339] The antibiotic-recombinant M13 phage conjugate can effectively lower the minimal inhibitory concentration of the antibiotic by an order of magnitude in vitro compared with the antibiotic in its free drug formulation. Through achieving a higher local concentration, bacteria that were previously resistant to the antibiotic become sensitive to the antibiotic-recombinant bacteriophage conjugate. Due to the order of magnitude change in the minimal inhibitory concentration of the antibiotic needed to achieve bactericidal activity on the same level, it is expected that the antibiotic-recombinant M13 bacteriophage conjugate would have lower nephrotoxicity while achieving the same therapeutic efficacy.

Example 7: Production of a Polymyxin B-Recombinant M13 Bacteriophage Conjugate and Methods of Use

[00340] A recombinant M13 bacteriophage is designed to target and bind to lipopolysaccharide (LPS), a common structure on Gram negative bacteria and a critical element forming their bacterial outer membranes of bacteria species including, but not limited to *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumonia*, *Burkholderia cepacia*, and more. Because these recombinant anti-LPS M13 phages are able to bind, but not infect or replicate, polymyxin B (PolyB), which has a mechanism of action outside the bacterial cells and one that is facing challenges of dose

limitation, is selected as an antibiotic. The PolyB molecules are conjugated at a high concentration on the recombinant M13 phage major capsid proteins to yield a polymyxin B-recombinant M13 phage (PMB-Phage), retaining the binding capability of the phage and the antimicrobial activity of the PolyB, and are delivered to the bacteria cell surface via the protein binding of the recombinant M13 phage receptor to the bacteria cell surface LPS.

[00341] As shown in the schematic diagram in **FIGURE 1B**, PolyB molecules, each composed of a peptide ring (bound proximal to phage) and a bacterial cell lysing fatty acid tail (distal to phage) that is able to lyse bacterial surfaces, are conjugated to M13 bacteriophage major capsid proteins, through the formation of an amide bond. The conjugated molecules are delivered to the proximity of target bacteria cell through the recombinant M13 phage binding to a bacterial cell (**FIGURE 1A**, a non-limiting example of an *E. coli* cell) through the Fab domain of the anti-LPS antibody (between cylindrical portion and bacterial cell) expressed on the recombinant M13 phage minor capsid proteins. (The schematic is not drawn to scale.)

[00342] The PMB-Phage conjugate can effectively lower the minimal inhibitory concentration and the minimal bactericidal concentration of the PolyB by an order of magnitude *in vitro* compared with PolyB in its free drug formulation. Through achieving a higher local concentration, bacteria that were previously resistant to PolyB become sensitive to the PMB-Phage conjugate. Due to the order of magnitude change in the minimal inhibitory concentration of PolyB needed to achieve bactericidal activity on the same level, it is expected that the PMB-Phage conjugate would have lower nephrotoxicity while achieving the same therapeutic efficacy.

Example 8: Production of a Polymyxin E-Recombinant M13 Bacteriophage Conjugate and Methods of Use

[00343] A recombinant M13 bacteriophage is designed to target and bind to lipopolysaccharide (LPS), a common structure on Gram negative bacteria and a critical element forming their bacterial outer membranes of bacteria species including, but not limited to *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumonia*, *Burkholderia cepacia*, and more. Because these recombinant anti-LPS M13 phages are able to bind, but not infect or replicate, polymyxin E (PolyE; Colistin), which has a mechanism of action outside the bacterial cells and one that is facing challenges of dose limitation, is selected as an antibiotic. The PolyE molecules are conjugated at a high

concentration on the recombinant M13 phage major capsid proteins to yield a polymyxin B-recombinant M13 phage (PolyE-Phage) and are delivered to the bacteria cell surface via the protein binding of the recombinant M13 phage receptor to the bacteria cell surface LPS. The PolyE molecules are conjugated to the recombinant M13 phage major capsid proteins in a manner similar to that shown for PolyB molecules.

[00344] The PolyE-Phage conjugate can effectively lower the minimal inhibitory concentration of the PolyE by an order of magnitude *in vitro* compared with PolyE in its free drug formulation. Through achieving a higher local concentration, bacteria that were previously resistant to PolyE become sensitive to the PolyE-Phage conjugate. Due to the order of magnitude change in the minimal inhibitory concentration of PolyE needed to achieve bactericidal activity on the same level, it is expected that the PolyE-Phage conjugate would have lower nephrotoxicity while achieving the same therapeutic efficacy.

Example 9: Production of Recombinant M13 Bacteriophage Comprising a Fab Region for Targeting and Binding LPS and Methods of Use

[00345] A recombinant M13 bacteriophage was designed to target and bind to lipopolysaccharide (LPS), a common structure on Gram negative bacteria and a critical element forming their bacterial outer membranes of bacteria species including, but not limited to *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Burkholderia cepacia*, and more.

[00346] A phagemid vector was used in combination with helper phages to construct the recombinant bacteriophage. A phagemid vector is a smaller vector with size around 5 kbp that only encodes genes for receptor binding protein pIII and other elements of a vector, which is useful for cloning large fragments of proteins, such as an antibody Fab region (e.g., capable of targeting the LPS on the bacterial outer membrane). For example, the phagemid vector is generated from pADL-10b (Antibody Design Lab) through site directed mutagenesis to mutated the first S amino acid on the glycine rich linker connecting N2 and C terminus domain of M13 G3P into a T amino acid. The recombinant gene is inserted through NotI and AgeI restriction site. After cloning in gene of interest, the other gene was provided by a helper phage that contains all other gene but the pIII gene. Thus, *E. coli* host cells had all the genes to make new virions but only with the recombinant pIII genes.

[00347] The fragment antigen-binding region (Fab region, the region on an antibody that binds to its antigen) of antibody WN1 222-5, sequence obtained from protein data bank (PDB ID:3V0V), was cloned into a phagemid vector. Fabs with either the light chain at the N-terminus and heavy chain at the C-terminus ("LLH", SEQ ID NO:1; FIGURE 2), or the heavy chain at the N-terminus and the light chain at the C-terminus ("HLL", SEQ ID NO:3; FIGURE 2) were prepared. The antibody Fab region was cloned in replacement of the pIII protein N terminus domain instead of on top of it, which renders the recombinant M13 phage able to bind to LPS on bacteria cell surfaces but not be able to infect them or replicate because they are not able to enter the bacterial cells. The LLH-pIII is SEQ ID NO:2, and the HLL-pIII is SEQ ID NO:4. The phagemid sequence was confirmed by Sanger dideoxy sequencing. The phages were produced and purified, then tested for their binding affinity for a wide range of Gram negative bacteria, as described below.

Example 10: Production of Recombinant M13 Bacteriophage Comprising a Fab Region for Targeting and Binding LPS and Methods of Targeting and Binding to Various Gram Negative Bacteria

[00348] A recombinant M13 bacteriophage was designed to target and bind to lipopolysaccharide (LPS) by including an antigen-binding region (Fab region).

[00349] A phagemid vector was used in combination with helper phages to construct the recombinant bacteriophage. The fragment antigen-binding region (Fab region, the region on an antibody that binds to its antigen) of antibody WN1 222-5, sequence obtained from protein data bank (PDB ID:3V0V), was cloned into a phagemid vector, as described in **Example 9**. The phagemid sequence was confirmed by Sanger dideoxy sequencing, and the phages were produced and purified.

[00350] Using transmission electron microscopy, recombinant M13 bacteriophage attachment was shown to *E. coli* strain DH5-alpha (DH5 α), *E. coli* strain ATCC BAA 1161, *E. coli* Clonal Group A, Extraintestinal Pathogenic *E. coli* (ExPEC), and Virulence Factor Multidrug-Resistant Clonal Group A associated with urinary tract infection (UTI), *Pseudomonas aeruginosa* (*P. aeruginosa*) strain ATCC 25102, *Pseudomonas aeruginosa* (*P. aeruginosa*) strain ATCC 25102, polymyxin B- resistant *P. aeruginosa* strain PAK PMR B6, *Acinetobacter baumannii* (*A. baumannii*) ATCC strain 19606, *Burkholderia cepacia* (*B. cepacia*) ATCC strain 25416, *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC strain 700603,

and *Klebsiella pneumoniae* (*K. pneumoniae*) Clinical Strain A.

Example 11: Production of Recombinant M13 Bacteriophage Comprising a Fab Region for Targeting and Binding LPS and Binding Affinity Thereof to a Wide Range of Bacteria Species and Strains

[00351] A recombinant M13 bacteriophage was designed to target and bind to lipopolysaccharide (LPS) by including an antigen-binding region (Fab region).

[00352] A phagemid vector was used in combination with helper phages to construct the recombinant bacteriophage. The fragment antigen-binding region (Fab region, the region on an antibody that binds to its antigen) of antibody WN1 222-5, sequence obtained from protein data bank (PDB ID:3V0V), was cloned into a phagemid vector, as described in **Example 9**. The phagemid sequence was confirmed by Sanger dideoxy sequencing. The phages were produced and purified, then tested for their binding affinity for a wide range of Gram negative bacteria, as described below.

Example 12: Production of Gold-Labelled Recombinant M13 Bacteriophage Comprising a Fab Region for Targeting and Binding LPS and Methods of Targeting and Binding to E. coli strain DH5-alpha, E. coli strain ATCC BAA 1161, P. aeruginosa strain ATCC 25102, Polymyxin B Resistant P. aeruginosa strain PAK PMr B6, A. baumannii strain ATCC 19606, K. pneumoniae strain ATCC 700603, K. pneumoniae Clinical Strain A, and B. cepacia strain ATCC 25416

[00353] A recombinant M13 bacteriophage was designed to target and bind to lipopolysaccharide (LPS) by including an antigen-binding region (Fab region).

[00354] A phagemid vector was used in combination with helper phages to construct the recombinant bacteriophage. The fragment antigen-binding region (Fab region, the region on an antibody that binds to its antigen) of antibody WN1 222-5, sequence obtained from protein data bank (PDB ID:3V0V), was cloned into a phagemid vector, as described in **Example 9**. The phagemid sequence was confirmed by Sanger dideoxy sequencing, and the phages were produced and purified. Phage were labelled with gold using gold nanoparticles bound to an anti-M13 antibody.

[00355] The binding affinity of this recombinant phage was tested, and it demonstrated strong and specific binding to a wide range of Gram-negative bacteria including species previously not identified for antibody WN1 222-5: *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *A.*

baumanii, and *B. cepacia*. When quantifying the amount of phage DNA, it was determined that the amount of recombinant phage bound to these Gram-negative bacteria was orders of magnitude higher than the amount of phage binding to Gram-positive bacteria *Staphylococcus aureus*.

Example 13: Production of a Polymyxin B-Recombinant M13 Bacteriophage Conjugate Comprising a Fab Region for Targeting and Binding LPS

[00356] A recombinant M13 bacteriophage, which was designed to target LPS, as described above. Polymyxin B (PolyB) molecules were conjugated to bacteriophage major capsid proteins through amide bonds. A high concentration of polymyxin B molecules were conjugated on recombinant phage major capsid proteins to the recombinant M13 bacteriophage. The resulting polymyxin B-recombinant M13 phage conjugate (PMB-Phage) binds to the bacterial cell surface through phage receptor binding protein binding to cell surface LPS through the Fab domain of anti-LPS antibodies, expressed on phage minor capsid proteins, thereby delivering the PolyB antibiotic to the bacterial cell surface to utilize a mechanism of action outside of the cells and to circumvent dose limitations.

[00357] A recombinant M13 bacteriophage was designed to target and bind to lipopolysaccharide (LPS) by including an antigen-binding region (Fab region).

[00358] A phagemid vector was used in combination with helper phages to construct the recombinant bacteriophage. The fragment antigen-binding region (fab region, the region on an antibody that binds to its antigen) of antibody WN1 222-5, sequence obtained from protein data bank (PDB ID:3V0V), was cloned into a phagemid vector, as described in **Example 9**. The phagemid sequence was confirmed by Sanger dideoxy sequencing, and the phages were produced and purified.

[00359] Because these recombinant anti-LPS M13 phages are able to bind, but not infect or replicate, the payload (drug) to be delivered requires a mechanism of action that functions without being internalized. The polymyxin class of antibiotics is one of a number of antibiotic classes suitable for this purpose. Polymyxins are a class of natural antibiotics from *Paenibacillus polymyxa* that consist of a charged peptide ring covalently linked to a fatty acid. Among all members of the polymyxin family, Polymyxin B has been used as last line therapy out of nephrotoxicity concerns and rapid deterioration of kidney functions, because these

exhibit a significant inhibitory effect on Gram negative bacterial pathogens. Therefore, it has been used to target such pathogens resistant to frontline antibiotics such as β -lactams, aminoglycosides and fluoroquinolones.

[00360] Polymyxin B has a peptide ring fused to a fatty acid tail structure, and it differs from colistin (Polymyxin E) at the single amino acid residue in the peptide ring where D-Phe is replaced by D-Leu (**FIGURE 1B**). Polymyxin B's pentacationic peptide ring interacts with the negatively charged LPS electrostatically. Following binding, the hydrophobic fatty acid chain of polymyxin B interacts with the lipid A domain of LPS, which leads to displacement of membrane-stabilizing cations including Ca^{2+} and Mg^{2+} , which further lead to disruption of bacterial outer membrane permeability barrier, release of periplasmic proteins, membrane leakage and ultimately bacterial cell death.

[00361] Polymyxin B (PolyB), which has a mechanism of action outside the bacterial cells and one that is facing challenges of dose limitation, was selected as an antibiotic to be specifically delivered to the bacterial surfaces by replacing the nonspecific interaction between polymyxin B peptide ring and LPS with the recombinant M13 phage's specific interaction with LPS. In this design, the peptide ring of polymyxin B was covalently conjugated to the major capsid protein on recombinant M13 phage using EDC chemistry to link the primary amines on Dab amino acid on polymyxin B to the 3 solvent exposed carboxyl groups on phage major capsid protein. (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, EDAC or EDCI) is a water-soluble carbodiimide usually provided as the hydrochloride and generally used as a carboxyl activating agent for the coupling of primary amines to yield amide bonds. One potential concern was that polymyxin B might be unspecifically conjugated to the antibody Fab region expressed on receptor binding protein and interfere with phage binding affinity to their bacterial target. Nonspecific labeling of small molecules, including fluorescent dye FITC and N,N,N',N''-tetramethylrhodamine (TMR), reactive fluorescein, urease and drug molecules neomycin, does not prevent phages from binding to their targets. After this conjugation, the fatty acid tail would be facing away from the phage virions and be able to interact with LPS after phage binding to bring the drug molecules in proximity to bacterial cell surfaces. Comparing the PMB-Phage construct to polymyxin B in its free drug formulation, in addition to having the advantage of reducing nonspecific interaction between polymyxin B and mammalian cells, the PMB-Phage construct could potentially deliver higher

amount of active drug molecule to bacteria cell, because each phage virion could theoretically deliver approximately 8100 polymyxin B to each cell. Further advantages of PMB-Phage over PolyB in free drug formulation could be studied as follows: (1) Phages are commonly present in the human body and M13 phage has been shown to demonstrate no toxicity in vivo. (2) Because there is no free drug in the serum, an order of magnitude less of polymyxin B might be required to achieve the same inhibitory effect on bacteria, and less toxicity could be expected due to having less drug molecules in the system. (3) Due to toxicity issues, specifically nephrotoxicity, PolyB is currently being used as a last line of defense antibiotics not principally because of effectiveness against multiple resistant bacteria infection, but because its toxicity is such that infectious disease specialists would not prescribe it to patients due the risk of significant nephrotoxicity even after recovery from bacterial infection. Such principles are also applicable to other antibiotics and are fully embraced herein.

[00362] To maximize the efficacy of PolyB – Phage, an approach was selected that would conjugate as many polymyxin B molecules on each recombinant phage virion as possible so that as much polymyxin B as feasible could be delivered through binding of each phage virion to bacteria. However, one challenge to this approach is altering recombinant M13 phage surface properties. The peptide ring of polymyxin B was conjugated to the phage surface, leaving the fatty acid tail solvent exposed, neutralizing phage surface charges and reducing the stability of PMB-Phage in aqueous solution. Conditions were optimized for conjugating the lowest amount of polymyxin B (highest stability) to achieve sufficient efficacy. The molar ratio of polymyxin B to recombinant phages added in the EDC crosslinking reaction step was adjusted and optimized. A positive correlation was observed between minimal inhibitory concentration of PMB-Phage and this molar ratio at the beginning until a saturation point, and this saturation point was selected for synthesizing the PMB-Phage. After conjugation, .45 μ m filter was used to remove any large aggregates. The low degree of aggregation could provide PMB-Phage with a strategic advantage by delivering sufficient amounts of polymyxin B to one bacterium and by having any recombinant phage within an aggregate binding the cells, possibly enabling the PMB-Phage to remain in the system for a longer period of time to provide prolonged therapeutic effect, as discussed in later below.

[00363] After conjugation, a significant change of bacteriophage morphology was observed post-polymyxin B conjugation. The original phage had a smooth surface while the PMB-

Phage showed a zigzag morphology and higher tendency towards aggregation. This change may be due to conjugated polymyxin with exposed fatty acid tails altering the surface charge and creating local hydrophobic domains on phage surface.

[00364] The PolyB molecules were conjugated at a high concentration on the recombinant M13 phage major capsid proteins to yield a polymyxin B-recombinant M13 phage (PMB-Phage), retaining the binding capability of the phage and the antimicrobial activity of the PolyB, and are delivered to the bacteria cell surface via the protein binding of the recombinant M13 phage receptor to the bacteria cell surface LPS.

Example 14: Comparison of Treatment of Various Gram Negative Phage with Polymyxin B Versus the Polymyxin B-Recombinant M13 Bacteriophage Conjugate Demonstrated Inhibitory and Bactericidal Activity at a Lower Polymyxin B Concentration in vitro With Respect to the Polymyxin B-Recombinant M13 Bacteriophage Conjugate

[00365] A recombinant M13 bacteriophage was designed to target and bind to lipopolysaccharide (LPS) by including an antigen-binding region (Fab region).

[00366] A phagemid vector was used in combination with helper phages to construct the recombinant bacteriophage. The fragment antigen-binding region (fab region, the region on an antibody that binds to its antigen) of antibody WN1 222-5, sequence obtained from protein data bank (PDB ID:3V0V), was cloned into a phagemid vector, as described in **Example 9**. The phagemid sequence was confirmed by Sanger dideoxy sequencing, and the phages were produced and purified.

[00367] PolyB molecules were conjugated at a high concentration on the recombinant M13 phage major capsid proteins to yield a polymyxin B-recombinant M13 phage (PMB-Phage). The phages were produced and purified, then tested for their binding affinity for a wide range of Gram negative bacteria. The Gram negative bacteria species/strains included: *E. coli* (strains ATCC 25922, ATCC BAA 1161, and ATCC 700927); *P. aeruginosa* (strains ATCC 25102, Clinical Strain E, and PAKpmrB6); *K. pneumoniae* (strains ATCC 700603, Clinical Strain A, and Clinical Strain 326); *A. baumannii* (strain ATCC 19606); and *B. cepacia* (strain ATCC 25416).

[00368] Through conjugating polymyxin B to recombinant M13 phage specific to LPS, it

was found that the MIC of polymyxin B had been lowered to 0.027 µg/ml. This value was obtained by measuring the concentration of recombinant phages and taking an estimated maximum amount of polymyxin B (8100) that could be conjugated onto each phage virion. The PMB-Phage lowered the MIC by 1 to 2 orders of magnitude for different species and strains of Gram negative bacteria. *Burkholderia cepacia* is intrinsically resistant to polymyxin B, and it served as negative control, together with a polymyxin B-resistant *Klebsiella pneumoniae*. Both strains were resistant to PMB-Phage as well as polymyxin B as the active ingredients. However, one strain of *Pseudomonas aeruginosa* that was previously reported to be polymyxin resistant and a clinical *Klebsiella* strain A, which were resistant to polymyxin B (Miller *et al.* (2011) Antimicrob. Agents Chemother. 55(12): 5761-5769), have become sensitive again using PMB-Phage.

Example 15: Comparison of the Kinetics of Polymyxin B and the Polymyxin B-Recombinant M13 Bacteriophage Conjugate

[00369] A recombinant M13 bacteriophage was designed to target and bind to lipopolysaccharide (LPS) by including an antigen-binding region (Fab region).

[00370] A phagemid vector was used in combination with helper phages to construct the recombinant bacteriophage. The fragment antigen-binding region (fab region, the region on an antibody that binds to its antigen) of antibody WN1 222-5, sequence obtained from protein data bank (PDB ID:3V0V), was cloned into a phagemid vector, as described in **Example 9**. The phagemid sequence was confirmed by Sanger dideoxy sequencing, and the phages were produced and purified.

[00371] PolyB molecules were conjugated at a high concentration on the recombinant M13 phage major capsid proteins to yield a polymyxin B-recombinant M13 phage (PMB-Phage), as described in **Example 14**.

[00372] Similar to polymyxin B, PMB-Phage has a rapid acting mechanism of action with a >3-log reduction of viable cells within 1 hour of time. The results shown indicate similar decreases in the number of colony-forming units (CFU) (normalized) over time both for PolyB and for PMB-Phage.

Example 16: Comparison of Hemolysis Activity of Polymyxin B and the Polymyxin B-Recombinant M13 Bacteriophage Conjugate in Sheep Erythrocytes Demonstrated

Lack of Significant Mammalian Cell Hemolysis in vitro

[00373] A recombinant M13 bacteriophage was designed to target and bind to lipopolysaccharide (LPS) by including an antigen-binding region (Fab region).

[00374] A phagemid vector was used in combination with helper phages to construct the recombinant bacteriophage. The fragment antigen-binding region (Fab region, the region on an antibody that binds to its antigen) of antibody WN1 222-5, sequence obtained from protein data bank (PDB ID:3V0V), was cloned into a phagemid vector, as described in **Example 9**. The phagemid sequence was confirmed by Sanger dideoxy sequencing, and the phages were produced and purified.

[00375] PolyB molecules were conjugated at a high concentration on the recombinant M13 phage major capsid proteins to yield a polymyxin B-recombinant M13 phage (PMB-Phage), as described in **Example 14**. A 2% blood solution of washed red blood cells was prepared and incubated with PMB-phage. Any sample with less than 2% hemolysis activity is considered non-hemolytic. Hemolysis activity of polymyxin B has been previously reported to not have any hemolytic activity on red blood cells. Similarly, PMB-Phage at 3 different concentrations (1x to 32x concentration of PolyB – Phage MIC for *E. coli* strain 25922 and *E. coli* strain ATCC BAA 1161 used for *in vivo* experiments) are non-hemolytic.

Example 17: Comparison of Human Embryonic Kidney (HEK) 293 Cell Viability when Exposed to Polymyxin B or the Polymyxin B-Recombinant M13 Bacteriophage Conjugate Demonstrated Lack of Significant Mammalian Cell Toxicity in vitro

[00376] A recombinant M13 bacteriophage was designed to target and bind to lipopolysaccharide (LPS) by including an antigen-binding region (Fab region).

[00377] A phagemid vector was used in combination with helper phages to construct the recombinant bacteriophage. The fragment antigen-binding region (Fab region, the region on an antibody that binds to its antigen) of antibody WN1 222-5, sequence obtained from protein data bank (PDB ID:3V0V), was cloned into a phagemid vector, as described in **Example 9**. The phagemid sequence was confirmed by Sanger dideoxy sequencing, and the phages were produced and purified.

[00378] PolyB molecules were conjugated at a high concentration on the recombinant M13 phage major capsid proteins to yield a polymyxin B-recombinant M13 phage (PMB-Phage),

as described in **Example 14**.

[00379] PolyB treatment of a Gram negative bacterial infection can result in hepatotoxicity. An experiment was performed to compare the viability of human embryonic kidney (HEK) 293 cells exposed to various concentrations of either PolyB alone or PMB-Phage. HEK 293 cells were cultured in 24 wells plate in DMEM culture containing 32, 8 and 2 µg/ml of polymyxin B or 2e10, 1e10 and 5e9 virions/ml of PolyB - Phage for 5 days. On day 1, 3 and 5, cell viability was examined. The percentage cell viability was evaluated as the ratio between sample and negative control for which cells were incubated under standard conditions. A greater than 80% cell viability is considered non-toxic. Aside from the highest concentration of polymyxin B on day 1, all other samples demonstrated cell viability that is greater than 80%. Furthermore, no obvious morphological changes were observed for control samples, samples incubated with polymyxin B and samples incubated with PMB-Phage and cells possess intact structure, indicating intact cell viability.

Example 18: Comparison of the Effects on Body Weight by Dosing with Polymyxin B or the Polymyxin B-Recombinant M13 Bacteriophage Conjugate

[00380] A recombinant M13 bacteriophage was designed to target and bind to lipopolysaccharide (LPS) by including an antigen-binding region (Fab region).

[00381] A phagemid vector was used in combination with helper phages to construct the recombinant bacteriophage. The fragment antigen-binding region (Fab region, the region on an antibody that binds to its antigen) of antibody WN1 222-5, sequence obtained from protein data bank (PDB ID:3V0V), was cloned into a phagemid vector, as described in **Example 9**. The phagemid sequence was confirmed by Sanger dideoxy sequencing, and the phages were produced and purified.

[00382] PolyB molecules were conjugated at a high concentration on the recombinant M13 phage major capsid proteins to yield a polymyxin B-recombinant M13 phage (PMB-Phage), as described in **Example 14**.

[00383] An experiment was performed in mice to study the effects of drug dosing on body weight, as a percentage of body weight change, over time with data points taken daily on Days 0-7. No toxicity to animals leading to body weight change was shown.

Example 19: The Polymyxin B-Recombinant M13 Bacteriophage Conjugate is Predicted to Be Stable When Stored

[00384] A recombinant M13 bacteriophage was designed to target and bind to lipopolysaccharide (LPS) by including an antigen-binding region (Fab region).

[00385] A phagemid vector was used in combination with helper phages to construct the recombinant bacteriophage. The fragment antigen-binding region (Fab region, the region on an antibody that binds to its antigen) of antibody WN1 222-5, sequence obtained from protein data bank (PDB ID:3V0V), was cloned into a phagemid vector, as described in **Example 9**. The phagemid sequence was confirmed by Sanger dideoxy sequencing, and the phages were produced and purified.

[00386] PolyB molecules were conjugated at a high concentration on the recombinant M13 phage major capsid proteins to yield a polymyxin B-recombinant M13 phage (PMB-Phage), as described in **Example 14**.

[00387] M13 phages have been reported to have half-life greater than 120 days. Polymyxin B in 0.9% NaCl stored under 4 C has been reported to retain >75% stability in 7 days. Using the MIC of PMB-Phage for *E. coli* strain ATCC 25922 as indication of drug stability, PMB-Phage stored at 4°C in 1xPBS is predicted to be stable for at least 5 weeks. The potential extended stability adds value to PMB-Phage over polymyxin B free drug formulation.

Example 20: Comparison of the Effects of Exposure to Polymyxin B or the Polymyxin B-Recombinant M13 Bacteriophage Conjugate on Kidney and Liver Function Tests Demonstrates Lack of Apparent Toxicity in Mice in vivo

[00388] A recombinant M13 bacteriophage was designed to target and bind to lipopolysaccharide (LPS) by including an antigen-binding region (Fab region).

[00389] A phagemid vector was used in combination with helper phages to construct the recombinant bacteriophage. The fragment antigen-binding region (Fab region, the region on an antibody that binds to its antigen) of antibody WN1 222-5, sequence obtained from protein data bank (PDB ID:3V0V), was cloned into a phagemid vector, as described in **Example 9**. The phagemid sequence was confirmed by Sanger dideoxy sequencing, and the phages were produced and purified.

[00390] PolyB molecules were conjugated at a high concentration on the recombinant M13 phage major capsid proteins to yield a polymyxin B-recombinant M13 phage (PMB-Phage), as described in **Example 14**.

[00391] PolyB treatment of a Gram negative bacterial infection can result in hepatotoxicity. An experiment was performed to study the effects of drug dosing on standard liver and kidney function tests for levels of total protein, blood urea nitrogen (BUN), and creatinine. No kidney or liver toxicity was observed. In addition, histology was performed and shows no obvious toxicity at these concentrations for kidney, liver and spleen.

Example 21: Production of an LPS-targeted Recombinant M13 Bacteriophage Conjugated to a Gram Negative Antibiotic, and Methods of Use

[00392] **Materials.** All chemical reagents including polymyxin B sulfate were purchased from Millipore Sigma unless otherwise specified. All enzymes and buffers used for restriction enzyme digestion, PCR, cloning, and Gibson assembly were purchased from New England Biolabs. Helper phage CM13d3 and phagemid vector pADL-10b were purchased from Antibody Design Labs. All oligonucleotides used as primers were purchased from Integrated DNA Technologies and listed in Table 1.

Table 1. Primers for PCR or synthetic gene fragments.

Primer Name	5' - 3' Sequence
S239T Forward	GCTGGCGGCGGCACCGGTGGTGGTTCTGGT (SEQ ID NO:9)
S239T Reverse	CGTCTGACCTGCCTCAACCTCCTGTCAAT (SEQ ID NO:10)
pADL-sequencing Forward	AGTGAGCGGTACCCGATAAAAG (SEQ ID NO:11)
pADL-sequencing Reverse	ACCAGCGCCAAAGACATAAG (SEQ ID NO:12)
UpstreamInsert	GAAGTGCCATTCCGCCTGACCTGCGGCCGCTGGATTGTTATTA CTCGCGGCCAGCCGGCCATGGCT (SEQ ID NO:13)
Downstream Insert	GGCGGCGGCACCGGTAGGCTAGGTGGAGGCTCAGTG (SEQ ID NO:14)
qPCR-upstream	TTGGTGACGTTTCCGGCCTTGC (SEQ ID NO:15)
qPCR-downstream	TCACCGTCACCGACTTGAGCCA (SEQ ID NO:16)
OriF1longForward	GGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGG (SEQ ID NO:17)
OriF1longReverse	CCAAAAAAGTTGATTAGGGTGATGGTTCACGTA (SEQ ID NO:18)

[00393] **Bacterial cultures.** Strains used for this project and their sources are listed in Table 2. Bacterial strains were revived from glycerol stocks stored at -80°C by streaking on LB agar plates and culturing overnight in 37°C incubator. A single colony from each plate was inoculated into corresponding liquid culture and incubated at 37°C with shaking at 220 rpm. To ensure bacteria were fully revived from the dormant state, cells were subcultured >3 times by inoculating 100 µL of overnight culture into 10 mL fresh media prior to use.

Table 2: List of species, strains and sources for microorganisms used in this study.

Species	Strain name
<i>Escherichia coli</i>	ATCC BAA1161
<i>Escherichia coli</i>	ATCC 700927
<i>Escherichia coli</i>	DH5alpha
<i>Escherichia coli</i>	ATCC 25922
<i>Pseudomonas aeruginosa</i>	ATCC 25102
<i>Pseudomonas aeruginosa</i>	PAKpmrB6 ²⁶
<i>Pseudomonas aeruginosa</i>	Clinical Strain A

Species	Strain name
<i>Pseudomonas aeruginosa</i>	Clinical Strain B
<i>Pseudomonas aeruginosa</i>	Clinical Strain C
<i>Pseudomonas aeruginosa</i>	Clinical Strain E
<i>Pseudomonas aeruginosa</i>	Clinical Strain F
<i>Pseudomonas aeruginosa</i>	Clinical Strain G
<i>Pseudomonas aeruginosa</i>	Clinical Strain J
<i>Pseudomonas aeruginosa</i>	GFP-PAO1*
<i>Pseudomonas aeruginosa</i>	ATCC 27853
<i>Pseudomonas aeruginosa</i>	BCCM/LMG 27624
<i>Pseudomonas aeruginosa</i>	AR Bank #0246
<i>Pseudomonas aeruginosa</i>	AR Bank #0266
<i>Klebsiella pneumoniae</i>	ATCC 700603
<i>Klebsiella pneumoniae</i>	Clinical Strain A
<i>Klebsiella pneumoniae</i>	Clinical Strain 326
<i>Acinetobacter baumannii</i>	ATCC 19606
<i>Burkholderia cepacia</i>	ATCC 25416
<i>Staphylococcus aureus</i>	ATCC 25904

* Minns, M. S. et al. NLRP3 selectively drives IL-1 β secretion by *Pseudomonas aeruginosa* infected neutrophils and regulates corneal disease severity. Nat. Commun. 14, 5832 (2023)

[00394] **Anti-LPS phagemid constructs.** The sequences of the heavy chain and the light chain of the antibody WN1 222-5 Fab domain were obtained from RCSB Protein Data Bank entry 3V0V. Two single-chain constructs of the antibody Fab domain were designed with each of the following protein domain orders: light chain - linker - heavy chain (LLH) and heavy chain - linker - light chain (HLL), with the linker sequence being (GGGGS)₃ (SEQ ID NO: 19). The sequences were codon-optimized by the vendor (Twist Bioscience) for *E. coli* expression and obtained as synthetic DNA. PCR of each synthetic gene fragment was performed with two primers (UpstreamInsert and DownstreamInsert; Table 1), adding NotI and AgeI restriction sites for cloning.

[00395] Phagemid vector pADL-10b was mutated to insert an AgeI restriction site in the glycine-rich linker between the N2 domain and C-terminal domain of g3p, using S239T Forward and S239T Reverse primers (Table 1) and the Q5 Site Directed Mutagenesis Kit (New England Biolabs). The construct was transformed into Mix & Go! Competent Cells-TG1 (Zymo Research) and purified with QIAprep Spin Miniprep Kit (Qiagen), resulting in new phagemid vector pADL-10bS239T. The two synthetic gene inserts were digested with NotI and AgeI restriction enzymes, run on a 1% agarose gel, and purified using a Qiaquick gel extraction kit (Qiagen). The vector pADL-10bS239T was digested and purified in the

same way, and then ligated to each of the digested inserts using ElectroLigase, followed by transformation into Mix & Go! Competent Cells-TG1 (Zymo Research). Clones were confirmed by Sanger sequencing using sequencing primers pADL-sequencing Forward and pADL-sequencing Reverse.

Recombinant M13 Phage Receptor-Binding Protein Constructs Verified by Sanger Sequencing

Receptor Binding Protein HLL Construct with signal sequence

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ATGAAATACCTATTGCCTACGGCGGCCGCTGGATTGTTATTACTCGCGGCC
CAGCCGGCCATGGCTGAAGTCAAACCTTGTCTGAATCCGGTGGAGGGCTGGTA
CAGCCTGGTGGTTCCCTGAGTCTGTCTTGTGCTGCGTCTGGTTTTACTTTTTTC
AGATTATTACATGACTTGGGTGCGTCAGGCCCGCGAAAAGCCCCAGAATG
GCTCGCATTAAATAAGAAATAAACGGAATGGTGATACAGCTGAGTATTCCGC
GAGTGTGAAAGGCCGCTTTACGATTTCCCGTGATTATTCACGGAGCATTCT
GCATCTTCAAATGAATGCACTTCGCACTGAAGATTCGGCGACATACTATTG
TGTGAGACAGGGTCGCGGTTACACCTTAGATTATTGGGGCCAGGGTACCTC
AGTGACCGTTTCTTCCGCCAAAACGACCGCTCCATCAGTGTATCCTTTAGCT
CCGGTTTGTGGCGATACCACCGGGTCCTCGGTTACGCTGGGCTGCCTGGTA
AAAGGTTACTTTCCGGAACCCGTTACACTGACATGGAACAGCGGCTCCTTG
TCAAGTGGTGTTCACACATTTCCCGCCGTGTTACAGTCTGGTCTCTACACGC
TGAGTAGCTCTGTACCGTTACATCTTCTACATGGCCTAGCCAGTCTATTAC
CTGCAATGTAGCGCATCCTGCGTCAAGCACGAAAGTTGATAAGAAAATCG
AACCAGGTGGCGGGGGATCAGGAGGCGGTGGGTGAGGCGGGGAGGTTTCG
GACATACAAATGAATCAGAGCCCCGAGCTCTTTGTCTGCAAGCCTGGGCGAT
ACCATCAGTATTACTTGTGCGGCCAGTCAGAATATTAATATCTGGTTAAGCT
GGTACCAACAGAAGCCTGGTAATGTGCCTAACTTCTGATCTACAAAGCTA
GCAATCTTCATACAGGAGTACCGTCACGTTTCAGTGGCTCAGGAAGCGGCA
CCGACTTCACGTTGATTATCTCATCGCTCCAACCAGAGGATATTGCAACTTA
TTATTGCTTGCAGGGACAGAGCTATCCGCGGACATTTGGGGGTGGTACCAA
ACTGGAGATTAAACGAGGCGACGCCGACCGACTGTTTCAATCTTCCACC
GTCGAGTGAACAATTAACCTTCTGGGGGCGCGTCCGTCGTGTGTTTCCTCAA
CAACTTTTATCCCAAGGACATTAACGTAAAGTGGAAGATAGATGGGTCCGA
ACGTCAAAACGGAGTCTTGAACAGTTGGACTGACCAGGATTCAAAGGATA
GCACGTATAGCATGAGTTCGACGCTGACACTGACGAAAGACGAGTATGAG
CGACATAATTTCGTATACCTGCGAAGCAACACACAAAACCTCTACTAGTCCG
ATAGTTAAAAGTTTTAACCGTGGCGGGGGCACCGGTGGTGGTCTGGTGGC
GGCTCTGAGGGTGGCGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGCTCT
GAGGGTGGCGGTTCCGGTGGCGGCTCCGGTCCGGTGATTTTGATTATGAA
AAAATGGCAAACGCTAATAAGGGGGCTATGACCGAAAATGCCGATGAAAA
CGCGCTACAGTCTGACGCTAAAGGCAAACTTGATTCTGTGCTACTGATTA
CGGTGCTGCTATCGATGGTTTTCATTGGTGACGTTTCCGGCCTTGCTAATGGT
AATGGTGTCTACTGGTGATTTTGCTGGCTCTAATTCCCAAATGGCTCAAGTCG
GTGACGGTGATAATTCACCTTTAATGAATAATTTCCGTCAATATTTACCTTC
TTTGCTCAGTCGGTTGAATGTGCGCCTTATGTCTTTGGCGCTGGTAAACCA

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TATGAATTTTCTATTGATTGTGACAAAATAAACTTATTCCGTGGTGTCTTTG
CGTTTCTTTTATATGTTGCCACCTTTATGTATGTATTTTCGACGTTTGCTAAC
ATACTGCGTAATAAGGAGTCTTAA (SEQ ID NO:20)

Receptor Binding Protein LLH Construct with signal sequence

ATGAAATACCTATTGCCTACGGCGGCCGCTGGATTGTTATTACTCGCGGCC
CAGCCGGCCATGGCTGATATTCAAATGAATCAGTCCCCTTCTTCCTTATCAG
CCTCTTTGGGTGATACAATCAGCATTACATGCCGAGCGTCACAGAATATCA
ACATCTGGCTGTCATGGTATCAACAAAAGCCGGGTAATGTTCCAAAACGTG
TGATCTACAAAGCTTCCAATCTTCATACTGGTGTTCCTTCGCGTTTCAGTGG
TAGCGGATCGGGTACTGATTTTACACTGATTATTTTCGAGCCTGCAGCCTGA
AGACATTGCTACCTACTATTGCCTCCAAGGTCAGAGCTACCCGCGTACATT
CGGCGGCGGCACATAAACTTGAGATAAAACGGGGCGACGCCGCCCTACAG
TATCAATCTTTCCGCCCTCCAGTGAACAGCTTACGAGTGGCGGTGCATCAG
TTGTCTGTTTTTTGAACAACCTTCTACCCAAAGGATATAAACGTAAAGTGGA
AGATTGACGGTTCAGAAAGACAGAATGGCGTGCTGAACTCTTGACGGAC
CAGGATAGCAAAGACAGCACGTATAGTATGTCATCAACTTTAACACTCACT
AAAGATGAGTATGAAAGACATAACTCTTATACATGCGAAGCAACCCACAA
GACCAGTACCTCTCCGATTGTGAAAAGTTTCAATCGGGGAGGAGGGGGTA
GTGGAGGTGGTGGCTCCGGAGGGGGCGGCTCTGAAGTGAAGCTTGTAGAA
TCTGGTGGCGGCTTAGTGCAGCCCCGGGGTTCTCTCAGTCTCTCCTGTGCAG
CCTCAGGTTTTACGTTTAGCGATTATTATATGACATGGGTACGTCAGGCGCC
AGGGAAAGCCCCGAATGGCTGGCATTGATTTCGCAATAAACGAAATGGGG
ATACGGCCGAGTACAGTGCTTCAGTAAAAGGGCGGTTTACTATATCTCGTG
ATTATTCACGCAGCATTCTGCATCTTCAAATGAATGCACTGCGCACTGAAG
ATACGCTACCTATTATTGTGTCCGCCAGGGACGTGGGTACACCTTAGATT
ATTGGGTCAAGGCACCTCCGTTACCGTCAGTTCCGCAAAAACGACCGCGC
CGTCTGTGTATCCACTGGCGCCTGTTTGCAGTGACACGACGGGGAGTTCCG
TGACCTGGGTTGTTTGGTTAAAGGATATTTTCCCGAGCCTGTCACGCTGAC
CTGGAATAGCGGCAGCCTGTCGAGCGGGGTTTCATACATTTCCAGCTGTCTT
ACAGTCTGGCCTGTACACATTATCGAGCTCAGTTACTGTGACCTCGTCTACT
TGGCCGTGCGAGTCCATAACGTGTAACGTGGCGCACCCGGCGAGTAGCACC
AAAGTTGATAAAAAAATCGAGCCGGGCGGAGGAACCGGTGGTGGTTCTGG
TGGCGGCTCTGAGGGTGGCGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCG
GCTCTGAGGGTGGCGGTTCCGGTGGCGGCTCCGGTTCGGGTGATTTTGATT
ATGAAAAAATGGCAAACGCTAATAAGGGGGCTATGACCGAAAAATGCCGAT
GAAAACGCGCTACAGTCTGACGCTAAAGGCAAACCTTGATTCTGTGCTACT
GATTACGGTGCTGCTATCGATGGTTTTCATTGGTGACGTTTCCGGCCTTGCTA
ATGGTAATGGTGCTACTGGTGATTTTGCTGGCTCTAATTCCTCAAATGGCTCA
AGTCGGTGACGGTGATAATTCACCTTTAATGAATAATTTCCGTCAATATTTA
CCTTCTTTGCCTCAGTCGGTTGAATGTGCCCCCTATGTCTTTGGCGCTGGTA
AACCATATGAATTTTCTATTGATTGTGACAAAATAAACTTATTCCGTGGTGT
CTTTGCGTTTCTTTTATATGTTGCCACCTTTATGTATGTATTTTCGACGTTTG
CTAACATACTGCGTAATAAGGAGTCTTAA (SEQ ID NO:21)

[00396] **Production and purification of recombinant M13 phages.** Phage virions were produced following the helper phage CM13d3 user manual (Antibody Design Laboratories, San Diego, CA) as follows. *E. coli* TG1 cell strains with phagemid vectors of interest were inoculated in 5 mL of 2xYT media (100 µg/mL ampicillin, 1% w/v glucose) and grown overnight at 37°C under shaking at 250 rpm. The overnight culture was sub-cultured into 50 mL of 2xYT media (100 µg/mL ampicillin) and incubated with shaking until OD₆₀₀ reached 0.5. Helper phage CM13d3 was defrosted and vortexed. 1 µL helper phage was added per 1 mL of cell culture for superinfection. The culture was incubated for 1 h at 25°C, followed by addition of stock solutions to reach 50 µg/mL kanamycin and 200 µM IPTG concentration. The phages were produced by allowing the culture to grow for 8 h to overnight by incubating at 25°C and shaking at 200 rpm. The cell solution was spun down at 5,000x g for 5 minutes in a countertop centrifuge and the supernatant was collected. The produced phages were purified following a revised version of a published protocol (Reitinger, S., Petriv, O. I., Mehr, K., Hansen, C. L. & Withers, S. G. Purification and quantitation of bacteriophage M13 using desalting spin columns and digital PCR. *J. Virol. Methods* **185**, 171–174 (2012)). The supernatant containing phages was filtered through a 0.22 µm membrane filter to remove the majority of cell debris. The filtrate was then concentrated using a 30 kilodalton MWCO Amicon protein desalting column (Millipore Sigma) and washed with 1 x PBS buffer 5 times by resuspending the concentrate in 1 x PBS buffer after each round of centrifugation. The retentate was then collected and resuspended in a desired volume of 1 x PBS buffer for further characterization. The concentration of phage virions was determined by UV absorbance spectrometry at 269 nm and 320 nm, using the equation

$$\frac{\text{virions}}{\text{mL}} = \frac{(A_{269} - A_{320}) \times 6 \times 10^{16}}{\frac{\text{number of bases}}{\text{phage genome}}}$$

and confirmed by qPCR quantification as described below. For short-term storage (less than 12 weeks), phage solutions were stored at 4°C.

[00397] The nucleic acid and amino acid sequences of the Fab fragments LLH and HLL, as well as those of the expressed LLH-g3p and HLL-g3p, without signal sequences, are shown below.

LLH Fab SEQ ID NO:1	DIQMNQSPSSLSASLGDITISITCRASQNINIWLWSWYQQK PGNVPKLLIYKASNLHTGVPSRFGSGSGTDFTLISSLQ PEDIATYYCLQGQSYPRTFGGGKLEIKRGDAAPTVSIF PPSSEQLTSGGASVVCFLNNFYPKDINVWKIDGSERQ NGVLNSWTDQDSKDYSTYSMSSTLTTLTKDEYERHNSYT CEATHKTSTSPIVKSFNRRGGGGSGGGSGGGGSEVKLV ESGGGLVQPGGSLSLSCAASGFTFSDYYMTWVRQAPG KAPEWLALIRNKRNGDTAEYSASVKGRFTISRDRYSRIL HLQMNALRTEDSATYYCVRQGRGYTLDYWGQGTSTV VSSAKTTAPSVYPLAPVCGDITGSSVTLGCLVKGYFPEP VTLTWNSGSLSSGVHTFPAVLQSGLYTL SSSVTVTSSTWPSQSIITCNVAHPASSTKVDKKIE
LLH + G3P SEQ ID NO:2	DIQMNQSPSSLSASLGDITISITCRASQNINIWLWSWYQQKPG NVPKLLIYKASNLHTGVPSRFGSGSGTDFTLISSLQPED IATYYCLQGQSYPRTFGGGKLEIKRGDAAPTVSIFPPSSE QLTSGGASVVCFLNNFYPKDINVWKIDGSERQNGVLNS WTDQDSKDYSTYSMSSTLTTLTKDEYERHNSYTCEATHKTS TSPIVKSFNRRGGGGSGGGSGGGGSEVKLVESGGGLVQ PGGSLSLSCAASGFTFSDYYMTWVRQAPGKAPEWLALIR NKRNGDTAEYSASVKGRFTISRDRYSRILHLQMNALRTE DSATYYCVRQGRGYTLDYWGQGTSTVSSAKTTAPSV YPLAPVCGDITGSSVTLGCLVKGYFPEPVTLTWNSGSL SSGVHTFPAVLQSGLYTLSSSVTVTSSTWPSQSIITCNVA HPASSTKVDKKIEPGGGTGGGSGGGGSEGGGSEGGGSE GGSEGGGSGGGSGGDFDYKMANANKGAMTENADE NALQSDAKGKLDSVATDYGAADGFIGDVSGLANGNG ATGDFAGSNSQMAQVGDGDNPLMNNFRQYLPSLPQSV ECRPYVFGAGKPYEFSIDCDKINLFRGVFAFLLYVATFMY VFSTFANILRNKES
HLL Fab SEQ ID NO:3	EVKLVESGGGLVQPGGSLSLSCAASGFTFSDYYMTWVR QAPGKAPEWLALIRNKRNGDTAEYSASVKGRFTISRDRY SRILHLQMNALRTEDSATYYCVRQGRGYTLDYWGQ GTSVTVSSAKTTAPSVYPLAPVCGDITGSSVTLGCLVK GYFPEPVTLTWNSGSLSSGVHTFPAVLQSGLYTLSSSVTV TSTWPSQSIITCNVAHPASSTKVDKKIEPGGGGSGGGGSG GGGSDIQMNQSPSSLSASLGDITISITCRASQNINIWLWSWY QKPGNVPKLLIYKASNLHTGVPSRFGSGSGTDFTLISSL QPEDIATYYCLQGQSYPRTFGGGKLEIKRGDAAPTVSIF PPSSEQLTSGGASVVCFLNNFYPKDINVWKIDGSERQN GVLNSWTDQDSKDYSTYSMSSTLTTLTKDEYERHNSYTCE ATHKTSTSPIVKSFN
HLL + G3P SEQ ID NO:4	EVKLVESGGGLVQPGGSLSLSCAASGFTFSDYYMTWVR QAPGKAPEWLALIRNKRNGDTAEYSASVKGRFTISRDRY SRILHLQMNALRTEDSATYYCVRQGRGYTLDYWGQGT SVTVSSAKTTAPSVYPLAPVCGDITGSSVTLGCLVKGYF PEPVTLTWNSGSLSSGVHTFPAVLQSGLYTLSSSVTVTS TWPSQSIITCNVAHPASSTKVDKKIEPGGGGSGGGGSGGG GSDIQMNQSPSSLSASLGDITISITCRASQNINIWLWSWYQQ KPGNVPKLLIYKASNLHTGVPSRFGSGSGTDFTLISSLQPE DIATYYCLQGQSYPRTFGGGKLEIKRGDAAPTVSIFPPS

	SEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVL NSWTDQDSKDYSTYSMSSTLTLTKDEYERHNSYTCEATHK TSTSPIVKSFNRGGGTGGGSGGGSEGGGSEGGGSEGGGS EGGGSGGGSGSGDFDYEKMANANKGAMTENADENAL QSDAKGKLDSVATDYGAAIDGFIGDVSGLANGNGATGD FAGSNSQMAQVGDGDN SPLMNNFRQYLPSLPQSVECRP YVFGAGKPYEFSIDCDKINLFRGVFAFLLYVATFMYVFS TFANILRNKES
LLH SEQ ID NO:5	GATATTCAAATGAATCAGTCCCCTTCTTCCTTATCAGCC TCTTTGGGTGATACAATCAGCATTACATGCCGAGCGTC ACAGAATATCAACATCTGGCTGTCTGGTATCAACAAA AGCCGGGTAATGTTCCAAAAGTGTGATCTACAAAGCT TCCAATCTTCATACTGGTGTTCCTTCGCGTTTCAGTGGT AGCGGATCGGGTACTGATTTTACACTGATTATTTTCGAG CCTGCAGCCTGAAGACATTGCTACCTACTATTGCCTCC AAGGTCAGAGCTACCCGCGTACATTTCGGCGGCGGCAC TAAACTTGAGATAAAACGGGGCGACGCCGCCCTACA GTATCAATCTTTCCGCCCTCCAGTGAACAGCTTACGAG TGGCGGTGCATCAGTTGTCTGTTTTTTGAACAACCTCTA CCCAAAGGATATAAACGTAAAGTGGAAGATTGACGGT TCAGAAAGACAGAATGGCGTGCTGAACTCTTGACGG ACCAGGATAGCAAAGACAGCACGTATAGTATGTCATCA ACTTTAACACTCACTAAAGATGAGTATGAAAGACATAA CTCTTATACATGCGAAGCAACCCACAAGACCAGTACCT CTCCGATTGTGAAAAGTTTCAATCGGGGAGGAGGGGGT AGTGGAGGTGGTGGCTCCGGAGGGGGCGGCTCTGAAGT GAAGCTTGTAGAATCTGGTGGCGGCTTAGTGCAGCCG GGGGTTCTCTCAGTCTCTCCTGTGCAGCCTCAGGTTTTA CGTTTAGCGATTATTATATGACATGGGTACGTCAGGCGC CAGGGAAAGCCCCGAATGGCTGGCATTGATTTCGCAAT AAACGAAAATGGGGATACGGCCGAGTACAGTGCTTCAGT AAAAGGGCGGTTTACTATATCTCGTGATTATTCACGCAG CATTCTGCATCTTCAAATGAATGCACTGCGCACTGAAGA TAGCGCTACCTATTATTGTGTCCGCCAGGGACGTGGGTA CACCTTAGATTATTGGGGTCAAGGCACCTCCGTTACCGT CAGTTCCGCAAAAACGACCGCGCCGTCTGTGTATCCACT GGCGCCTGTTTGCGGTGACACGACGGGGAGTTCCGTGAC CCTGGGTTGTTTGGTTAAAGGATATTTCCCGAGCCTGTC ACGCTGACCTGGAATAGCGGCAGCCTGTGAGCGGGGTT CATACATTTCCAGCTGTCTTACAGTCTGGCCTGTACACAT TATCGAGCTCAGTTACTGTGACCTCGTCTACTTGGCCGTC GCAGTCCATAACGTGTAACGTGGCGCACCCGGCGAGTAG CACCAAAGTTGATAAAAAAATCGAGCCG
LLH + G3P SEQ ID NO:6	GATATTCAAATGAATCAGTCCCCTTCTTCCTTATCAGCCT CTTTGGGTGATACAATCAGCATTACATGCCGAGCGTCACA GAATATCAACATCTGGCTGTCTGGTATCAACAAAAGCC GGGTAATGTTCCAAAAGTGTGATCTACAAAGCTTCCAA TCTTCATACTGGTGTTCCTTCGCGTTTCAGTGGTAGCGGA TCGGGTACTGATTTTACACTGATTATTTTCGAGCCTGCAGC CTGAAGACATTGCTACCTACTATTGCCTCCAAGGTCAGAG CTACCCGCGTACATTTCGGCGGCGGCACTAAACTTGAGATA

	AAACGGGGCGACGCCGCCCTACAGTATCAATCTTTCCGC CCTCCAGTGAACAGCTTACGAGTGGCGGTGCATCAGTTGT CTGTTTTTTGAACAACCTTCTACCCAAAGGATATAAACGTA AAGTGGAAGATTGACGGTTCAGAAAGACAGAATGGCGTG CTGAACTCTTGGACGGACCAGGATAGCAAAGACAGCACG TATAGTATGTCATCAACTTTAACTCACTAAAGATGAG TATGAAAGACATAACTCTTATACATGCGAAGCAACCCAC AAGACCAGTACCTCTCCGATTGTGAAAAGTTTCAATCGGG GAGGAGGGGGTAGTGGAGGTGGTGGCTCCGGAGGGGGCG GCTCTGAAGTGAAGCTTGTAGAATCTGGTGGCGGCTTAGT GCAGCCCGGGGTTCTCTCAGTCTCTCCTGTGCAGCCTCAG GTTTTACGTTTAGCGATTATTATATGACATGGGTACGTCAG GCGCCAGGGAAGCCCCGAATGGCTGGCATTGATTCCGA ATAAACGAAATGGGGATACGGCCGAGTACAGTGCTTCAGT AAAAGGGCGGTTTACTATATCTCGTGATTATTCACGCAGCA TTCTGCATCTTCAAATGAATGCACTGCGCACTGAAGATAG CGCTACCTATTATTGTGTCCGCCAGGGACGTGGGTACACCT TAGATTATTGGGGTCAAGGCACCTCCGTTACCGTCAGTTCCG CAAAAACGACCGCGCCGTCTGTGTATCCACTGGCGCCTGTTT GCGGTGACACGACGGGGAGTTCCGTGACCTGGGTTGTGTTGG TTAAAGGATATTTTCCCGAGCCTGTCACGCTGACCTGGAATA GCGGCAGCCTGTGAGCGGGGTTACATATTTCCAGCTGTCTT ACAGTCTGGCCTGTACACATTATCGAGCTCAGTTACTGTGACC TCGTCTACTTGGCCGTGCGAGTCCATAACGTGTAACGTGGCGC ACCCGGCGAGTAGCACCAAAGTTGATAAAAAAATCGAGCCGG GCGGAGGAACCGGTGGTGGTTCTGGTGGCGGCTCTGAGGGTG GCGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGCTCTGAGG GTGGCGGTTCCGGTGGCGGCTCCGGTTCGGTGATTTTGATTA TGAAAAAATGGCAAACGCTAATAAGGGGGCTATGCCGAAAA TGCCGATGAAAACGCGCTACAGTCTGACGCTAAAGGCAAACCT GATTCTGTGCTACTGATTACGGTGCTGCTATCGATGGTTTCAT TGGTGACGTTTCCGGCCTTGCTAATGGTAATGGTGCTACTGGTG ATTTTGCTGGCTCTAATTCCCAAATGGCTCAAGTCGGTGACGGT GATAATTCACCTTTAATGAATAATTTCCGTCAATATTTACCTTCT TTGCCTCAGTCGGTTGAATGTCGCCCTTATGTCTTTGGCGCTGGT AAACCATATGAATTTTCTATTGATTGTGACAAAATAAACTTA TTCCGTGGTGTCTTTGCGTTTCTTTTATATGTTGCCACCTTTAT GTATGTATTTTCGACGTTTGCTAACATACTGCGTAATAAGGA GTCT
HLL SEQ ID NO:7	GAAGTCAAACCTTGTGCAATCCGGTGGAGGGCTGGTACAG CCTGGTGGTTCCCTGAGTCTGTCTTGTGCTGCGTCTGGTTT TACTTTTTCAGATTATTACATGACTTGGGTGCGTCAGGCC CCCGGAAAAGCCCCAGAATGGCTCGCATTAATAAGAAAT AAACGGAATGGTGATACAGCTGAGTATCCGCGAGTGTC AAAGGCCGCTTTACGATTTCCTCGTGATTATTCACGGAGC ATTCTGCATCTTCAAATGAATGCACTTCGCACTGAAGATT CGGCGACATACTATTGTGTGAGACAGGGTTCGCGGTTACA CCTTAGATTATTGGGGCCAGGGTACCTCAGTGACCGTTTC TTCCGCCAAAACGACCGCTCCATCAGTGATCCTTTAGCT CCGGTTTGTGGCGATACCACCGGTCCTCGGTTACGCTGG GCTGCCTGGTAAAAGGTTACTTTCCGGAACCGTTACACT GACATGGAACAGCGGCTCCTTGTCAAGTGGTGTTCACAC

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HLL + G3P SEQ ID NO:8	GAAGTCAAACCTTGTCGAATCCGGTGGAGGGCTGGTACA GCCTGGTGGTTCCCTGAGTCTGTCTTGTGCTGCGTCTGG TTTTACTTTTTTCAGATTATTACATGACTTGGGTGCGTCAG GCCCCCGGAAAAGCCCCAGAATGGCTCGCATTAATAAG AAATAAACGGAAATGGTGATACAGCTGAGTATTCCGCGA GTGTGAAAGGCCGCTTTACGATTTCCCGTGATTATTCAC GGAGCATTCTGCATCTTCAAATGAATGCACTTCGCACTG AAGATTTCGGCGACATACTATTGTGTGAGACAGGGTCGC GGTTACACCTTAGATTATTGGGGCCAGGGTACCTCAGT GACCGTTTCTTCCGCCAAAACGACCGCTCCATCAGTGT ATCCTTTAGCTCCGGTTTGTGGCGATACCACCGGTCCCT CGGTTACGCTGGGCTGCCTGGTAAAAGGTTACTTTCCG GAACCCGTTACACTGACATGGAACAGCGGCTCCTTGTC AAGTGGTGTTACACATTTCCCGCCGTGTTACAGTCTG GTCTCTACACGCTGAGTAGCTCTGTACCCGTTACATCT TCTACATGGCCTAGCCAGTCTATTACCTGCAATGTAGC GCATCCTGCGTCAAGCACGAAAGTTGATAAGAAAATC GAACCAGGTGGCGGGGGATCAGGAGGCGGTGGGTCA GGCGGGGAGGTTCCGACATACAAATGAATCAGAGCC CGAGCTCTTTGTCTGCAAGCCTGGGCGATACCATCAG TATTACTTGTGCGGCCAGTCAGAATATTAATATCTGGT TAAGCTGGTACCAACAGAAGCCTGGTAATGTGCCTAA ACTTCTGATCTACAAAGCTAGCAATCTTCATACAGGAG TACCGTCACGTTTCAGTGGCTCAGGAAGCGGCACCGA CTTCACGTTGATTATCTCATCGCTCCAACCAGAGGATA TTGCAACTTATTATTGCTTGCAGGGACAGAGCTATCCG CGGACATTTGGGGGTGGTACCAAACTGGAGATTAAACG AGGCGACGCCGCACCGACTGTTTCAATCTTCCCACCG TCGAGTGAACAATTAACCTTCTGGGGGCGCGTCCGTCTG GTGTTTCTCAACAACCTTTATCCCAAGGACATTAACGT AAAGTGGAAGATAGATGGGTCCGAACGTCAAAACGGA

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[00398] **Binding assay for phage and bacterial cells.** To quantify the amount of phage binding to different bacterial cell targets, 1×10^{10} virions in 100 μ L volume were mixed with 1 mL of bacterial cells in the exponential growth phase at a concentration of $OD_{600}=1$. The cells and phages were incubated at room temperature (RT) on a rotator for 30 minutes and then spun down at 4,000x g for 5 minutes. The cells were washed with 1 x PBS buffer to remove unbound or non-specifically bound phage and resuspended in 1 x PBS buffer. Bound phage DNA in each sample was extracted using a miniprep procedure and quantified using qPCR as described below.

[00399] **Quantitative polymerase chain reaction (qPCR) assay.** To quantify the number of phage virions in a sample, 100 μ L of phage sample was processed following standard miniprep protocol using QIAprep Spin Miniprep Kit (Qiagen), and the phage DNA was eluted in 50 μ L of MilliQ water. The quantity of phagemid present in eluted samples was quantified using qPCR with a standard curve made from a known concentration standard of purified phagemid vector pADL10b. The qPCR assay was carried out using SYBR Green Master Mix (Bio-Rad Laboratories) with primer set qPCR-upstream and qPCR-downstream on a Bio-Rad C1000 PCR machine. PCR conditions were: 95 °C for 30 seconds followed by 45 cycles of denaturation for 30 seconds at 95 °C and extension for 15 seconds at 60°C.

[00400] **Transmission Electron Microscopy (TEM).** Negatively stained TEM samples were prepared following a published protocol (Yang, Y. & Chen, I. A. Visualization of engineered M13 phages bound to bacterial targets by transmission electron microscopy. *Methods Mol. Biol. Springer* (2024)). Bacterial cell culture in stationary phase was spun down at 4,000 rpm for 5 minutes, washed once with 1 x PBS buffer and resuspended in 1 x PBS buffer to OD₆₀₀ value between 2-3. 100 µL of the cells were incubated with either 1 x PBS as control, or 10 µL of phages at a concentration of 1×10^{11} virions/mL, for 30 minutes at RT. The samples were spun down and washed twice with 1 x PBS buffer to remove any unbound or non-specifically bound phages and resuspended in 1 x PBS buffer to the original volume. 8 µL of samples were loaded on the shiny side of Formvar/Carbon 200 Mesh, Ni TEM grid (Electron Microscopy Sciences) and incubated for 2 minutes. The grid was washed 4 times in filter-sterilized wash buffer composed of 1% bovine serum albumin in 1 x PBS buffer. The sample was then blocked with blocking buffer, composed of filter-sterilized 0.1% gelatin in 1 x PBS buffer, at RT for 1 h, washed once, and incubated with 100 µL of 1:100 dilution anti-M13 major coat protein monoclonal antibody (ThermoFisher Scientific) or Polymyxin B Monoclonal Antibody (ThermoFisher Scientific) for 1 h at RT. The samples were then washed with the same wash buffer 4 times, blocked with blocking buffer, and incubated with 100 µL of 1:20 dilution of pre-adsorbed secondary antibody-coated 6 nm gold nanoparticles (Abcam) for 1 h at RT. The sample was washed with MilliQ water 5 times and stained with 8 µL of 1% uranium acetate for 1.5 minutes, dried and imaged on a FEI Tecnai T12 transmission electron microscope (California NanoSystems Institute at UCLA).

[00401] **Conjugation of polymyxin B to anti-LPS phage to produce PMB-Phage.** Polymyxin B (PMB; FIG. 1B) molecules were conjugated on the surface of recombinant M13 phages using carbodiimide crosslinker chemistry. Surface-exposed primary amines of anti-LPS phages were first blocked with sulfo-NHS-acetate to prevent phage crosslinking. The reacted product was dialyzed (1:45 volume ratio) 2 times in 1 x PBS buffer and 3 times in 0.1x MES buffer (pH 5.5) for 4 h per dialysis cycle at 4°C, using a Slide-A-Lyzer Mini Dialysis Device (20K MWCO, Fisher Scientific, Waltham, MA). The dialyzed product was incubated with 100 µL of EDC (4 mg/mL) and Sulfo-NHS (11 mg/mL) mixture dissolved in water for 20 minutes. The reacted product was dialyzed 3 times in 1 x PBS buffer (pH 7.2) for 40 minutes per dialysis cycle at 4°C. The dialyzed product was transferred to a 15 mL tube and 1 x PBS buffer was added to reach a final volume of 10 mL. 0.75 mg of PMB was then

mixed and incubated with each 1 mL ($\sim 1 \times 10^{12}$ virion/mL) of phage for 2 h. The samples were then dialyzed 2 times with 1x TBS buffer (pH 7.2) to quench the reaction and 3 times with 1x PBS buffer (pH 7.4) to remove excess unreacted PMB. Amino acid composition analysis was performed on 434.6 μ g of PMB-Phage (pooled from 5 independently synthesized batches) by Creative Proteomics (Shirley, New York).

[00402] **Minimal Inhibitory Concentration and Minimal Bactericidal Concentration assays.** The minimal inhibitory concentrations (MIC) of PMB and PMB-Phage for different pathogen species and strains were determined following published broth microdilution protocols in triplicate samples. In brief, in non-treated and sterile U-Shaped-Bottom 96 well plates (Fisher Scientific), 50 μ L of PMB at concentrations ranging from 1 to 256 μ g/mL, or PMB-Phage at concentrations ranging from 2.5×10^9 to 8×10^{10} virions/mL, in two-fold serial dilutions with Muller-Hinton broth (MH broth), were mixed with 50 μ L of bacterial cells (5×10^5 colony forming units (CFU)/mL) in each well. 100 μ L of MH broth with or without the same concentration of bacteria were used as growth controls and sterility controls, respectively. The sterilely covered 96-well plate was incubated at 37°C overnight without shaking and cell growth in each well was examined by spectrophotometry at 600 nm on a plate reader (Tecan Infinite M200 PRO, Tecan Group Ltd., Switzerland). The well with the lowest antibiotic concentration without significant growth (OD_{600} increase < 0.1 compared to sterility control) indicated the value of the MIC. Subsequently, 50 μ L of culture from each well having no significant growth was spread uniformly on a Muller-Hinton plate and incubated overnight. The lowest concentration of PMB or PMB-Phage yielding a plate having no cell colonies indicated the minimal bactericidal concentration (MBC). The MIC assays for *P. aeruginosa* strains ATCC 27853, LES 431, AR-Bank #0246, and AR-Bank #0266 were conducted by Eurofins.

[00403] ***P. aeruginosa* biofilm MBC assay.** The MBC of PMB and PMB-Phage for biofilm-forming strain *P. aeruginosa* strain ATCC 25102 was determined based on published broth microdilution protocols in quadruplicate samples. In brief, in non-treated and sterile U-Shaped-Bottom 96 well plates (Fisher Scientific), 150 μ L of MH broth with 5×10^5 CFU of *P. aeruginosa* strain ATCC 25102 were incubated in each well for three days under room temperature, covered by a sterile lid. By day 3, a bacterial floc(s) could be observed near the bottom of each well. 150 μ L of PMB (200, 240, 280, 320, 360, or 400 μ g/mL) or PMB-Phage

(1.25×10^{11} to 4×10^{12} virions/mL in two-fold serial dilutions) in MH broth were added to each well without disturbing the bacterial flocs. The sterilely covered 96-well plate was incubated at room temperature overnight without shaking, and cell viability in each well was examined by spreading uniformly on a MH plate with incubation overnight at 37°C. The lowest concentration of PMB or PMB-Phage yielding a plate having no cell colonies indicated the MBC for the biofilm.

[00404] **Time-kill kinetics assay.** Following established protocols (Adusei, E. B. A., Adosraku, R. K., Oppong-Kyekyeku, J., Amengor, C. D. K. & Jibira, Y. Resistance Modulation Action, Time-Kill Kinetics Assay, and Inhibition of Biofilm Formation Effects of Plumbagin from *Plumbago zeylanica* Linn. J. Trop. Med. 2019, 1250645 (2019); Bergen, P. J., Li, J., Rayner, C. R. & Nation, R. L. Colistin methanesulfonate is an inactive prodrug of colistin against *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 50, 1953–1958 (2006)), in 96-well plates, cultures containing PMB and PMB-Phage at the concentration equal to the MIC and 5×10^5 CFU/mL of bacterial cells were incubated at 37°C. 100 µL of cultures from three triplicate wells were plated on individual Muller-Hinton plates at 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 h time points. The plates were incubated at 37°C overnight and the number of CFUs was determined for each time point.

[00405] **Biocompatibility with mammalian cells assayed with MTT.** Cell metabolic activity was measured by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay as an indication of cell viability for human embryonic kidney (HEK-293) cells (ATCC CRL-1573) following published protocols (Bang, S., Jung, U.-W. & Noh, I. Synthesis and Biocompatibility Characterizations of in Situ Chondroitin Sulfate-Gelatin Hydrogel for Tissue Engineering. Tissue Eng. Regen. Med. 15, 25–35 (2018); Kılıç, H., Ceylan Tuncaboğlu, D., Argun, A. & Öztürk Civelek, D. Design of Biocompatible Multifunctional Hydrogels with Stearyl Methacrylate and Vinylpyrrolidone. ACS Appl. Polym. Mater. 4, 1717–1727 (2022)). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin) using Falcon® polystyrene tissue culture flasks with ventilated caps (Corning). The cells were incubated at 37°C with 5% CO₂ until confluency. Then, cells were detached from the culture flask and seeded at a density of 1×10^3 cells/well in 1 mL culture volume in each well of 24-well plates (Corning). Five hours after seeding, the media was

changed to DMEM containing 1% antibiotics (penicillin/streptomycin) supplemented with either PMB (at concentrations of 2, 8, and 32 µg/mL), PMB-Phage (at concentrations of 5×10^9 , 1×10^{10} , and 2×10^{10} virions/mL) or no supplements as the negative control. Triplicate samples were prepared for each condition and media was refreshed every 2 days. HEK-293 cell metabolic activity was quantified using an MTT assay according to the manufacturer's protocol. After 1, 3, and 5 days of culture, 100 µL of MTT stock solution (5 mg/mL) was added to each well. After 4 h of incubation at 37°C with 5% CO₂, 1 mL of solubilization solution was added and left overnight. The next day, the absorbance of the wells was read at 550 nm (reference wavelength at 650 nm) using a Tecan Infinite M200 PRO plate reader.

[00406] HEK-293 cell spreading and morphology for cells incubated under all conditions described above were observed using Actin/DAPI staining. All procedures were conducted at RT. After 1, 3, and 5 days of culture, the cells were fixed using 4% (v/v) paraformaldehyde for 10 min. Then, the cells were washed with 0.3% (v/v) Triton X-100 for 10 min and blocked with 1% bovine serum albumin (BSA) for 30 min. A light-sensitive mixture of 0.1% (v/v) phalloidin and 0.05% (v/v) DAPI in 1 x PBS was added to stain the cells for 20 min. After washing with DPBS, the cells were imaged with an AxioObserver Z1 inverted microscope (Zeiss).

[00407] **In vitro hemolysis assay.** Hemolysis assays were performed following previously established protocols to determine and compare the hemolytic activity of PMB and PMB-Phage at serially diluted concentrations. 5 mL of 10% sheep red blood cells (MP Biomedicals) were washed five times by spinning down at 500x g for 5 minutes, removing the supernatant, and refilling to the original volume with 1 x PBS buffer for gentle resuspension. After the last washing step, cells were resuspended in 1 x PBS buffer to obtain a 4% red blood cells solution. 100 µL of the 4% solution was added into each 1.5 mL sterile Eppendorf tube and mixed with 100 µL of each sample, 1 x PBS for negative control, or 2% Triton X-100 for positive control (N=3). The mixtures were incubated for 1 hour at 37 °C. The samples were then spun down at 500x g for 5 minutes and 100 µL of supernatant was transferred into 96-well plates. Absorbance at 405 nm was measured using a plate reader and the hemolytic activity was determined using the following equation:

$$\% \text{ hemolysis} = (A_{\text{sample}} - A_{\text{PBS}}) / (A_{\text{Triton X-100}} - A_{\text{PBS}})$$

A_{sample} , A_{PBS} , and $A_{Triton X-100}$ are absorbance values at 405 nm of the sample, negative control, and positive control correspondingly.

[00408] ***In vivo* maximum tolerated dose and toxicity.** These experiments were conducted under protocol ARC-2020-044-AM approved by the UCLA Animal Research Committee (IACUC). Male 10-week-old C57BL/6J mice (24-30 g; The Jackson Laboratory), were used for this study. Mice were housed in rigid, non-porous, leak-proof containers (maximum 4 animals per cage) that were compatible with biohazardous animals. Intravenous (IV) tail vein injections were performed using BD Ultra-Fine Insulin Syringes 30G 3/10 cc 1/2 inch (Becton Dickinson). 100 μ L of PMB-Phage or PMB at different concentrations in 1 x PBS buffer were administered to each animal through IV tail vein injection once a day for seven consecutive days (N=3). 24 h after the last injection, 350 μ L of blood was collected through retro-orbital bleeding from each animal under 2% isoflurane anesthesia, into a BD Microcontainer Tube with Serum Separator (Becton Dickinson). The animals were euthanized through decapitation under anesthesia. The left kidney, spleen, and liver samples from each animal (size approximately 5 mm x 5 mm x 3 mm) were collected. The blood samples were spun down at 1,000 x g for 10 minutes after allowing the blood to clot for 30 minutes at 4°C. The serum was collected in sterile 1.5 mL Eppendorf tubes, stored at 4°C, and sent for biomarker analysis (Standard Tox Panel 62794 analysis performed by IDEXX Bioanalytics, USA). The tissues were soaked in formalin for 24 h before being transferred into 70% ethanol for downstream H&E staining.

[00409] **μ PET/CT study of PMB-Phage biodistribution.** These experiments were conducted under protocol ARC-2020-044-AM approved by the UCLA Animal Research Committee. Positron emission tomography (PET) imaging was used to characterize the biodistribution of PMB-Phage in male 10-week-old C57BL/6J mice, 24-30 g (The Jackson Laboratory), at time points across a 72 h period, for conditions with and without bacteria in the bloodstream (N=4). PMB-Phage virions were radioactively labeled with ^{89}Zr by first covalently conjugating with chelator desferrioxamine (DFO). The isothiocyanate-bearing derivative of DFO p-SCN-Bn-deferoxamine B (DFO-NCS) was dissolved in dry DMSO at a concentration of 5 mg/mL. 5 mL of PMB-Phage at a concentration of 5×10^{11} virions/mL in 1 x PBS were adjusted to pH 9 with small aliquots of 0.1 M Na_2SO_4 . 25 μ L of DFO-NCS were added to PMB-Phage and incubated for 1 h on a rotator at RT⁴². The product DFO-PMB-

Phage was purified from unreacted NCS-DFO and DMSO through 5 x dialysis (4 h each cycle in 1 x PBS buffer using a Slide-A-Lyzer dialysis cassette). DFO-PMB-Phage was further conjugated with ^{89}Zr and purified similarly. Radioactivity was determined using radio-thin layer chromatography. For the group of animals with bacteria, 1×10^7 CFU of the *E. coli* strain ATCC BAA 1161 in 1 x PBS, diluted from samples with $\text{OD}_{600} = 0.5$ (5×10^7 CFU/mL) were injected in 50 μL volume via tail vein, 2 h before injection of ^{89}Zr - DFO-PMB-Phage with 100 μCi radioactivity ($\sim 5 \times 10^{10}$ virions). The mice were scanned using $\mu\text{PET/CT}$ at time points of 10 and 30 min, and 1, 2, 4, 6, 24, 48, and 72 h. One animal in the bacterial injection group died after 24 hours and one injection failed for an animal in the group without bacterial injection, leaving $N=3$ for each group. The images were acquired with an energy window between 350 to 650 keV, generated using 3D-OSEM/MAP method, and analyzed using the software AMIDE.

[00410] ***In vivo* effect of PMB-Phage in a *P. aeruginosa* mouse lung infection model.**

Pathogen-free 7 weeks old female BALB/c mice (specific pathogen-free (SPF)) were obtained from BioLASCO Taiwan, an AAALAC-certified Charles River Licensee and rodent breeder. Mice were quarantined for three days after receipt in an SPF facility before transfer to the vivarium. During infection studies, animals were housed in a separate room in negative-pressure individually ventilated cages (GM500 IVC seal safe cage system; Tecniplast, Italy) and supplied with sterile bedding and gamma-irradiated food. *P. aeruginosa* strain AR Bank #0266 was prepared from a 0.2 mL aliquot of a single-use glycerol stock seeding 20 mL Tryptic Soy Broth and then incubated at $35\text{--}37^\circ\text{C}$ with shaking (250 rpm) for 16 h. 0.5 mL of the culture was used to seed 49.5 mL of fresh media and incubated in the same manner for 3 h. The cells were then pelleted, resuspended in 10 mL of 1x PBS, and quantified by optical density at 620 nm. The cells were then diluted to desired concentration using 1 x PBS and inoculum was administered within 1 h of time. The inoculum size ($2\text{--}4 \times 10^7$ CFU) was estimated to be a lethal dose (LD90) in a titration study. On Day 0, after anesthesia with pentobarbital (50 mg/kg, IP), animals were infected with 2.8×10^7 CFU of pathogen suspension (*P. aeruginosa* strain AR Bank #0266) by intranasal (IN) administration in 0.05 mL volume. Each mouse was held upright and 25 μL of the bacterial suspension was gradually released into each nostril using a micropipette. The mouse were held in an upright position until the breathing rate and depth renormalized (approximately two minutes) and then placed into the cage for recovery. The test articles (PMB, PMB-Phage, anti-LPS phage and wild-type

M13 phage) were formulated in 1× PBS. One hour past infection, animals used to count disease baseline CFUs were sacrificed with CO₂ asphyxiation for aseptic lung tissue collection, and other animals were subject to treatment. For treated animals, an additional anesthetic dose (pentobarbital 25 mg/kg at 30 min post-infection) was administered IP and the treatment administered IN in the manner described. Animals were observed at 30 min and 12 h after IN dosing for signs of acute toxicity; none were found to have reached humane endpoints prior to the scheduled time for euthanasia. 25 hours post-infection, treated animals were sacrificed with CO₂ asphyxiation for aseptic lung tissue collection. The lung tissues were weighed and homogenized in 1 mL sterile 1 x PBS buffer using a Polytron homogenizer. The bacterial burden in the tissue homogenates was determined by performing 10-fold serial dilutions and plating 0.1 mL of each onto MacConkey II agar. Colonies were counted after 18-24 h incubation, and the dilution plate yielding the most colonies (10-300) was used for calculation. The CFU value per tissue was calculated with the following equation:

$$\frac{CFU}{Lung} = \frac{\frac{CFU}{plate}}{\frac{mL \text{ sample plated}}{plate}} \times \text{dilution factor} \times \text{total sample volume} \left(\frac{mL}{tissue} \right)$$

Bacterial burden (CFU/tissue) of treated animal groups was compared to the baseline bacterial count at 1 h after infection and to animals treated with 1 x PBS as a negative control. The significance of effects was assessed with ANOVA followed by Dunnett's test using GraphPad Prism software.

[00411] ***In vivo* effect of PMB-Phage on a *P. aeruginosa* corneal infection model.** Age-matched male and female 6-8 week old C57BL/6/J (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred and housed in a UC Irvine vivarium. *P. aeruginosa* GFP-expressing strain (PAO1-GFP) bacteria were grown from frozen stock using a sterile inoculating loop, with high-salt Luria Bertani broth (BD Biosciences) supplemented with 50 µg/mL carbenicillin. The culture was incubated at 37°C overnight. 100 µL of the overnight culture was sub-cultured into new culture media and grown to mid-log phase at 37 degrees C with 5% CO₂ with agitation until an OD of 0.2 was reached. Bacteria were then spun down at 3,000 x g for 10 minutes, resuspended in sterile PBS, and diluted to the desired concentration (2.5 x 10⁷ cells/mL). Mice were anesthetized according to IACUC approved standards using ketamine/xylazine and were monitored through the duration of experiments.

The corneal epithelium was abraded using a sterile 30-gauge needle (BD Biosciences) to create three parallel 1 mm scratches. After the corneas were abraded, 2 μ L of bacteria (as prepared above) was applied topically. Test articles were formulated one hour after infection. At 2, 20, and 24 hours post-infection, mice were given treatments by dropping 10 μ L of each treatment onto the cornea. Mice were euthanized at 48 hours post-infection and eyes were imaged for corneal opacity and GFP quantification. Animal eyes were imaged using a Leica MZ10 F Modular Stereo Microscope for Fluorescent Imaging with a Leica DFC450 C camera, the brightfield and GFP fluorescent images were taken using the following settings: Zoom: 3.2x, brightfield exposure: 40 ms, fluorescence exposure: 2 s. For CFU quantification, whole eyes were collected and placed in 1 mL of sterile 1 x PBS containing a sterile steel ball bearing (5 mm). Eyes were homogenized using a Qiagen TissueLyser II at 30 Hz for 3 minutes. Homogenates were 10-fold serially diluted, and 10 μ L of each dilution was plated onto LB agar plates. Plates were incubated at 37 degrees C with 5% CO₂ overnight and colonies were counted manually the next day. CFUs were calculated following the equation of:

$$\frac{CFU}{Eye} = \frac{\frac{CFU}{plate}}{\frac{mL \text{ sample plated}}{plate}} \times dilution \text{ factor} \times total \text{ sample volume} \left(\frac{mL}{tissue} \right)$$

Results

[00412] **Construction, production, and initial binding tests of recombinant phages.** The gene fragments encoding two different constructs of antibody WN1 222-5 as single-chain fragment antigen-binding region (scFab) were cloned into phagemid vector pADL10b-S239T, as replacements of the wild-type pIII N-terminal domains (N1 and N2). The two constructs differed in ordering of the heavy and light chains, and a glycine linker (GGGGS)₄ (SEQ ID NO:19) was placed between the two chains (FIGURE 2). Construct HLL was ordered as heavy chain – linker – light chain (SEQ ID NO: 4), and construct LLH was ordered as light chain – linker – heavy chain (SEQ ID NO: 2). Clones of the constructed phagemids were confirmed by Sanger sequencing. Constructs with signal sequences, the scFab, linker and C-terminal domain of g3p are shown as SEQ ID NO:20 and SEQ ID NO:21, for HLL and LLH, respectively. Phage virions were produced by standard methods and verified by UV absorbance spectrum and TEM (FIGURE 3A-B).

[00413] Binding of recombinant phages to several species of Gram-negative bacterial cells was tested for both HLL and LLH constructs, by incubation of phages with cells, pelleting and washing of cell-phage complexes, and phagemid-specific qPCR of isolated DNA. In general, the HLL construct showed one order of magnitude more binding to each target pathogen (*E. coli* strain DH5 α ; *P. aeruginosa* strain ATCC 25102 and clinical strains B, C, E, F, G, J; *K. pneumoniae* strain ATCC 700603 and clinical strain A; *A. baumannii* ATCC 19606; *B. cepacia* ATCC 25416; and *C. sakazakii* ATCC 25944) compared to the LLH construct. Since the HLL construct demonstrated stronger binding than the LLH construct. The HLL construct was selected for downstream experiments and termed 'anti-LPS phage' hereafter.

[00414] **Binding of anti-LPS phage to various Gram-negative bacterial species.** LPS is composed of three distinct domains, namely a core oligosaccharide bridging lipid A in the outer membrane and an O-antigen exposed to the external environment. The O-antigen contributes greatly to LPS diversity, with more than 180 reported O-antigen serotypes for *E. coli* alone. In contrast, the core oligosaccharide, composed of an inner core composed of keto-deoxyoctulosonate and heptose residues and an outer core composed of hexoses, is relatively conserved. The antibody WN1 222-5 recognizes the inner core, and thus the anti-LPS phage was expected to bind a variety of Gram-negative organisms. Binding was visualized by TEM of phage incubated with bacterial cells, using an anti-M13 primary antibody and gold-nanoparticle-labeled secondary antibody to positively identify the filamentous phages (FIGURES 3C-F, FIGURE 6). The anti-LPS phages were observed to be bound to cell surface structures via the virion tip (not sidewall), consistent with the virion structure that should display the modified receptor-binding protein at the tip. In some cases, multiple virions were observed bound to a single cell, consistent with the expression of LPS across the cell wall.

[00415] The amount of anti-LPS phage bound to the cells was quantified by mixing 1 mL of cells ($OD_{600}=1$, or approximately 10^8 cells) with 100 μ L phage (1×10^5 to 1×10^{11} virions), pelleting of phage-cell complexes, washing, and qPCR of extracted DNA to determine the number of phagemid copies isolated. For comparison, a Gram-positive bacterial strain (*Staphylococcus aureus* strain ATCC 25904) was used as a negative control since Gram-positive organisms lack LPS. In general, for phage concentrations $\geq 10^7$ or 10^8 virions/mL, the amount of phage bound to Gram-negative strains exceeded the amount bound to *S. aureus*

by 1-2 orders of magnitude (FIGURES 4A-C, FIGURE 5A). Among the Gram-negative strains tested (*E. coli*, *P. aeruginosa*, *K. pneumoniae*, *A. baumannii*, and *B. cepacia*), some strains exhibited greater binding than others, but the saturating amounts bound were within the same order of magnitude (FIGURE 5A). In general, above 10^{10} virions/mL, approximately 1 or more anti-LPS phage virions were bound per cell on average, as detected by qPCR. These results indicate that the anti-LPS phage exhibits binding to a range of Gram-negative organisms, consistent with its design.

[00416] **Conjugation of Polymyxin B to anti-LPS phage.** To deliver PMB using the anti-LPS phage, polymyxin B (PMB) molecules were conjugated to the anti-LPS phage surface using carbodiimide crosslinking (EDC) chemistry. PMB contains multiple primary amines from Dab side chains of amino acids on the peptide ring, and the major phage coat protein pVIII contains three solvent-exposed carboxylic acid residues (Glu2, Asp4 and Asp5) per copy. Since pVIII also contains primary amines, phage crosslinking was prevented by first blocking the anti-LPS phage using sulfo-NHS-acetate. The ratio of phage to PMB in the crosslinking reaction was empirically optimized to obtain the highest potency, i.e., the lowest minimum inhibitory concentration (MIC), for PMB-Phage (see next section).

[00417] The amount of PMB conjugated per virion in PMB-Phage was determined using amino acid composition analysis to measure the mole percentage of amino acids (Asx (Asn + Asp), Thr, Ser, Glx (Gln + Glu), Pro, Gly, Ala, Val, Ile, Leu, Trp, Phe, His, Lys, Arg, Cys, and Tyr). The measurements were compared to the expected mole percentages calculated for varying ratios of PMB molecules conjugated per g8p protein. The analysis indicated that on average, ~2 PMB molecules were conjugated to each copy of pVIII, or ~5400 PMB carried by each virion ($\sim 1.1 \times 10^{-11}$ μ g of PMB per virion).

[00418] Higher PMB loading on anti-LPS phages would give higher efficacy. Given three surface-accessible carboxylic acids on each of approximately 2,700 copies of pVIII per virion, up to ~8,100 molecules of PMB could be conjugated up virion based on the number of reactive groups. However, since the phage surface is negatively charged while PMB is positively charged, increased loading would, in some embodiments, also cause colloidal instability as the particle charge is neutralized. Consistent with this expectation, conjugation to PMB altered the phage morphology, showing more compaction compared to unconjugated anti-LPS phage (FIGURE 7 and FIGURE 3A). The PMB-conjugated anti-LPS phage, termed PMB-Phage

hereafter, was filtered (0.22 μm) to remove large aggregates. Another potential problem with high PMB loading is that nonspecific conjugation of PMB might interfere with the PMB-phage binding to LPS. The literature indicates that nonspecific conjugation of small and large molecules (e.g., FITC, urease, neomycin) can occur without affecting phage binding to their targets. Regardless, these concerns regarding PMB loading were addressed by empirical optimization of the conjugation reaction to maximize the *in vitro* antibacterial effect of PMB-Phage (see below).

[00419] **Bactericidal effect of PMB-Phage in vitro.** According to the recommended breakpoints for PMB (standard form: PMB sulfate salt; PMBSO₄) published by the United States Committee on Antimicrobial Susceptibility Testing in 2020, an MIC ≤ 2 $\mu\text{g/mL}$ is considered susceptible, and an MIC ≥ 4 $\mu\text{g/mL}$ is considered resistant. Based on the conversion factor from amino acid composition analysis of PMB-Phage, we calculated the equivalent PMB concentrations and MIC values from PMB-Phage concentrations. PMB-Phage concentrations were determined by UV absorbance. MICs in liquid culture were determined by a standard broth microdilution assay in which cells were cultured in the presence of varying concentrations of PMB or PMB-Phage. We first tested the commonly accepted *E. coli* reference strain ATCC 25922 (acceptable MIC range for PMB: 0.25-2 $\mu\text{g/mL}$) and found the MIC for PMB (MIC_{PMBSO₄}) to be 2 $\mu\text{g/mL}$, validating the assay. Using PMB-Phage, the MIC (MIC_{PMB-Phage}) for this strain was found to be 5×10^9 virions/mL (equivalent to 0.054 $\mu\text{g/mL}$ PMB), indicating that PMB-Phage indeed lowers the MIC. We then determined MIC and MBC values for multiple Gram-negative strains.

[00420] In general, PMB-Phage effectively lowered the MIC by 1-2 orders of magnitude compared to PMB sulfate, for several different species and strains of Gram-negative bacteria, including clinical isolates (**Table 3**). For *P. aeruginosa* strains, MIC_{PMB-Phage} was 19-74-fold lower than MIC_{PMBSO₄}. For some strains that are resistant to PMB (MIC_{PMBSO₄} above the breakpoint), namely *P. aeruginosa* PAKpmrB6 and *K. pneumoniae* clinical strain A, the MIC_{PMB-Phage} value was well within the susceptible range, confirming the greater potency of PMB-Phage. Two strains were included that are extremely resistant to PMB (with MIC_{PMBSO₄} > 100 times the breakpoint), namely *K. pneumoniae* clinical strain 326 and *B. cepacia* ATCC 25416. For these, the MIC_{PMB-Phage} value was higher than the tested concentration range, indicating a limitation for the potency of PMB-Phage.

Table 3. Minimum inhibitory concentration (MIC) of anti-LPS phage, PMB sulfate, and PMB-Phage, determined *in vitro* for several Gram-negative organisms.

Species	Strain	MIC _{anti-LPS phage} (virions/mL)	MIC _{PMB-SO₄} (μg/mL)	MIC _{PMB-phage} (virions/mL)	MIC _{PMB-phage} (μg/mL)	$\frac{\text{MIC}_{\text{PMB-SO}_4}}{\text{MIC}_{\text{PMB-phage}}}$ (n-fold reduction in MIC)
<i>E. coli</i>	ATCC 25922	$> 5 \times 10^{12}$	2	5×10^9	0.054	37
<i>E. coli</i>	ATCC BAA 1161	$> 5 \times 10^{12}$	2	5×10^9	0.054	37
<i>E. coli</i>	ATCC 700927	$> 5 \times 10^{12}$	2	5×10^9	0.054	37
<i>P. aeruginosa</i>	ATCC 25102	$> 5 \times 10^{12}$	2	5×10^9	0.054	37
<i>P. aeruginosa</i>	Clinical Strain E	$> 5 \times 10^{12}$	2	2.5×10^9	0.027	74
<i>P. aeruginosa</i>	PAKpmrB6	$> 5 \times 10^{12}$	8*	4×10^{10}	0.43	19
<i>P. aeruginosa</i>	GFP-PAO1	$> 5 \times 10^{12}$	0.5	2.5×10^9	0.027	19
<i>P. aeruginosa</i>	ATCC 27853		2	5×10^9	0.054	37
<i>P. aeruginosa</i>	LES 431		1	2.5×10^9	0.027	37
<i>P. aeruginosa</i>	AR Bank #0246		2	5×10^9	0.054	37
<i>P. aeruginosa</i>	AR Bank #0266		1	2.5×10^9	0.027	37
<i>K. pneumoniae</i>	ATCC 700603	$> 5 \times 10^{12}$	2	5×10^9	0.054	37
<i>K. pneumoniae</i>	Clinical Strain A	$> 5 \times 10^{12}$	4*	1×10^{10}	0.107	61
<i>K. pneumoniae</i>	Clinical Strain 326	$> 5 \times 10^{12}$	$> 256^*$	$> 8 \times 10^{10}$	> 0.859	
<i>A. baumannii</i>	ATCC 19606	$> 5 \times 10^{12}$	2	5×10^9	0.054	37
<i>B. cepacia</i>	ATCC 25416	$> 5 \times 10^{12}$	$> 256^*$	$> 8 \times 10^{10}$	> 0.859	

For PMB, an MIC of 4 μg/mL or greater is defined as resistant (designated by *). N-fold reduction in MIC was calculated as the ratio $\text{MIC}_{\text{PMB-SO}_4} / \text{MIC}_{\text{PMB-Phage}}$ (both in units of μg/mL).

[00421] To verify that PMB was the active component of PMB-Phage, anti-LPS phage (without PMB conjugation) was tested in the same MIC assay. As expected, anti-LPS phage alone did not show bactericidal activity within the tested concentration range (Table 3). In addition, PMB is known to kill cells rapidly through membrane disruption. PMB-Phage also killed cells, as verified by plating and colony counting to determine the minimum bactericidal concentrations (MBC). As observed for the MIC values, $\text{MBC}_{\text{PMB-Phage}}$ values were generally 19-74 lower than $\text{MBC}_{\text{PMB-SO}_4}$ values (Table 4). PMB-Phage also showed a rapid mechanism of action, with a $>1,000$ -fold reduction of viable bacterial cells within 1 h in a time-kill kinetics assay (FIGURE 5B), comparable with PMB kinetics. Thus, PMB-Phage substantially lowers the MIC and MBC while maintaining the bactericidal effect and rapid kill-time of PMB.

Table 4. Minimum bactericidal concentration (MBC) of anti-LPS phage, PMB and PMB-Phage, determined in vitro for several Gram-negative organisms.

Species	Strain	MBC			$\frac{MBC_{PMB-SO_4}}{MBC_{PMB-phage}}$ (n-fold reduction in MBC)
		PMB ($\mu\text{g/mL}$)	PMB-Phage		
			(virions/mL)	($\mu\text{g/mL}$)	
<i>E. coli</i>	ATCC 25922	2	5.0E+09	0.054	37
<i>E. coli</i>	ATCC BAA 1161	2	5.0E+09	0.054	37
<i>E. coli</i>	ATCC 700927	2	5.0E+09	0.054	37
<i>P. aeruginosa</i>	ATCC 25102	2	1.0E+10	0.107	19
<i>P. aeruginosa</i>	Clinical Strain E	2	1.0E+10	0.107	19
<i>P. aeruginosa</i>	PAKpmrB6	32	> 8.0E+10	> 0.859	
<i>P. aeruginosa</i>	GFP-PAO1	2	2.5E+09	0.027	74
<i>K. pneumoniae</i>	ATCC 700603	4	1.0E+10	0.107	37
<i>K. pneumoniae</i>	Clinical Strain A	4	1.0E+10	0.107	37
<i>K. pneumoniae</i>	Clinical Strain 326	> 256	> 8.0E+10	> 0.859	
<i>A. baumannii</i>	ATCC 19606	2	1.0E+10	0.107	19
<i>B. cepacia</i>	ATCC 25416	>.256	> 8.0E+10	> 0.859	

[00422] Biofilms are a major contributing factor for prolonged virulence in nosocomial infections, especially in *P. aeruginosa*, and can significantly limit antibiotic exposure of bacterial cells. We grew *P. aeruginosa* biofilms undisturbed for 3 days and then added various concentrations of PMB or PMB-Phage to assay bactericidal activity on cells in the biofilms. We found that both MBC_{PMB-SO_4} and $MBC_{PMB-Phage}$ were higher for biofilm cells compared to planktonic cells by approximately 2 orders of magnitude. Regardless, a higher potency of PMB-Phage was also observed for the biofilm, with an $MBC_{PMB-Phage}$ of 11 $\mu\text{g/mL}$ (10^{12} virions/mL) compared to MBC_{PMB-SO_4} of 200 $\mu\text{g/mL}$ for PMB, or a ~20-fold improvement.

[00423] **Stability of PMB-Phage in solution.** M13 phages have been reported to have a half-life of >120 days. PMB sulfate in 0.9% NaCl stored at 4°C retains >75% activity after 7 days. We measured the stability of PMB-Phage using the MIC for *E. coli* (ATCC 25922) to assay retention of antibacterial activity. 5 mL of PMB-Phage at 1×10^{12} virions/mL stored at 4°C in 1 x PBS for >12 weeks showed no changed in MIC. Thus PMB-Phage retains full activity for >3 months of refrigerated storage.

[00424] **Cytotoxicity testing of PMB-Phage on mammalian cells *in vitro*.** PMB-Phage was tested for hemolytic activity using sheep red blood cells. Materials demonstrating <2% loss of cell content are considered non-hemolytic. PMB-Phage was non-hemolytic at concentrations up to 8×10^{10} virions/mL (32 times the MIC for *E. coli* strain 25922) (FIGURE 8A).

[00425] Human embryonic kidney (HEK) 293 cells were cultured in media containing $0.5-2 \times 10^{10}$ virions/mL of PMB-Phage (2-8 times the MIC for *E. coli* strain 25922). Metabolic activity was assayed by MTT as an indicator of cell viability. All cultures exposed to PMB-Phage had >80% activity for the duration of the 5-day assay (Figure 8B), which is considered non-toxic. This result was verified by microscopy with Phalloidin/DAPI staining, which showed normal cell growth and morphology upon exposure to PMB-Phage. The results indicated a lack of toxicity for mammalian cells *in vitro*.

[00426] **Toxicity testing of PMB-Phage in mice.** M13 phage has been shown previously to demonstrate no toxicity *in vivo*. To test PMB-Phage, male mice were subjected to daily IV tail vein injections of PMB-Phage for 1 week, at daily doses of 6.9×10^9 , 1.4×10^{10} , 2.7×10^{10} , and 1.6×10^{11} virions. Given the PMB loading (1.1×10^{-11} μ g of PMB / virion) and a typical animal weight of 27 g, these doses are equivalent to 3-64 mg of PMB per kg of body weight per dose. Control animals were untreated or injected with 1x PBS or PMB sulfate solution (2.5, 21, or 72 mg/kg). Blood samples taken 24 h after the last injection showed no impairment of liver or kidney function as assessed by biomarkers. Blinded histological analysis also showed no observable toxicity at these PMB-Phage concentrations for kidney, liver, and spleen samples. All animals receiving PMB-Phage survived with no observable indication of sickness based on lethargy, fur condition, posture, and body temperature. In contrast, a single dose of PMB at 72 mg/kg led to death in all animals (3/3) on day 1. The lack of toxicity *in vivo* for PMB-Phage supported the *in vitro* cytotoxicity results.

[00427] **Biodistribution of PMB-Phage in mice.** To characterize PMB-Phage biodistribution, PMB-Phage was radioactively labeled with ^{89}Zr , using the chelator desferrioxamine (DFO), and 5×10^{10} virions of PMB-Phage (containing 100 μ Ci radioactivity) were injected into male mice. The mice were separated into 2 groups, with one group also having 1×10^7 CFU of *E. coli* BAA 1161 administered IV through tail vein injection 30 minutes before PMB-Phage injection, to investigate whether *E. coli* bacteremia would

impact biodistribution of PMB-Phage. At time points of 10 min, 30 min, and 1, 2, 4, 6, 24, 48 and 72 h after injection, the mice were imaged with μ PET co-registered with μ CT and the percentage of injected dose (ID) per volume (cc) was quantified from images after accounting for radioactive decay. PMB-Phage was found to be distributed to all organs tested within 1 hour and remained present for at least 6 hours, with an overall elimination half-life of approximately 1 day. The rate of PMB-Phage systemic clearance decreased after 24 hours, with ~50% injected dose remaining in the system after 72 hours. The circulation time and clearance rate were similar regardless of the bacterial injection. The ^{89}Zr -labeled PMB-Phage was substantially distributed to both kidneys, liver, and spleen, each with approximately 8-25% of the injected dose/cc after 24 hours. Bacteremia affected the distribution among organs, with increased PMB-Phage observed in the lungs and muscle and decreased PMB-Phage in the kidneys and spleen, with effects being generally within 5% change in ID/cc. Overall, distribution throughout the animal volume occurred quickly (within 1 hour), and PMB-Phage stayed within the body for a significant time (24 hours). Images are shown for one animals and kinetics for total, blood and left kidney in FIGURES 9A-9D.

[00428] **PMB-Phage efficacy in a multidrug-resistant *P. aeruginosa* pneumonia model in immunocompetent mice.** A mouse infection model was established based on a published model for lethal *P. aeruginosa* pneumonia (Patel, A. et al. An engineered bispecific DNA-encoded IgG antibody protects against *Pseudomonas aeruginosa* in a pneumonia challenge model. Nat. Commun. 8, 637 (2017)). Infection was initiated by intranasal inoculation of 2.8×10^7 CFU of *P. aeruginosa* strain AR Bank #0266, a clinical isolate strain that is resistant to multiple antibiotics including carbapenems and fluoroquinolones, into female 7-week-old BALB/c mice. One hour after infection, a 50 μL volume of each test article was formulated with 1 x PBS buffer and administered intranasally (N=5). Test articles were vehicle control (1 x PBS), wild type (WT)-M13 phage (4×10^{10} and 8×10^{10} virions/dose), anti-LPS phage (4×10^{10} and 8×10^{10} virions/dose), PMB sulfate (0.5, 80, and 160 μg /dose) and PMB-Phage (2×10^{10} , 4×10^{10} , and 8×10^{10} virions/dose, containing 0.2, 0.4, and 0.9 μg of PMB respectively). At 24 hours after treatment, animals were sacrificed and their lung tissues were collected to determine the bacterial burden (CFU count). The initial baseline CFU count was measured at one hour post bacterial inoculation (without treatment). When 1x PBS, WT-M13, or anti-LPS phage was injected, a 16-fold increase in bacterial CFU count was observed compared to baseline, reflecting bacterial growth during the 24-hour period.

[00429] In comparison, bacteriostasis and a dose-dependent CFU reduction was observed in animals receiving PMB-Phage and PMB. Treatment with PMB-Phage at 4×10^{10} and 8×10^{10} virions/dose (containing 0.4 and 0.9 μg of PMB) reduced the CFU count by 320-fold and 500-fold, respectively ($p < 0.05$ and $p < 0.001$, respectively) compared to 1x PBS (FIGURE 10). The lowest dose of PMB-Phage (2×10^{10} virions) did not cause a significant reduction in CFU compared to 1x PBS. For PMB, the 80 and 160 μg doses resulted in 500-fold and 20,000-fold decrease in CFU ($p < 0.001$), while PMB at 0.5 μg dose did not cause a significant decrease in CFU, compared to 1x PBS. 160 μg corresponds to a previously determined maximum tolerated dose of PMB. PMB-Phage containing 0.9 μg of PMB resulted in the same CFU reduction as PMB sulfate at 80 μg , indicating a ~90-fold increase in drug potency in vivo.

[00430] Animal mortality (death before 25-h sacrifice time point) was observed at 20-40% for groups given 1x PBS, WT-M13, or anti-LPS phage, consistent with an untreated infection from the bacterial inoculum. The lowest PMB dose resulted in 20% mortality, while the medium and high PMB doses resulted in no deaths. No animal deaths were observed in the groups treated by low, medium, or high doses of PMB-Phage. This work utilized NIAID's suite of preclinical services for *in vivo* assessment (Contract No. HHSN272201700020I/75N93023F00002).

[00431] **PMB-Phage efficacy in mouse *P. aeruginosa* corneal keratitis model.** The mouse keratitis model was developed by creating three parallel 1 mm scratches on the cornea using a 30-gauge needle, followed by topically inoculating 5×10^4 CFU of *P. aeruginosa* strain PAO1-GFP under anesthesia. The test articles (anti-LPS phage and wild-type M13 phage at 1×10^{10} virions/dose, PMB sulfate at 0.5 and 0.25 $\mu\text{g}/\text{dose}$, and PMB-Phage at 6.25×10^8 to 2.5×10^9 virions/dose (containing 0.007 to 0.027 μg of PMB) were formulated in 1x PBS. The test articles or 1x PBS (negative control) were administered topically by dropping 10 μL onto the cornea at 2, 20, and 24 hours post-infection. At 48 hours post-treatment, animals were sacrificed and their eyes tissues were aseptically harvested and homogenized to determine bacterial burden by plating and CFU counts.

[00432] Compared to the 1x PBS control (N=10, 1 animal died prior to data collection), treatment by anti-LPS phage (N=10) or wild-type M13 phage (N=10) phage at relatively high doses did not significantly reduce bacterial burden (FIGURE 11). In comparison, dose-dependent CFU reductions were observed with treatment by PMB-Phage or PMB. All doses

of PMB-Phage (6.25×10^8 (N=10), 1.25×10^9 (N=10), and 2.5×10^9 (N=5) virions/dose, containing 0.007, 0.013, and 0.027 μg of PMB per dose, respectively) yielded significant CFU reductions relative to 1x PBS ($p < 0.01$, $p < 0.0001$, and $p < 0.0001$ respectively). Similarly, topical dosing of PMB at 0.5 (N=10) and 0.25 (N=5) $\mu\text{g}/\text{dose}$ resulted in significant reductions in CFU compared to 1x PBS ($p < 0.0001$ for both). PMB-Phage containing 0.027 μg of PMB caused a CFU reduction similar to 0.5 μg of PMB sulfate, indicating a ~19-fold increase in drug efficacy.

Example 22: Creating a library of phages engineered to express bacterial binding proteins targeting specific cells, and conjugation to a bioactive agent

[00433] As noted in other examples and embodiments herein, a binding moiety to a target cell may comprise an antibody or antigen-binding fragment thereof, fused to the C-terminal domain of a minor capsid protein such as pIII. In this example, another exemplary method is described for identifying and preparing recombinant bacteriophages that bind to a target cell, and to which a bioactive agent may be conjugated for exerting an activity on the target cell by the bioactive agent bound to the bacteriophage. While this example is directed to identifying bacteriophages targeting Gram negative bacteria, as disclosed herein, such methods may be used to identify and prepare bacteriophages targeting any desired cell, such as a pathogen or pathogenic cell, e.g., any pathogenic bacterium or cancer cells. Moreover, the bioactive agent may be any of those described herein; in this example, colistin (polymyxin E; PME) was used as the antibiotic bioactive agent.

[00434] As will be detailed below, libraries were created of recombinant M13 phages with great genetic diversity by assembling oligonucleotides encoding for shorter genes with a tendency to target Gram-negative bacteria, referred to herein as “DNA shotgun assembly”. From these recombinant phage libraries, binding specifically to Gram-negative pathogens is readily performed. Using such bacteriophage as a drug delivery agent for the otherwise highly nephrotoxic lipopeptide antibiotic colistin, the minimal inhibitory concentration (MIC) of colistin was lowered by 2 orders of magnitudes for multiple strains of *Pseudomonas aeruginosa*. Moreover, the MIC for an originally colistin resistant strain was brought down to below to colistin breakpoint. Such “colistin-phage” showed biocompatibility, no cytotoxicity or hemolytic activities to mammalian cells.

[00435] **Materials.** All chemical reagents used in this study, including colistin sulfate, were purchased from Millipore Sigma, if not specified otherwise. Oligonucleotides used for library construction, PCR and qPCR were ordered from Integrated DNA Technologies. Sequences of primers named in this study are listed below.

Table 5. Primers for PCR or synthetic gene fragments

Primer Name	5' - 3' Sequence
S239T_Forward	GCTGGCGGCGGCACCGGTGGTGGTTCTGGT (SEQ ID NO:9)
S239T_Reverse	CGTCTGACCTGCCTCAACCTCCTGTCAAT (SEQ ID NO:10)
Sequencing_upstream	AGTGAGCGGTACCCGATAAAAG (SEQ ID NO:11)
Sequencing_downstream	ACCAGCGCCAAAGACATAAG (SEQ ID NO:12)
Gibson_upstream	GAAGTGCCATTCCGCCTGACCTGCGGCCGCTGG ATTGTTATTACTCGCGGCCAGCCGGCCATGGCT (SEQ ID NO:13)
Gibson_downstream	GGCGGCGGCACCGGTAGGCTAGGTGGAGGCTCAGTG (SEQ ID NO:14)
qPCR-upstream	TTGGTGACGTTTCCGGCCTTGC (SEQ ID NO:15)
qPCR-downstream	TCACCGTCACCGACTTGAGCCA (SEQ ID NO:16)
OrfiF1longForward	GGTCGAGGTGCCGTAAAGCACTAAATCGGAAC CCTAAAGGG (SEQ ID NO:17)
OriF1longReverse	CCAAAAAAGTTGATTAGGGTGATGGTTCACGTA (SEQ ID NO:18)
Oligo_upstream	CGATTAGCAAGAGTCCTAGC (SEQ ID NO:22)
Oligo_downstream	CGTAGGACTCTGTGATACTC (SEQ ID NO:23)
InsertApm_upstream	GAAGTGCCATTCCGCCTGACCT (SEQ ID NO:24)
InsertApm_downstream	CACTGAGCCTCCACCTAGCCT (SEQ ID NO:25)
fill_up dummy sequence	ATGCTACGAATTGGCCTTAACCGGAAATTTGGGCCCTT TAAACCGGGTTTTCCCCAAAAGGGG (SEQ ID NO:26)

[00436] Phagemid vector pADL-10b and helper phage cm13d3 were ordered from Antibody Design Lab. All restriction enzymes, Q5 PCR reagents, and DNA HiFi assembly kit reagents were purchased from New England Biolabs. DNA purifications from enzymatic reaction steps (restriction enzyme digestion and PCR) were performed using the QIAquick PCR Purification Kit (Qiagen). DNA purifications from agarose gel electrophoresis were performed using the QIAquick Gel Extraction Kit (Qiagen). Purification of Colistin-Phage was conducted using Slide-A-lyzer (20K MWCO, Thermo Fisher Scientific). Mix & Go! Competent Cells-JM109 was used for all transformations (Zymo Research).

[00437] Microbial **strains**. The microbial strains used in this study and their sources are shown below.

Table 6. Microbial strains used in this example.

Species	Strain name
<i>Escherichia coli</i>	ATCC 700927
<i>Escherichia coli</i>	DH5alpha
<i>Escherichia coli</i>	ATCC 25922
<i>Pseudomonas aeruginosa</i>	ATCC 25102
<i>Pseudomonas aeruginosa</i>	PAKpmrB6
<i>Pseudomonas aeruginosa</i>	14 clinical isolates, designated A through M, and O
<i>Pseudomonas aeruginosa</i>	Clinical Strain 320
<i>Klebsiella pneumoniae</i>	ATCC 700603
<i>Klebsiella pneumoniae</i>	4 clinical isolates, designated A through D
<i>Acinetobacter baumannii</i>	ATCC 19606
<i>Burkholderia cepacia</i>	ATCC 25416
<i>Chronobacter sakazakii</i>	ATCC 25944
<i>Saccharomyces cerevisiae</i>	ATCC 204508 / S288c (Baker's yeast)

[00438] Bacterial and yeast strains were revived from glycerol stocks stored in -80°C by streaking on LB agar plates and culturing in 37°C incubator. A single colony from each plate was inoculated into the corresponding liquid culture and incubated at 37°C with shaking at 220 rpm. The ensure bacteria fully revived from a dormant stage into an active stage, the cells were further subcultured >3 times by inoculating 100 µL of overnight culture into 10mL fresh media prior to use for experiments.

[00439] **Receptor-binding protein library design from metagenomic data.** To create a phagemid library expressing potential phage RBDs fused to the C-terminal domain of M13 g3p, we used a published metagenomic database containing 10,293 inovirus-like genome sequences identified using machine learning (Secor, P. R. et al. Pf Bacteriophage and Their Impact on Pseudomonas Virulence, Mammalian Immunity, and Chronic Infections. Front. Immunol. 11, 244 (2020)). The inovirus-like genomes, organized in GenBank files with annotations, contain 75,325 open reading frames in total. A local database containing these ORFs (putative inovirus protein or “PIP” database) was created, labeled from “1” to “75,325”. The PIP database was used to design two different phagemid libraries. Each library was constructed using known inovirus receptor-binding proteins (RBPs) as reference sequences.

Putative receptor-binding domain (RBD) sequences from the PIP database were included based on similarity to the reference sequences. The two libraries were designed as follows.

[00440] The first library (“Pa-RBD” library) was focused on targeting *P. aeruginosa*. The PIP database was searched for sequences similar to the RBPs from *P. aeruginosa* filamentous phage Pf1. Two entries for the Pf1 RBP were found in GenBank, and both were used as reference sequences (GenBank protein ID: AAQ94686.1 and NP_039604.1). Protein sequences in the PIP database with high similarity to each reference sequence were identified using the Basic Local Alignment Search Tool (NCBI BLAST+ 2.11.0; default settings) and refined as follows. Database hits to each reference sequence were sorted by increasing E-value and those with E-value > 0.002 were removed. Given limitations on pool synthesis (described below) and the fact that the two reference RBPs were 437 and 439 amino acids in length, we removed sequences under 300 or over 550 amino acids long. To reduce redundancy, a pairwise alignment score was calculated among the remaining hits using the “Biopython” package “pairwise2” module. For any pair of hits having an alignment score $\geq$ 320, the hit with a greater E-value was removed. The remaining hits for the two reference RBPs were combined and any duplicates were removed.

[00441] To prepare sequences for phagemid expression, a starting Met was added to any sequences that lacked a starting Met. In addition, signal peptides are short N-terminal peptide sequences that direct protein translocation. Putative signal peptides were identified by SignalP 5.0, and sequences that lacked a signal peptide were considered less likely to be RBPs and were therefore removed. The phagemid vector contains a signal peptide that is compatible with phage production in *E. coli*, and therefore the native predicted signal peptides of database sequences were removed in anticipation of cloning. Similar to the *E. coli*-specific inoviruses M13 and If1, each Pf1 phage RBP contains a glycine-rich linker separating the C-terminal domain, which integrates into the capsid, and the exposed N-terminal RBD. We identified the glycine-rich linker in hits using a sliding window of 30 amino acids to identify the start of a region with the highest Gly, Glu, Asn, Thr, Asp, Ser, and Pro abundance in the sequence. The region N-terminal to this putative linker was designated as the putative RBD for each sequence (**Figure 12**). A hit was removed if it lacked a putative linker (i.e., had no window containing >9 of the listed amino acids). Putative RBDs that were shorter than 200 amino acids in length were removed; the reference RBDs have lengths of 265 and 257 amino acids. Each putative

RBD was manually inspected, in cases where the start of the glycine-rich linker is earlier than what was identified through the sliding window algorithm, the putative RBD protein length was adjusted correspondingly. The remaining 61 hits constituted the basis of the Pa-RBD library.

[00442] The amino acid sequences in Pa-RBD were reverse-translated using the Python library “DNA Chisel” following a previously described codon bias (<https://d3js.org>). Using the same tool, we adjusted codons in the designed DNA sequences to avoid restriction sites for enzymes MlyI, NcoI, and AgeI and to avoid consecutively repetitive 9-base pair stretches. Flanking upstream primer sequences (Gibson_upstream and Gibson_downstream) were added to the beginning and end of each hit for downstream PCR amplification and cloning, resulting in 61 DNA sequences designed to comprise the Pa-RBD library.

[00443] The DNA sequences would be assembled from synthetic oligonucleotides of fixed length (260 bp). Each oligonucleotide was designed as follows, from 5' to 3': a primer sequence Oligo_upstream (20 bp), followed by a designed sequence based on a Pa-RBD sequence (220 bp), and ending with the primer sequence Oligo_downstream (20 bp). The two primer sequences were used to amplify the oligonucleotides through PCR, and were then removed using the type IIS restriction enzyme MlyI. For Gibson assembly, adjacent oligonucleotides were designed to have a 30 bp overlap. Therefore, assembly of four oligonucleotides could give an RBD up to 229 amino acids. We designed two assembly reactions to create the Pa-RBD library: one comprising the 43 hits that could be assembled from four oligonucleotides (Pa4 assembly) and another comprising the 18 sequences that required five oligonucleotides (Pa5 assembly). Oligonucleotides were designed from the reverse-translated sequences by breaking up the desired RBD sequence into 220 bp-long sequences with 30 bp overlap between adjacent oligonucleotides. The last fragment of the putative RBD was not necessarily 220 bp. If the length was ≤ 64 bp, the sequence was first filled up to 64 bp using the fill_up dummy sequence at matching positions. Then, along with other last fragments under 220 bp, a series of T nucleotides was added to bring the fragment length to 220 bp (260 bp total including the PCR primers). This set of 172 (Pa4) and 90 (Pa5) oligonucleotides was synthesized commercially by Integrated DNA Technologies.

[00444] The second library (“Ino-200RBD” library) created a more diverse set sampling a larger fraction of the inovirus metagenome RBDs. We followed the same strategy as outlined

above for the PA-RBD library, with the following changes. We used 39 characterized inovirus RBPs (all known inovirus RBPs as of Sep. 2022) as reference sequences for BLAST. No hits were removed based on E-values. Based on the relatively poor assembly efficiency we observed from Pa5 compared to Pa4 (see below), we constrained the RBD design in Ino-200RBD to the N-terminal 229 amino acid residues of each RBP, which would be amenable to 4-fragment assembly. We remove RBDs with protein sequences containing “GGG” in the first 229 amino acids due to the difficulty in assembling such sequences. To limit the number of library members to a feasible number (given assembly of ~50 library members per batch), we ranked candidate RBDs by a similarity score. Each PIP database hit could match one or more than one of the 39 reference sequences with a given E-value. A similarity score was calculated as the sum of the reciprocals of E-values for matching reference sequences, and the top-scoring 200 candidates were identified. Oligonucleotides were designed and synthesized as described for the Pa-RBD library, noting that the dummy sequence was not needed. These 200 candidates were split into four batches (InoN, where N = 1 to 4) for assembly (200 oligonucleotides per batch).

[00445] **Construction of phagemid libraries.** The Pa4, Pa5, and four InoN assembly reactions were conducted as follows. 2 ng of the corresponding oligonucleotide pool (assuming 330 Da per nucleotide, approximately 7×10^7 copies of each oligonucleotide) was amplified by 15 PCR cycles, using primers Oligo_upstream and Oligo_downstream and purified using a QIAquick PCR purification kit (Qiagen). The purified pool was digested by type IIS restriction enzyme MlyI to remove the PCR primer sequences and purified using a QIAquick PCR purification kit. Each pool was assembled using the NEBUILDER® HiFi DNA Assembly Master Mix (New England Biolabs) following the manufacturer’s protocol. In brief, 10 µL of water containing 0.2 pmol of each digested and purified oligonucleotide was mixed with the reaction master mix and incubated at 50°C for 1 h. The reaction products were amplified by 20 PCR cycles, using primers InsertApm_upstream and InsertApm_downstream, run on a 1% agarose gel, and DNA from the desired band was purified using the QIAquick Gel Extraction Kit.

[00446] To clone the assembled RBD genes into phagemids for sequencing, expression, and display, an AgeI restriction site was introduced on the phagemid vector pADL-10b at the beginning of the pIII glycine-rich linker by mutating amino acid Ser239 to Thr using site-

directed mutagenesis as follows. The vector pADL-10b was amplified for 15 PCR cycles using mutagenic primers S239T_Foward and S239T_Reverse, treated with KLD Enzyme Mix, and transformed into chemically competent cells. The cells were grown and plasmid was purified using a miniprep kit. The resulting phagemid vector pADL10b-S239T and each assembly pool (Pa4, Pa5, and four InoN assembly pools) were double-digested with restriction enzymes NotI and AgeI, purified, ligated using ElectroLigase, and transformed into competent cells. The phagemid libraries were amplified by growth in *E. coli* and collected from cells using the QIAprep miniprep kit (Qiagen). The cells containing phagemid libraries for the four InoN assembly reactions were pooled, making the final Ino-200RBD phagemid library. The Pa4, Pa5, and Ino-200RBD phagemid libraries were single-digested with NcoI, purified, and characterized using PacBio Sequencing (CD Genomics). The Pa4 and Pa5 libraries were pooled together, creating the Pa-RBD phagemid library.

[00447] **Characterization of phagemid libraries.** To analyze the Pa4, Pa5, and Ino-200RBD phagemid assembly from PacBio sequencing data, all reads were first extracted into FASTA file format using the SMRT Link software (version 10.1.0.119588). Each read was screened by identifying the signal peptide and glycine-rich linker to isolate the intervening RBD sequence using the local software version of SignalP (Version 5.0b, Platform: Linux). Only sequences with both the signal peptide and the AgeI restriction site were considered valid phagemids from the library. Since PacBio sequencing reads could occur in either direction, the reads in which the glycine-rich linker was found as a reverse complement were converted to the forward strand. The sequences with codons in-frame were translated in silico, and sequences that were out-of-frame were removed from the libraries. Sequencing read databases (SR databases Pa4_read, Pa5_read, and Ino200_read) were generated using BLAST+, containing the translated putative RBDs from PacBio reads of each library.

[00448] The number of times each unique protein sequence was found in each SR database was determined. To match each sequence read in the SR databases to sequences in the original design, each read was compared to the corresponding designed phagemid library using BLAST+ and the three designed sequences with the lowest E-values were recorded. As a control, the same analysis was performed on the set of reference sequences to established the distribution expected if the sequence reads conformed perfectly to the design. To represent the sequencing data, force-directed graphs were created using the Javascript D3 graphing

library. The force governing the placement of the nodes was proportional to the E-value between the reference and RBD sequences. Due to the large distribution of E-values in the dataset, these inputted E-values were first normalized using the logarithm of the E-value divided by the most common E-value in the dataset. Then using the minimum E-value's logarithm, all of these computed values were shifted such that the minimum normalized E-value is mapped to a value of 0, representing the minimum distance between a reference and protein sequence node, following the equation:

$$E_value_{normalized} = \log\left(\frac{E_{value}}{\text{Most common } E_{value}}\right) - \min\left(\log\left(\frac{E_{value}}{\text{Most common } E_{value}}\right)\right)$$

[00449] **Production and purification of phage libraries.** 1 mL of frozen glycerol stock of *E. coli* cells transformed with each phagemid library (5×10^8 cells/mL) was inoculated in 10 mL of 2xYT media with 100 µg/mL ampicillin and 1% w/v glucose, and grown at 37 degrees C at 250 rpm until $OD_{600} = 2$. Then 1 mL of the culture was subcultured in 45 mL of the same media until $OD_{600} = 0.5$. 50 µL of helper phage cm13d3 at 1×10^{13} virions/mL were added to the culture for superinfection and cultured for 30 minutes, followed by supplementation of IPTG and kanamycin to 200 µM and 50 µg/mL respectively, to induce RBD expression. The cells were cultured overnight at 25°C and shaken at 200 rpm for phage production. The cells were pelleted by centrifugation at 7,000g for 10 minutes and the supernatant was filtered through a 0.22 µm nitrocellulose filter to remove any remaining cells or cell debris. The filtrate was concentrated to 2 mL volume and buffer-exchanged to 1 x PBS buffer using an Amicon Ultra-15 Centrifugal Filter 50kDA MWCO (Millipore). The phage concentration was determined by spectrometry following the equation

$$\frac{\text{virions}}{\text{mL}} = \frac{(A_{269} - A_{320}) \times 6 \times 10^{16}}{\frac{\text{number of bases}}{\text{phage genome}}}$$

and confirmed by qPCR using primer pair qPCR_upstream and qPCR_downstream. The phages were diluted to 1×10^{13} virions/mL with 1 x PBS buffer and stored at 4°C for short-term storage.

[00450] **Phage selection for binding bacterial cells.** To identify members of the Pa-RBD and Ino-200-RBD phagemid libraries that bind target pathogens, 2 rounds of selection were performed as follows. 4 mL of log-phase cell culture ($OD_{600}=1$) of the target pathogens (Table 6) were grown in LB media at 37 degrees (*P. aeruginosa* strains: ATCC 25102, engineered strain PAK *pmrB6* (Pm^r), and 15 clinical strains; *E. coli* strains: DH5 α and ATCC 700927; *A. baumannii* strain ATCC 19606; *B. cepacia* strain: ATCC 25416; *C. sakazakii* strain: ATCC 25944; *K. pneumoniae* strains: ATCC 700603, and four clinical strains). Cells were pelleted by centrifugation at 5000g for 5 minutes, washed twice in 1 x PBS buffer and resuspended in 1x PBS at the original volume. 100 μ L of purified phage at 1×10^{13} virions/mL was mixed with the cells and incubated on a rotator for 30 minutes at room temperature. The cells were spun down at 3,000g and washed with 5 mL of 1 x PBS buffer with 1% w/v bovine serum albumin (BSA) three times. The cells and any bound phages were pelleted and the DNA of bound phages was extracted from the pellet using a Qiagen miniprep kit. The DNA was eluted in 25 μ L of water and amplified by 15 PCR cycles using back-to-back primers Orif1longForward and Orif1longReverse. The PCR products were purified using a QIAquick PCR Purification Kit, circularized by ligation, and transformed into competent cells (*E. coli* strain TG1). The transformed cells were cultured to produce phages and the selection steps were repeated as described above. After the second round of selection, the transformed cells were plated on LB agar plates supplemented with 100 μ g/mL ampicillin and incubated at 37°C overnight. 60 colonies were picked from each library selection and sent for Sanger sequencing using primers Sequencing_upstream and Sequencing_downstream.

[00451] **Clinical isolates of *P. aeruginosa* and *K. pneumoniae*.** Clinical strains were obtained from routine aerobic bacterial cultures performed in the UCLA Clinical Microbiology Laboratory. All clinical isolates in this study were obtained from lower respiratory tract specimens including sputum and bronchoalveolar lavage. Samples were inoculated on sheep blood agar plates (Becton Dickinson, MD, USA) and incubated at 35°C in 6–10% CO₂ for 12 to 24 h. Isolates were identified to species level using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF, VITEKMS, BioMérieux, NC, USA).

[00452] **Quantitative PCR (qPCR) phage-bacteria binding assay.** The number of phages binding to different bacterial targets in each sample and the concentration of phage stocks

were determined using qPCR. The number of phagemid copies of in the samples was treated as a proxy of the number of phage virions present. To characterize phage concentration in the stock solution, 100 μL of phages was used as the sample. To quantify the amount of phage binding to different bacterial cell targets, a known quantity of phages in 100 μL volume was mixed with 1 mL of bacterial cells in the exponential growth phase at a concentration of $\text{OD}_{600} = 1$ and allowed to incubate for 30 minutes at room temperature. The cell samples with phages were washed for two times with 1 x PBS to remove any unbound phages or non-specifically bound phages through spinning down at 4,000x g for 5 minutes and resuspended in 1 x PBS buffer back to the original volume. For both assays, the samples were further processed following the standard miniprep protocol using QIAprep Spin Miniprep Kit (Qiagen) to lyse bacterial cells and phage capsid to collect the single-stranded phagemids DNA from phages within the sample. In the final elution step to recover DNA from the spin column, the genetic materials were eluted in 50 μL of MilliQ water instead of elution buffer provided in the Miniprep kit. The quantity of phagemid present in eluted samples was quantified using qPCR with a standard curve made from a known concentration standard of purified phagemid vector pADL10b. The qPCR assay was carried out in 10 μL reactions with triplicates for samples and duplicates for standards using SYBR Green Master Mix (Bio-Rad Laboratories) with primer set qPCR-upstream and qPCR-downstream on a Bio-rad C1000 PCR machine. PCR conditions: 95 °C for 30 seconds, then 45 cycles of denaturation for 30 seconds at 95 °C and extension for 15 seconds at 60°C followed by melt curve analysis (10 seconds at each increment using 0.5 °C increments up to 95°C).

[00453] **Transmission electron microscopy.** Samples were prepared for microscopy imaging of bacterial cells, phages, and phages bound to bacterial cells following a previously developed protocol (Yang, Y. & Chen, I. A. Visualization of engineered M13 phages bound to bacterial targets by transmission electron microscopy. *Methods Mol. Biol.* Springer (2024) vol. 2793, pp. 175-183. Cell samples were prepared by inoculating an overnight culture into 10 mL fresh MH broth (1:20 v/v) and allowing the culture to grow to $\text{OD}_{600}=0.5$. The cells were washed twice with 1 x PBS buffer and resuspended to 1/5 of the original volume. Phage samples were prepared at 1×10^{13} virions/mL. To image phages bound to cells, 100 μL each suspension were mixed and incubated for 30 minutes, washed two times using 1 x PBS buffer with 1% w/v (BSA), and resuspended to 100 μL volume. The TEM sample grids were prepared at room temperature by loading 8 μL of each sample on a Formvar/Carbon 200

Mesh, Ni TEM grid (Electron Microscopy Science) and incubating for 2 min. The sample grid was washed four times with washing solution (1 x PBS buffer with 1% BSA), and blocked with blocking solution (1 x PBS buffer with 0.5% w/v gelatin) for 1 hour at room temperature. To label the phages, the sample grid was incubated with primary anti-M13pVIII antibody (ThermoFisher Scientific) for 1 h, washed with washing solution four times, incubated with gold nanoparticles (6 nm dia.) coated with donkey anti-mouse IgG (Abcam) for 1 h, and washed with water five times. The sample was negatively stained with 8 μ L 1% uranyl acetate for 1.5 min, dried with a Kimwipe, and imaged on a FEI Tecnai T12 transmission electron microscope.

[00454] **Synthesis of Colistin-Phage.** Primary amines on PABinder phage were blocked by mixing 1 mL of phage at 1×10^{13} virions/mL in 1 x PBS buffer with 10 nM Sulfo-NHS-Acetate on a rotator for two hours at room temperature. The sample was dialyzed for 2 h three times using 1 x PBS buffer and two times with 0.1 M MES (pH 5.5) buffer at 4°C, using a Slide-A-Lyzer dialysis cassette (20K MWCO, Thermo Fisher Scientific). The phage product was then incubated with a mixture of EDC (20 mM) and Sulfo-NHS (50 mM) in 100 μ L of water for 20 minutes at room temperature for activation. The sample was dialyzed three times in 1 x PBS (pH 7.2) buffer for 40 minutes in each dialysis at 4°C and was mixed with 1 x PBS to reach 1×10^{12} virions/mL concentration. 100 μ L of colistin sulfate (0.75 mg/mL) was mixed with 1 mL of activated phage and incubated at room temperature for 2 hours on a rotator. The product, Colistin-Phage, was purified using dialysis for 4 hours two times with 1 x TBS buffer and three times with 1 x PBS buffer at 4°C, then filtered through a 0.22 μ m nitrocellulose membrane filter. The concentration of Colistin-Phage was determined using UV spectrometry as described above, and the sample was stored at 4°C for short-term storage up to 12 weeks. For amino acid composition analysis, 1×10^{13} virions of Colistin-Phage in total were synthesized in four separate batches and pooled together into one sample tube. The sample was shipped to and the amino acid composition analysis was performed by Creative Proteomics (Shirley, New York). The samples were hydrolyzed with 200 μ L 6 N HCl-1% phenol at 100°C for 24 h and dried under nitrogen. Aminoethanethiol-cysteine buffer were added to the hydrolysis tube to achieved a final volume of 1 mL. 50 μ L of the material were loaded into column for analysis using a Hitachi LA8080 Amino Acid Analyzer.

[00455] **Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) Assay.** To characterize the colistin and Colistin-Phage MIC and MBC, we followed an established microdilution protocol (Wiegand, I., Hilpert, K. & Hancock, R. E. W. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* 3, 163–175 (2008); Salas-Ambrosio, P., Vexler, S., Rajalakshmi, P. S., Chen, I. A. & Maynard, H. D. Caffeine and Cationic Copolymers with Antimicrobial Properties. *ACS Bio Med Chem Au* 3, 189–200 (2023). In a non-treated, U-shaped bottom 96 well plate (Fisher Scientific), 5×10^5 CFU of log-phase bacterial cells in Mueller-Hinton (MH) broth were added to each well except wells in the last column. Colistin or Colistin-Phage, serially diluted in MH broth in 2-fold dilutions, was added to each well (N=3 replicates for each reagent at each concentration). In the second to last column, 50 μ L of MH broth without antibiotics was added to wells as a growth control (N=3). In the last column, 100 μ L of MH broth was added to wells as sterility controls (N=3). The sterile covered plates were incubated overnight without shaking at 37°C. The cell growth in each well was measured using spectrometry at 600 nm on a plate reader (Tecan Group Ltd). A lack of visible cell pellet was observed to always correspond to a < 0.2 increase of OD₆₀₀ value, and these measures were interpreted as no cell growth in a well. The well containing the lowest concentration of each antibiotic resulting in no growth indicated the MIC. 50 μ L of the culture at the MIC and higher reagent concentrations were spread onto MH agar plates and incubated overnight at 37°C. The plates without colony growth indicated the reagent was bactericidal at that concentration, and the lowest bactericidal concentration gave the MBC.

[00456] **In Vitro Hemolysis Assay.** To characterize the hemolysis activity of colistin and Colistin-Phage at serially diluted concentrations, an established hemolysis assay protocol was followed ((Salas-Ambrosio, P., Vexler, S., Rajalakshmi, P. S., Chen, I. A. & Maynard, H. D. Caffeine and Cationic Copolymers with Antimicrobial Properties. *ACS Bio Med Chem Au* 3, 189–200 (2023); Evans, B. C. et al. Ex Vivo Red Blood Cell Hemolysis Assay for the Evaluation of pH-responsive Endosomolytic Agents for Cytosolic Delivery of Biomacromolecular Drugs. *J. Vis. Exp.* 7–11 (2013). doi:10.3791/50166). 5 mL of 10% sheep red blood cells (MP Biomedicals) were washed five times with 1 x PBS buffer by spinning down at 500 g for 5 minutes on a benchtop centrifuge, discarding supernatant, gently resuspending to the original volume in 1 x PBS buffer. After the last washing step, the sample was diluted to a 4% red blood cell solution and aliquoted into 1.5 mL Eppendorf tubes (100

μL in each tube). 100 μL of Colistin or Colistin-Phage at 2x the desired final concentration, 1 x PBS for negative control, or 2% Triton X-100 for positive control, was added to each tube (N=3). The mixtures were incubated for 1 hour at 37 °C. After incubation, samples were spun down at 500 g for 5 minutes, and 100 μL of supernatant from each tube was transferred into a 96-well plate. Absorbance at 405 nm was measured using a plate reader and the hemolytic activity was determined using the following equation:

$$\% \text{ hemolysis} = (A_{\text{sample}} - A_{\text{PBS}}) / (A_{\text{Triton X-100}} - A_{\text{PBS}})$$

where A_{sample} , A_{PBS} , and $A_{\text{Triton X-100}}$ are absorbance values at 405 nm of samples, negative and positive controls, respectively.

[00457] ***In Vitro* Mammalian Cell Toxicity Assay.** The toxicity of colistin and Colistin-Phage on mammalian cell cultures was assessed using human embryonic kidney (HEK-293) cells (ATCC CRL-1573) following a previously established protocol (Bang, S., Jung, U.-W. & Noh, I. op. cit.; Kılıç, H., Ceylan Tuncaboylu, D., Argun, A. & Öztürk Civelek, D., op. cit.). The cells were revived from liquid N₂ storage (90% FBS, 10% DMSO) by thawing in a 37°C water bath, pelleting, and resuspending in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin) in Falcon® polystyrene tissue culture flasks with ventilated caps (Corning). The cells were incubated at 37°C with 5% CO₂ until 80-90% confluency. Then, cells were detached from the culture flask using trypsin-EDTA and seeded at a density of 1 x 10³ cells/well in 24-well plates (Corning). DMEM with 1% antibiotics (penicillin/streptomycin) was supplemented with either colistin sulfate (2, 8, or 64 $\mu\text{g/mL}$) or Colistin-Phage (5x10⁹, 1x10¹⁰, or 2x10¹⁰ virions/mL). HEK-293 cells were also cultured in DMEM with or without penicillin/streptomycin, as controls (N=3). The media was refreshed every 2 days.

[00458] HEK-293 cell viability, indicated by metabolic activity, was quantified using an MTT assay (Millipore Sigma) following the manufacturer's protocol. After 1, 3, and 5 days of culture, 100 μL of 5 mg/mL MTT stock solution was added to the wells, incubated for 4 hours at 37°C, and mixed with 1 mL of solubilization solution and incubated at 37°C overnight. The absorbance of the samples was read at 550 nm (reference wavelength at 650 nm) using a Tecan Infinite M200 PRO plate reader (Tecan Group Ltd).

[00459] HEK-293 cell spreading and morphology were observed by staining the cells with phalloidin and DAPI at room temperature. After 1, 3, and 5 days of culture, the cells were fixed using 4% (v/v) paraformaldehyde for 10 min, washed with 0.3% (v/v) Triton-X for 10 min, and blocked with 1 x PBS buffer containing 1% bovine serum albumin (BSA) for 30 min at room temperature. A light-sensitive mixture of 0.1% (v/v) phalloidin and 0.05% (v/v) DAPI in 1 x PBS was added to stain the cells for 20 min. After washing with 1 x PBS for two times, the cells were imaged with an AxioObserver Z1 inverted microscope (Zeiss). Actin filaments were stained green while nuclei were stained blue (N=2).

Results

[00460] **Construction of phagemid libraries.** To construct libraries of recombinant M13 phages with RBD sequences from the *Inoviridae* family, we searched for sequences with similarity to known inovirus RBDs in a putative inovirus protein database (“PIP” database). The PIP database was created from protein sequences encoded by 10,295 inovirus-like genomes previously identified using machine learning from published metagenomic databases. Using two *P. aeruginosa* filamentous phage RBPs as references, we designed a “Pa-RBD” library to theoretically contain 61 protein sequences. Using all known inovirus RBPs (39 sequences as of Sep. 2022) as reference sequences, we identified the top 200 candidates for an “Ino-200RBD” library design.

[00461] To synthesize the sequences, we conducted several pooled reactions. Each reaction contained overlapping gene fragments that would be assembled into multiple full-length genes using Gibson assembly. Two assembly reactions were used to synthesize the Pa-RBD library: one for the 43 sequences that could be assembled from four oligonucleotides (Pa4 library) and one for the 18 sequences that required five oligonucleotides (Pa5 library). The larger Ino-200RBD library was synthesized using only four-oligonucleotide assembly, truncating sequences past the first 229 amino acids of each RBD, using four reactions with each reaction theoretically assembling 50 sequences. The pooled Gibson assembly reactions resulted in DNA of approximately the expected lengths. The assembled products were amplified by PCR, digested, cloned into phagemid vector pADL10b-S239T, and transformed into *E. coli* competent cells.

[00462] DNA from the Pa4, Pa5 and Ino-200RBD phagemid libraries was characterized using PacBio sequencing. Valid reads were defined as reads demonstrating an in-frame RBD placed between the signal peptide and the glycine-rich linker of the phagemid vector. While the Pa4 and Ino-200RBD libraries gave a high yield of valid reads (~80%), the Pa5 library gave a lower yield (7.5%), possibly owing to inefficient assembly of five fragments and/or sequencing errors. Thus, assembly from four fragments was more efficient than assembly from five fragments.

[00463] The length distribution of valid reads roughly matched the design for the three libraries, but most reads did not exactly match a designed sequence. For each read, a Levenshtein distance <100 compared to the reference is considered to be the correct assembly since that is less than half the length of each oligonucleotide (220 bp). Using this criteria, for the Pa5, Pa4, and Ino-200RBD libraries, 66.7%, 83.7%, and 34.5% of the designed sequences have correct assembly in the corresponding library. However, the majority of reads in each library did not exactly match a designed sequence, and presumably resulted from unintended fragment combinations and/or mutations. In general, the non-designed sequences were similar to one or more of the designed sequences, and the library as a whole showed uneven coverage of the different variants. Of the valid reads, the vast majority were unique (>99.9%; 29,047 out of 29,056 reads) in the Pa5 library, compared to 55.7% (16,956 out of 30,420 reads) in the Pa4 library and 23.8% (15,380 out of 64,583 reads) in the Ino-200RBD library. Pa4 and Pa5 were combined into a single library, Pa-RBD, for further experiments. Overall, library synthesis using pooled assembly resulted in an unexpectedly diversified set of sequences based on the inovirus metagenome. Most of the designed sequences were indeed observed in the library, and at the same time, most of the generated sequences increased the library complexity beyond the original design.

[00464] **In vitro selection of recombinant phage binding to *P. aeruginosa* strains.** To identify *P. aeruginosa*-binding clones, phages were produced from the Pa-RBD library (1×10^{12} virions) and incubated with $\sim 4 \times 10^8$ cfu of bacteria from each of 15 different *P. aeruginosa* strains (ATCC 25102 and 14 clinical isolates; Table 6). Cells were spun down and unbound phages were discarded. Phagemid DNA from the pellet was extracted, amplified, and transformed into competent *E. coli* cells to produce phages for a second round of selection. After the second round, phages binding to *P. aeruginosa* strain ATCC 25102 and nine of the

clinical isolate strains (A, B, C, E, F, G, J, K, L) were detected by PCR amplifying the phagemid. However, five clinical isolates (D, H, I, M, O) did not yield a visible PCR band, indicating no detectable phage was bound to these cells after two rounds of selection. For each of the 10 strains exhibiting bound phages, we randomly sampled 60 phagemid clones through Sanger sequencing. A single member of the Pa-RBD library (designated “PAbinder”, RBD length of 92 amino acids) dominated the bound phages across strains, constituting >90% of sampled clones, indicating that PAbinder could recognize multiple strains of *P. aeruginosa*. PAbinder phages were produced, purified, and verified for binding to eight of the *P. aeruginosa* strains, including clinical strains A, B, C, E, F, for which PAbinder was selected as described above and clinical strain E, for which PAbinder was not initially selected. PAbinder phage exhibited >100-fold more binding to each *P. aeruginosa* strain compared to a negative control (*Saccharomyces cerevisiae*). TEM images confirmed that PAbinder phage was bound to the bacterial cell wall through one terminus of the virion structure, and not bound via the sidewall, consistent with binding mediated by g3p (FIGURES 13A-13B-13C-13D-13E-13F).

[00465] **Effect of sequence deletions on PAbinder phage binding activity.** We further characterize the N-terminus domain of PAbinder receptor binding protein, a 92-mer (MW: 10 kDa) peptide termed the “PAB” protein (EVFWWQVNGIDSLRDKPFGSPGEGCSAVLAYFQSQSGSGYSFTFNLYLRNSDVEFS CSLKRLDQGEFVGEVCAPGTTDPDAPPPDPDAPPGTP; SEQ ID NO: 28). Similar to the receptor binding proteins of a few other *P. aeruginosa*-specific phages which mainly target the bacterial type IV pili, the PAB binding protein is overall negatively charged and having the characteristics of globular, hydrophilic proteins. Through machine learning tools RoseTTAFold and AlphaFold2, the PAB protein was predicted to be composed of 4 distinct domains, an unstructured N-terminal domain (residues 1-20), an α -helix (residues 21-35), a β -sheet (41-73), followed by a disordered C-terminal region (residues 74-92). To further identify the domain contributing to *P. aeruginosa*-binding of PAbinder, four deletion mutants of PAbinder were created: PAbinder-dNT (deleting N-terminal domain), PAbinder-d α (deleting alpha helix), PAbinder-d β (deleting beta sheet), and PAbinder-dCT (deleting C-terminal domain). The mutants were confirmed by whole-plasmid DNA sequencing and the expression of each mutant phage virions were confirmed by UV-vis absorption spectrometry. We compared the binding activity of wild-type and each mutant PAbinder phages by

incubating 1×10^{11} virions of each phage with 1×10^8 of *P. aeruginosa* strain ATCC 25102. The number of virions of binding WT-PAbinder is similar to that in Figure 2, the PAbinder- α , PAbinder- β , and PAbinder-dCT demonstrated similar binding strength as WT-PAbinder while PAbinder-dNT had ~ one order of magnitude less phages binding, indicating the N-terminal domain is responsible recognizing the *P. aeruginosa* surface antigen.

[00466] **Conjugation of colistin to PAbinder phage.** To test the ability of PAbinder phage to deliver antimicrobial cargo, we conjugated colistin to synthesize “Colistin-Phage”. Colistin sulfate contains primary amines from the five 2,4-diaminobutyric acid (Dab) residues, while the major coat protein of M13 and PAbinder phage, g8p, has three solvent-accessible carboxyl groups. PAbinder phage was pre-treated to block reactive amines and then conjugated to colistin via EDC chemistry. Amino acid composition analysis on the phage product was used to determine the amount of colistin conjugation. The experimentally determined fractions of Thr and Leu residues were compared to the theoretical values for varying stoichiometries of colistin to g8p. Analysis showed that an average of 2.5 colistin molecules were conjugated per copy of g8p, or ~6,750 colistin molecules per virion. This measurement of colistin:g8p ratio was used to calculate the bulk-equivalent concentration of colistin in Colistin-Phage.

[00467] **Antimicrobial activity of Colistin-Phage on *P. aeruginosa*.** The antimicrobial effect of Colistin-Phage was tested on several strains of *P. aeruginosa*, including seven clinical isolate strains, and compared to colistin sulfate. The minimal inhibitory concentration (MIC) was determined as the lowest concentration of colistin or Colistin-Phage that prevented visible cell growth. From each culture lacking observable cell growth, 50 μ L were plated on a MH agar plate to determine the minimal bactericidal concentration (MBC). To validate the MIC test, a reference strain of *E. coli* (ATCC 25922) was tested and found to have an MIC of 0.5 μ g/mL, which is within the acceptable range (0.25-1 μ g/mL). For colistin, strains with $\text{MIC} \leq 2$ μ g/mL are considered sensitive, and strains with $\text{MIC} \geq 4$ are considered resistant.

[00468] The MIC measurements showed that the colistin-sensitive *P. aeruginosa* strains, such as ATCC 25102, a common host strain for Pf phages, were also sensitive to Colistin-Phage. More importantly, comparing colistin concentrations with bulk-equivalent Colistin-Phage concentrations, Colistin-Phage reduced the MIC and MBC for these strains by >100-fold. For the colistin-resistant strain PAK pmrB6 , Colistin-Phage decreased the MIC by >400-fold, bringing the bulk-equivalent MIC value into the sensitive range (Tables 7 and 8).

Colistin-Phage exhibits potentiated antimicrobial effect, as shown by reduced MIC and MBC compared to colistin, determined *in vitro* for several strains of *P. aeruginosa*. Equivalent colistin concentrations for Colistin-Phage concentrations were calculated from the measured colistin:g8p ratio. The heat map corresponds to the MIC or MBC values as shown in the legend. * = resistant. ND = not determined due to technical limitation on phage concentration.

Table 7. Colistin-Phage exhibits potentiated antimicrobial effect, as shown by reduced MIC compared to colistin, determined *in vitro* for several strains of *P. aeruginosa*.

Species	Strain	MIC _{PA-binder phage} (virions/mL)	MIC _{Colistin} (µg/mL)	MIC _{Colistin-phage}		MIC _{PMBSO4} MIC _{Colistin-phage} (n-fold reduction in MIC)
				(virions/mL)	(µg/mL)	
<i>E. coli</i>	ATCC 25922		0.5			
<i>P. aeruginosa</i>	ATCC 25102	> 1 x 10 ¹²	1	1.25 x 10 ⁹	0.016	63
<i>P. aeruginosa</i>	PAKpmrB6	> 1 x 10 ¹²	64	8 x 10 ¹⁰	1.036	63
<i>P. aeruginosa</i>	Clinical Strain A	> 1 x 10 ¹²	0.5	1.25 x 10 ⁹	0.016	31
<i>P. aeruginosa</i>	Clinical Strain B	> 1 x 10 ¹²	0.5	6.25 x 10 ⁸	0.008	63
<i>P. aeruginosa</i>	Clinical Strain C	> 1 x 10 ¹²	0.5	6.25 x 10 ⁸	0.008	63
<i>P. aeruginosa</i>	Clinical Strain E	> 1 x 10 ¹²	0.5	6.25 x 10 ⁸	0.008	63
<i>P. aeruginosa</i>	Clinical Strain F	> 1 x 10 ¹²	0.5	6.25 x 10 ⁸	0.008	63
<i>P. aeruginosa</i>	Clinical Strain G	> 1 x 10 ¹²	0.5	6.25 x 10 ⁸	0.008	63
<i>P. aeruginosa</i>	Clinical Strain L	> 1 x 10 ¹²	0.5	1.25 x 10 ⁹	0.016	31
<i>P. aeruginosa</i>	Clinical Strain 320	> 1 x 10 ¹²	0.5	6.25 x 10 ⁸	0.008	125

Table 8. Colistin-Phage exhibits potentiated antimicrobial effect, as shown by reduced MBC compared to colistin, determined *in vitro* for several strains of *P. aeruginosa*.

Species	Strain	MBC _{PA-binder phage} (virions/mL)	MBC _{Colistin} (µg/mL)	MBC _{Colistin-phage}		MBC _{PMBSO4} / MBCColistin-phage (n-fold reduction in MBC)
				(virions/mL)	(µg/mL)	
<i>E. coli</i>	ATCC 25922		0.5			
<i>P. aeruginosa</i>	ATCC 25102	> 1 x 10 ¹²	1	1.25 x 10 ⁹	0.016	63
<i>P. aeruginosa</i>	PAKpmrB6	> 1 x 10 ¹²	> 128	> 8 x 10 ¹⁰	> 1.036	

<i>P. aeruginosa</i>	Clinical Strain A	$> 1 \times 10^{12}$	0.5	1.25×10^9	0.016	31
<i>P. aeruginosa</i>	Clinical Strain B	$> 1 \times 10^{12}$	0.5	6.25×10^8	0.008	63
<i>P. aeruginosa</i>	Clinical Strain C	$> 1 \times 10^{12}$	0.5	6.25×10^8	0.008	63
<i>P. aeruginosa</i>	Clinical Strain E	$> 1 \times 10^{12}$	0.5	6.25×10^8	0.008	63
<i>P. aeruginosa</i>	Clinical Strain F	$> 1 \times 10^{12}$	0.5	6.25×10^8	0.008	63
<i>P. aeruginosa</i>	Clinical Strain G	$> 1 \times 10^{12}$	0.5	6.25×10^8	0.008	63
<i>P. aeruginosa</i>	Clinical Strain L	$> 1 \times 10^{12}$	0.5	2.5×10^9	0.032	16
<i>P. aeruginosa</i>	Clinical Strain 320	$> 1 \times 10^{12}$	2	6.25×10^8	0.008	250

[00469] ***In vitro* hemolysis and cytotoxicity assays.** Hemolysis activity was tested for colistin (2 to 64 $\mu\text{g/mL}$) and Colistin-Phage (2.5×10^9 to 8×10^{10} virions/mL) by incubation with sheep red blood cells. Materials exhibiting less than 2% hemolysis are considered non-hemolytic. Similar to previous reports, we observed no hemolytic activity for colistin at the concentrations tested, although hemolysis approached 2% at the highest concentration of colistin (64 $\mu\text{g/mL}$). For Colistin-Phage, no hemolytic activity (<0.4%) was observed over the concentration range, which covers the range of MICs observed, including for the most resistant strain tested (PAK*pmrB6*).

[00470] The effect of colistin and Colistin-Phage on mammalian cell viability was tested by the MTT assay using human embryonic kidney cells (HEK-293; ATCC CRL-1573). >80% cell viability in the MTT assay is an accepted threshold for a condition to be considered non-toxic. No detectable *in vitro* toxicity was observed up to 64 $\mu\text{g/mL}$ colistin or up to 2×10^{10} virions/mL of Colistin-Phage, compared to the negative control, up to day 5. These results indicate that Colistin-Phage demonstrates apparent compatibility with mammalian cells *in vitro*.

[00471] **Stability of Colistin-Phage in refrigerated storage.** The MIC of the same batch of Colistin-Phage, stored in 1x PBS buffer at 4°C, was tested weekly on *P. aeruginosa* strain ATCC 25102 for 12 consecutive weeks. The MIC stayed within a factor of two over the 12 weeks (>50% activity retained).

[00472] ***In vitro* selection of recombinant phages binding to various Gram-negative organisms.** To test the generalizability of phage selection from a metagenome-derived library, we conducted two rounds of *in vitro* selection from the Ino-200RBD library for binding to

each of the following 11 Gram-negative strains: *E. coli* DH5 α , *E. coli* (ATCC 700927), *P. aeruginosa* (ATCC 25102), *A. baumannii* (ATCC 19606), *B. cepacia* (ATCC 25416), *C. sakazakii* (ATCC 25944), *K. pneumoniae* (ATCC 700603), and *K. pneumoniae* clinical strains (A, B, C, and D). Bound phages were isolated for each strain. The lengths of the RBDs of bound phages from Ino-200RBD ranged from 23 to 251 amino acids, with most RBDs being around 100 amino acids long. Multiple sequence alignments were conducted using COBALT⁴⁶ on RBDs of all selected phages in both libraries (Pa-RBD and Ino-RBD₂₀₀) to identify homology across the selected phages. A 70 amino acid consensus fragment was identified

(GSVPADTNCPGMTGPSLNRSTPPYCPTDPNGPPSDPDNDPGCPAQGAGYSWSGTT CVKTEPDPTDPEDPT; SEQ ID NO:27), and BLASTP (non-redundant protein sequences database) indicated homology of this fragment to *P. aeruginosa* attachment proteins. Overall, some phages were isolated from Ino-200RBD selections against multiple strains, suggesting a broad binding range among Gram-negative organisms. At the same time, other phages were only isolated from a single selection (e.g., phages IB2 and IB3), suggesting higher specificity.

[00473] Thus, the methods described for targeting bacteriophage using antigen-binding components of an anti-LPS antibody are applicable to other methods for identifying, preparing and using bacteriophages targeted to other pathogenic cells such as bacteria, as well as other target cells.

Example 23: Production of Recombinant Bacteriophage and Methods of Use

[00474] Following the guidance provided elsewhere herein, a recombinant bacteriophage is designed to target and selectively bind to a tumor marker but not normal cells from the same patient. A sequence encoding a protein (e.g., an antigen binding region that targets and binds to the tumor marker) is cloned into a bacteriophage.

Example 24: Production of Recombinant M13 Bacteriophage Using a Single Vector and Methods of Use

[00475] A recombinant bacteriophage is designed to target and selectively bind to tumor marker. A sequence encoding a protein (e.g., an antigen binding region that targets and binds to the tumor marker) is cloned in recombinant protein into a phage vector, a large single vector containing some or all of the 10 genes of M13 phage with size around 8 kbp including

antibiotic resistance selection markers.

Example 25: Production of Recombinant M13 Bacteriophage Using a Phagemid Vector and a Helper Phage and Methods of Use

[00476] A recombinant bacteriophage is designed to target and selectively bind to a tumor marker. A phagemid vector is used in combination with helper phages to construct the recombinant bacteriophage. A phagemid vector is a smaller vector with size around 5 kbp that only encodes genes for receptor binding protein pIII and other elements of a vector, which is useful for cloning large fragments of proteins, such as an antibody Fab region (e.g., capable of targeting the tumor marker). After cloning in gene of interest, the other gene is provided by a helper phage that contains all other gene but the pIII gene. Thus, *E. coli* host cells have all the genes to make new virions but only with the recombinant pIII genes.

Example 26: Production of Recombinant M13 Bacteriophage Comprising a Therapeutic Moiety and Methods of Use

[00477] A recombinant M13 bacteriophage is designed to target and selectively bind to a tumor marker expressed on tumor. The recombinant protein is constructed using one of the methods described above. An anticancer bioactive agent is conjugated at a high concentration on the recombinant M13 phage major capsid proteins and is delivered to the cancer cell surface via the binding of the phage receptor to the cell surface tumor marker. This method facilitates delivery of a high concentration of the anticancer agent to the tumor cell surface. Anticancer agents with small therapeutic indices, i.e., they are toxic to the patient near the concentrations needed for anticancer efficacy, are particularly amenable to the methods herein.

Example 27: Production of Anticancer agent-Recombinant Bacteriophage Conjugates and Methods of Use

[00478] A recombinant bacteriophage is designed to target and selectively bind to a tumor marker. An anticancer agent is bound thereto. The anticancer agent-recombinant phage conjugate can effectively lower the minimum effective dose of the anticancer agent by up to an order of magnitude or more *in vitro* compared with the anticancer agent in its free drug formulation. Through achieving a higher local concentration, tumors can be treated selectively with lower toxicity to the patient.

Example 28: Production of a Antitumor Agent-Recombinant M13 Bacteriophage Conjugate and Methods of Use

[00479] A recombinant M13 bacteriophage is designed to selectively target and bind to a tumor. An anticancer agent with a poor therapeutic index is conjugated at a high concentration on the recombinant M13 phage major capsid proteins and are delivered to the tumor cell surface.

Example 29: Production of a MMAE-Recombinant M13 Bacteriophage Conjugate and Methods of Use

[00480] A recombinant M13 bacteriophage is designed to target and specifically bind to a patient's tumor. The anticancer agent monomethyl auristatin E (MMAE) is conjugated to M13 bacteriophage major capsid proteins (cylindrical surface). The conjugated molecules are delivered to the proximity of a tumor cell through the recombinant M13 phage binding through the Fab domain of the antitumor antibody (between cylindrical portion and bacterial cell) expressed on the recombinant M13 phage minor capsid proteins.

[00481] The MMAE-Phage conjugate can effectively raise the therapeutic index of the anticancer agent by targeting the agent to the tumor cells and avoiding offtarget sites responsible for toxicity. Due to an up to or more than an order of magnitude increase in the therapeutic index, it is expected that the MMAE-Phage conjugate would have antitumor efficacy with lower toxicity.

Example 30: Production of a MMAE-Recombinant M13 Bacteriophage Conjugate and Methods of Use

[00482] A recombinant M13 bacteriophage is designed to target and bind to non-small cell lung cancer cells using an Fab of trastuzumab

[00483] The MMAE-Phage conjugate can effectively lower the minimal inhibitory concentration of the MMAE by an order of magnitude *in vitro* compared with MMAE in its free drug form. Through achieving a higher local concentration, tumor cells that were previously resistant to MMAE become sensitive to the MMAE-Phage conjugate.

CLAIMS

1. A filamentous phage comprising:
 - a. a binding moiety to a target cell, said binding moiety expressed on a minor capsid protein of the filamentous phage; and
 - b. a bioactive agent bound to a plurality of major capsid proteins of the filamentous phage, said bioactive agent exerting an activity on the target cell.
2. The filamentous phage of claim 1, wherein the target cell is an infectious organism or a pathological cell.
3. The filamentous phage of claim 2, wherein the infectious organism is a bacterium, fungus or parasite.
4. The filamentous phage of claim 1 wherein the binding moiety binds to peptidoglycan.
5. The filamentous phage of claim 3, wherein the bacterium is a Gram positive bacterium.
6. The method of claim 5, wherein the Gram positive bacterium is *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Clostridium difficile*, *Clostridium botulinum*, *Clostridium tetani*, *Listeria monocytogenes*, *Bacillus anthracis* or *Corynebacterium diphtheriae*.
7. The filamentous phage of claim 1 wherein the binding moiety binds to lipopolysaccharide (LPS).
8. The filamentous phage of claim 3 wherein the bacterium is a Gram negative bacterium.
9. The filamentous phage of claim 8 wherein the Gram negative bacterium is *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Burkholderia*

cepacia, *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Salmonella* spp. *Shigella* spp. and *Vibrio cholerae*.

10. The filamentous phage of claim 1, wherein the binding moiety binds to a yeast cell surface structure, protein and/or cell wall component.
11. The filamentous phage of claim 3, wherein the fungus is a yeast.
12. The filamentous phage of claim 2, wherein the pathological cell is a cancer cell, an infected cell, an immune cell or an autoreactive immune cell.
13. The filamentous phage of claim 12, wherein the cancer cell is a solid tumor cancer cell.
14. The filamentous phage of any one of claims 1-13, wherein the binding moiety is expressed on minor capsid protein pIII, pVI, pVII or pIX of the filamentous phage.
15. The filamentous phage of claim 1, wherein the binding moiety expressed on a minor capsid protein of the filamentous phage replaces the N-terminal domain of the minor capsid protein.
16. The filamentous phage of any ones of claim 1-15, wherein the binding moiety to the target cell comprises an antibody, binding fragment thereof, or cell-targeted receptor binding domain.
17. The filamentous phage of claim 16 wherein the binding moiety comprises a Fab, scFab, scFv or dAb.
18. The filamentous phage of claim 16, wherein the antibody or binding fragment thereof comprises a scFab domain or binding fragment thereof.
19. The filamentous phage of claim 18, wherein the infectious organism is a Gram negative bacterium and the scFab domain comprises WN1 222-5.
20. The filamentous phage of claim 1 wherein the filamentous phage is a Ff phage.

21. The filamentous phage of claim 20 wherein the filamentous phage is a M13 phage.
22. The filamentous phage of claim 21, wherein the minor capsid protein is pIII, pVI, pVII or pIX.
23. The filamentous phage of claim 22, wherein the minor capsid protein is pIII.
24. The filamentous phage of claim 23 wherein the binding moiety expressed on pIII comprises SEQ ID NO:2 (HLL-pIII) or SEQ ID NO:4 (LLH-pIII).
25. The filamentous phage of claim 1, wherein the phage does not predominantly bind to bacterial pili.
26. The filamentous phage of claim 3, 5 or 8, wherein the filamentous phage is unable to enter bacterial cells.
27. The filamentous phage of claim 1, wherein the phage is non-replicating.
28. The filamentous phage of claim 1, wherein the major capsid protein is pVIII.
29. The filamentous phage of claim 1, wherein the bioactive agent is a cytotoxic agent, anticancer agent, immune checkpoint inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme.
30. The filamentous phage of claim 1, wherein the bioactive agent is an antibiotic, antimicrobial, antifungal, antiparasitic, immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent.
31. The filamentous phage of claim 29 or 30, wherein the bioactive agent is bound covalently to the major capsid protein.
32. The filamentous phage of claim 31, wherein the bioactive agent is covalently bound via reactive groups thereon to a reactive group on the major capsid protein by means of direct conjugation or a bifunctional cross-linking agent.

33. The filamentous phage of claim 30, wherein the antibiotic is a Gram positive bacterium targeting antibiotic.
34. The filamentous phage of claim 33, wherein the antibiotic is daptomycin, penicillin, tetracycline, erythromycin, vancomycin, or an analogue thereof.
35. The filamentous phage of claim 30, wherein the antibiotic is a fungus targeting antibiotic.
36. The filamentous phage of claim 35, wherein the fungus targeting antibiotic is amphotericin B, an echinocardin or an analogue thereof.
37. The filamentous phage of claim 30, wherein the antibiotic is a Gram negative bacterium targeting antibiotic.
38. The filamentous phage of claim 37, wherein the Gram negative bacterium is *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Salmonella* spp. *Shigella* spp. or *Vibrio cholerae*.
39. The filamentous phage of claim 30, wherein the antibiotic is streptomycin or an analogue thereof.
40. The filamentous phage of claim 37, wherein the Gram negative antibiotic is a lipopeptide.
41. The filamentous phage of claim 40 wherein the lipopeptide is a cyclic lipopeptide.
42. The filamentous phage of claim 41, wherein the cyclic lipopeptide is a polymyxin.
43. The filamentous phage of claim 42, wherein the polymyxin is polymyxin B or polymyxin E.
44. The filamentous phage of claim 43, wherein the major capsid protein is pVIII.
45. The filamentous phage of claim 44, wherein the pVIII is bound to polymyxin B by amide linkages.

46. The filamentous phage of claim 30, wherein the ratio of antibiotic to major coat protein is less than or equal to 3:1.
47. The filamentous phage of claim 30, wherein the minimal inhibitory concentration of the filamentous phage is at least 2X, 5X, 10X, 20X, 50X or 100X lower than the minimal inhibitory concentration of the antibiotic alone.
48. The filamentous phage of claim 30, wherein the nephrotoxicity thereof is reduced compared to that of the antibiotic alone.
49. The filamentous phage of claim 30, wherein an infectious organism that is resistant to the antibiotic alone is sensitive to the filamentous phage.
50. The filamentous phage of claim 35, wherein the antibiotic is an amphotericin B, an echinocandin or an analogue thereof.
51. The filamentous phage of claim 30, wherein the anticancer agent is a cytotoxic agent, immune checkpoint inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme.
52. The filamentous phage of claim 51, wherein the cytotoxic agent is an antibiotic, antimicrobial, antifungal, antiparasitic, immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent.
53. A method for treating a target cell in a subject comprising exposing a subject in need thereof to filamentous phage comprising:
 - a. a binding moiety to the target cell, said binding moiety expressed on a minor capsid protein of the filamentous phage; and
 - b. a bioactive agent that exerts an activity on the target cell, the bioactive agent bound to a plurality of major capsid proteins of the filamentous phage.
54. The method of claim 53, wherein the target cell is an infectious organism or a pathological cell.

55. The method of claim 53, wherein the pathological cell is a cancer cell, an infected cell, an immune cell or an autoreactive immune cell.
56. The method of claim 53, wherein the binding moiety is expressed on minor capsid protein pIII, pVI, pVII or pIX of the filamentous phage.
57. The method of claim 56, wherein the binding moiety expressed on a minor capsid protein of the filamentous phage replaces the N-terminal domain of the minor capsid protein.
58. The method of claim 53, wherein the binding moiety comprises a Fab, scFab, scFv or dAb.
59. The method of claim 53, wherein the binding domain comprises a scFab domain or binding fragment thereof.
60. The method of claim 53, wherein the filamentous phage is a Ff phage.
61. The method of claim 53, wherein the filamentous phage is a M13 phage.
62. The method of claim 61, wherein the minor capsid protein is pIII, pVI, pVII or pIX.
63. The method of claim 62, wherein the minor capsid protein is pIII.
64. The method of claim 53, wherein the filamentous phage does not predominantly bind to bacterial pili.
65. The method of claim 53, wherein the filamentous phage is unable to enter bacterial cells.
66. The method of claim 53, wherein the filamentous phage is non-replicating.
67. The method of claim 53, wherein the major capsid protein is pVIII.
68. The method of claim 53, wherein the bioactive agent is a cytotoxic agent, anticancer agent, immune checkpoint inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme.

69. The method of claim 68, wherein the cytotoxic agent is an antibiotic, antimicrobial, antifungal, antiparasitic, immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent.
70. The method of claim 53, wherein the bioactive agent is bound covalently to the major capsid protein.
71. The method of claim 70, wherein the bioactive agent is covalently bound via reactive groups thereon to a reactive group on the major capsid protein by means of direct conjugation or a bifunctional cross-linking agent.
72. A method for treating a pathogen or pathogenic cell comprising exposing a subject in need thereof to filamentous phage comprising:
- a. a binding moiety to the pathogen or pathogenic cell, said binding moiety expressed on a minor capsid protein of the filamentous phage; and
 - b. a bioactive agent that exerts an activity against the pathogen or pathogenic cell, the bioactive agent bound to a plurality of major capsid proteins of the filamentous phage.
73. The method of claim 72, wherein the pathogen or pathogenic cell is an infectious organism or a pathological cell.
74. The method of claim 73, wherein the pathogen is an infectious organism is a bacterium, fungus or parasite.
75. The method of claim 72, wherein the binding moiety binds to peptidoglycan.
76. The method of claim 74, wherein the bacterium is a Gram positive bacterium.
77. The method of claim 76, wherein the Gram positive bacterium is *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Clostridium difficile*, *Clostridium botulinum*, *Clostridium tetani*, *Listeria monocytogenes*, *Bacillus anthracis* or *Corynebacterium diphtheriae*.

78. The method of claim 72, wherein the binding moiety binds to lipopolysaccharide (LPS).
79. The method of claim 74 wherein the bacterium is a Gram negative bacterium.
80. The method of claim 79, wherein the Gram negative bacterium is *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Salmonella* spp. *Shigella* spp. and *Vibrio cholerae*.
81. The method of claim 74, wherein the binding moiety binds to a yeast cell surface structure, protein and/or cell wall component.
82. The method of claim 74, wherein the fungus is a yeast.
83. The method of claim 73, wherein the pathological cell is a cancer cell, an infected cell, an immune cell or an autoreactive immune cell.
84. The method of claim 83, wherein the cancer cell is a solid tumor cancer cell.
85. The method of claim 72, wherein the binding moiety is expressed on minor capsid protein pIII, pVI, pVII or pIX of the filamentous phage.
86. The method of claim 85, wherein the binding moiety expressed on a minor capsid protein of the filamentous phage replaces the N-terminal domain of the minor capsid protein.
87. The method of claim 72, wherein the binding moiety to the pathogen or pathogenic cell comprises an antibody, binding fragment thereof, or cell-targeted receptor binding domain.
88. The method of claim 72, wherein the binding moiety comprises a Fab, scFab, scFv or dAb.
89. The method of claim 88, wherein the antibody or binding fragment thereof comprises a scFab domain or binding fragment thereof.

90. The method of claim 73, wherein the infectious organism is a Gram negative bacterium and the scFab domain comprises WN1 222-5.
91. The method of claim 72, wherein the filamentous phage is a Ff phage.
92. The method of claim 91, wherein the filamentous phage is a M13 phage.
93. The method of claim 92, wherein the minor capsid protein is pIII, pVI, pVII or pIX.
94. The method of claim 93, wherein the minor capsid protein is pIII.
95. The method of claim 94, wherein the binding moiety expressed on pIII comprises SEQ ID NO:2 (HLL-pIII) or SEQ ID NO:4 (LLH-pIII).
96. The method of claim 72, wherein the phage does not predominantly bind to bacterial pili.
97. The method of claim 72, wherein the filamentous phage is unable to enter bacterial cells.
98. The method of claim 72, wherein the phage is non-replicating.
99. The method of claim 72, wherein the major capsid protein is pVIII.
100. The method of claim 72, wherein the bioactive agent is a cytotoxic agent, anticancer agent, immune checkpoint inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme.
101. The method of claim 100, wherein the cytotoxic agent is an antibiotic, antimicrobial, antifungal, antiparasitic, immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent.
102. The method of claim 72, wherein the bioactive agent is bound covalently to the major capsid protein.

103. The method of claim 102, wherein the bioactive agent is covalently bound via reactive groups thereon to a reactive group on the major capsid protein by means of direct conjugation or a bifunctional cross-linking agent.
104. The method of claim 72, wherein the pathogen is a Gram negative bacterium, and the bioactive agent is an antibiotic.
105. A method for treating an microbial infection comprising exposing a subject in need thereof to filamentous phage comprising:
- a. a binding moiety to an infectious organism, said binding moiety expressed on a minor capsid protein of the filamentous phage; and
 - b. an antibiotic active against the infectious organism, the antibiotic bound to a plurality of major capsid proteins of the filamentous phage.
106. The method of claim 105, wherein the infectious organism is a bacterium, fungus or parasite.
107. The method of claim 105, wherein the binding moiety binds to peptidoglycan.
108. The method of claim 106, wherein the bacterium is a Gram positive bacterium.
109. The method of claim 108, wherein the Gram positive bacterium is *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Clostridium difficile*, *Clostridium botulinum*, *Clostridium tetani*, *Listeria monocytogenes*, *Bacillus anthracis* or *Corynebacterium diphtheriae*.
110. The method of claim 105, wherein the binding moiety binds to lipopolysaccharide (LPS).
111. The method of claim 106, wherein the bacterium is a Gram negative bacterium.
112. The method of claim 111, wherein the Gram negative bacterium is *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Burkholderia cepacia*,

Acinetobacter baumannii, *Neisseria gonorrhoeae*, *Neisseria meningitidis*,
Salmonella spp. *Shigella* spp. and *Vibrio cholerae*.

113. The method of claim 105, wherein the binding moiety binds to a yeast cell surface structure, protein and/or cell wall component.
114. The method of claim 106, wherein the fungus is a yeast.
115. The method of claim 105, wherein the binding moiety is expressed on minor capsid protein pIII, pVI, pVII or pIX of the filamentous phage.
116. The method of claim 115, wherein the binding moiety expressed on a minor capsid protein of the filamentous phage replaces the N-terminal domain of the minor capsid protein.
117. The method of claim 105, wherein the binding moiety to the target cell comprises an antibody, binding fragment thereof, or cell-targeted receptor binding domain.
118. The method of claim 105, wherein the binding moiety comprises a Fab, scFab, scFv or dAb.
119. The method of claim 118, wherein the antibody or binding fragment thereof comprises a scFab domain or binding fragment thereof.
120. The method of claim 119, wherein the infectious organism is a Gram negative bacterium and the scFab domain comprises WN1 222-5.
121. The method of claim 105, wherein the filamentous phage is a Ff phage.
122. The method of claim 121 wherein the filamentous phage is a M13 phage.
123. The method of claim 105, wherein the minor capsid protein is pIII, pVI, pVII or pIX.
124. The method of claim 123, wherein the minor capsid protein is pIII.
125. The method of claim 124, wherein the binding moiety expressed on pIII comprises SEQ ID NO:2 (HLL-pIII) or SEQ ID NO:4 (LLH-pIII).

126. The method of claim 105, wherein the phage does not predominantly bind to bacterial pili.
127. The method of claim 105, wherein the filamentous phage is unable to enter bacterial cells.
128. The method of claim 105, wherein the phage is non-replicating.
129. The method of claim 105, wherein the major capsid protein is pVIII.
130. The method of claim 105, wherein the antibiotic is bound covalently to the major capsid protein.
131. The method of claim 130, wherein the antibiotic is covalently bound via reactive groups thereon to a reactive group on the major capsid protein by means of direct conjugation or a bifunctional cross-linking agent.
132. The method of claim 105, wherein the antibiotic is a Gram positive bacterium targeting antibiotic.
133. The method of claim 132, wherein the antibiotic is daptomycin, penicillin, tetracycline, erythromycin, vancomycin, or an analogue thereof.
134. The method of claim 105, wherein the antibiotic is a fungus targeting antibiotic.
135. The method of claim 134, wherein the fungus targeting antibiotic is amphotericin B, an echinocardin or an analogue thereof.
136. The method of claim 105, wherein the antibiotic is a Gram negative bacterium targeting antibiotic.
137. The method of claim 105, wherein the antibiotic is streptomycin or an analogue thereof.
138. The method of claim 136, wherein the Gram negative antibiotic is a lipopeptide.
139. The method of claim 138, wherein the lipopeptide is a cyclic lipopeptide.

140. The method of claim 139, wherein the cyclic lipopeptide is a polymyxin.
141. The method of claim 140, wherein the polymyxin is polymyxin B or polymyxin E.
142. The method of claim 141, wherein the major capsid protein is pVIII.
143. The method of claim 142, wherein the pVIII is bound to polymyxin B or polymyxin E by amide linkages.
144. The method of claim 105, wherein the ratio of antibiotic to major coat protein is less than or equal to 3:1.
145. The method of claim 105, wherein the minimal inhibitory concentration of the filamentous phage is at least 2X, 5X, 10X, 20X, 50X or 100X lower than the minimal inhibitory concentration of the antibiotic alone.
146. The method of claim 105, wherein the nephrotoxicity of the filamentous phage is reduced compared to that of the antibiotic alone.
147. The method of claim 105, wherein the infectious organism that is resistant to the antibiotic alone is sensitive to the filamentous phage.
148. The method of claim 105, wherein the antibiotic is an amphotericin B, an echinocandin or an analogue thereof.
149. A method for treating cancer comprising exposing a subject in need thereof to filamentous phage comprising:
 - a. a binding moiety to the cancer, said binding moiety expressed on a minor capsid protein of the filamentous phage; and
 - b. a bioactive agent that exerts activity against the cancer, the bioactive agent bound to a plurality of major capsid proteins of the filamentous phage.
150. The method of claim 149, wherein the cancer cell is a solid tumor cancer cell.

151. The method of claim 149, wherein the binding moiety is expressed on minor capsid protein pIII, pVI, pVII or pIX of the filamentous phage.
152. The method of claim 149, wherein the binding moiety expressed on a minor capsid protein of the filamentous phage replaces the N-terminal domain of the minor capsid protein.
153. The method of claim 149, wherein the binding moiety to the cancer comprises an antibody, binding fragment thereof, or cell-targeted receptor binding domain.
154. The method of claim 149, wherein the binding moiety comprises a Fab, scFab, scFv or dAb.
155. The method of claim 154, wherein the antibody or binding fragment thereof comprises a scFab domain or binding fragment thereof.
156. The method of claim 149, wherein the filamentous phage is a Ff phage.
157. The method of claim 156, wherein the filamentous phage is a M13 phage.
158. The method of claim 157, wherein the minor capsid protein is pIII, pVI, pVII or pIX.
159. The method of claim 158, wherein the minor capsid protein is pIII.
160. The method of claim 149, wherein the filamentous phage does not predominantly bind to bacterial pili.
161. The method of claim 149, wherein the filamentous phage is unable to enter bacterial cells.
162. The method of claim 149, wherein the phage is non-replicating.
163. The method of claim 149, wherein the major capsid protein is pVIII.
164. The method of claim 149, wherein the bioactive agent against the cancer is a cytotoxic agent, anticancer agent, immune checkpoint inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme.

165. The method of claim 149, wherein the bioactive agent against the cancer is an immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent.
166. The method of claim 149, wherein the bioactive agent is bound covalently to the major capsid protein.
167. The method of claim 166, wherein the bioactive agent is covalently bound via reactive groups thereon to a reactive group on the major capsid protein by means of direct conjugation or a bifunctional cross-linking agent.
168. The method of claim 166, wherein the ratio of bioactive agent to major coat protein is less than or equal to 3:1.
169. The method of claim 149, wherein the minimal effective dose of the filamentous phage is at least 2X, 5X, 10X, 20X, 50X or 100X lower than the minimal effective dose of the bioactive agent alone.
170. The method of any one of claims 53-169, wherein the filamentous phage is administered topically, intranasally, parenterally or by inhalation.
171. The method of claim 170, wherein parenterally is paracancerally, intramuscularly, intravenously, intradermally, subcutaneously, intraperitoneally, intraventricularly, intracranially or intratumorally.
172. The method of claim 105, wherein the subject is in septic shock.
173. The method of claim 105, wherein the subject has a community acquired infection or community-onset bacteremia
174. The method of claim 105, wherein the subject has a urogenital infection.
175. The method of claim 105, wherein the subject has a systemic fungal infection.
176. The method of claim 105, wherein the subject has cancer.

177. A method for inhibiting microorganism growth on a surface comprising treating the surface with the filamentous phage of claim 2.
178. The method of claim 177, wherein the surface is a biological surface, implantable medical device, catheter, surgical or medical instrument, implantable prosthesis, artificial joint, or dental post.
179. A method for making a filamentous phage comprising a binding moiety to a target cell expressed on a minor capsid protein thereof, and comprising a bioactive agent bound to a plurality of major capsid proteins thereof, wherein said bioactive agent exerting an activity on the target cell, the method comprising the steps of:
- a. producing filamentous phages that express the minor capsid protein comprising the binding moiety to the target cell; and
 - b. binding the bioactive agent to the major capsid protein thereof.
180. The method of claim 179, wherein the target cell is an infectious organism or a pathological cell.
181. The method of claim 180, wherein the infectious organism is a bacterium, fungus or parasite.
182. The method of claim 179, wherein the binding moiety binds to peptidoglycan.
183. The method of claim 181, wherein the bacterium is a Gram positive bacterium.
184. The method of claim 183, wherein the Gram positive bacterium is *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Clostridium difficile*, *Clostridium botulinum*, *Clostridium tetani*, *Listeria monocytogenes*, *Bacillus anthracis* or *Corynebacterium diphtheriae*.
185. The method of claim 179, wherein the binding moiety binds to lipopolysaccharide (LPS).
186. The method of claim 181, wherein the bacterium is a Gram negative bacterium.

187. The method of claim 186, wherein the Gram negative bacterium is *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Salmonella* spp. *Shigella* spp. and *Vibrio cholerae*.
188. The method of claim 179, wherein the binding moiety binds to a yeast cell surface structure, protein and/or cell wall component.
189. The method of claim 181, wherein the fungus is a yeast.
190. The method of claim 180, wherein the pathological cell is a cancer cell, an infected cell, an immune cell or an autoreactive immune cell.
191. The method of claim 190, wherein the cancer cell is a solid tumor cancer cell.
192. The method of any one of claims 179-191, wherein the binding moiety is expressed on minor capsid protein pIII, pVI, pVII or pIX of the filamentous phage.
193. The method of claim 179, wherein the binding moiety expressed on a minor capsid protein of the filamentous phage replaces the N-terminal domain of the minor capsid protein.
194. The filamentous phage of any ones of claim 179-193, wherein the binding moiety to the target cell comprises an antibody, binding fragment thereof, or cell-targeted receptor binding domain.
195. The method of claim 194, wherein the binding moiety comprises a Fab, scFab, scFv or dAb.
196. The method of claim 195, wherein the antibody or binding fragment thereof comprises a scFab domain or binding fragment thereof.
197. The method of claim 196, wherein the infectious organism is a Gram negative bacterium and the scFab domain comprises WN1 222-5.
198. The method of claim 179, wherein the filamentous phage is a Ff phage.

199. The method of claim 198, wherein the filamentous phage is a M13 phage.
200. The method of claim 199, wherein the minor capsid protein is pIII, pVI, pVII or pIX.
201. The method of claim 200, wherein the minor capsid protein is pIII.
202. The method of claim 201, wherein the binding moiety expressed on pIII comprises SEQ ID NO:2 (HLL-pIII) or SEQ ID NO:4 (LLH-pIII).
203. The method of claim 179, wherein the major capsid protein is pVIII.
204. The method of claim 179, wherein the bioactive agent is a cytotoxic agent, anticancer agent, immune checkpoint inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme.
205. The method of claim 179, wherein the bioactive agent is an antibiotic, antimicrobial, antifungal, antiparasitic, immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent.
206. The method of claim 204 or 205, wherein the bioactive agent is bound covalently to the major capsid protein.
207. The method of claim 206, wherein the bioactive agent is covalently bound via reactive groups thereon to a reactive group on the major capsid protein by means of direct conjugation or a bifunctional cross-linking agent.
208. The method of claim 205, wherein the antibiotic is a Gram positive bacterium targeting antibiotic.
209. The method of claim 208, wherein the antibiotic is daptomycin, penicillin, tetracycline, erythromycin, vancomycin, or an analogue thereof.
210. The method of claim 205, wherein the antibiotic is a fungus targeting antibiotic.
211. The method of claim 210, wherein the fungus targeting antibiotic is amphotericin B, an echinocardin or an analogue thereof.

- 212. The method of claim 205, wherein the antibiotic is a Gram negative bacterium targeting antibiotic.
- 213. The method of claim 212, wherein the Gram negative bacterium is *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Salmonella* spp. *Shigella* spp. or *Vibrio cholerae*.
- 214. The method of claim 212, wherein the antibiotic is streptomycin or an analogue thereof.
- 215. The method of claim 212, wherein the Gram negative antibiotic is a lipopeptide.
- 216. The method of claim 215 wherein the lipopeptide is a cyclic lipopeptide.
- 217. The method of claim 216, wherein the cyclic lipopeptide is a polymyxin.
- 218. The method of claim 217, wherein the polymyxin is polymyxin B or polymyxin E.
- 219. The method of claim 218, wherein the major capsid protein is pVIII.
- 220. The method of claim 219, wherein the pVIII is bound to polymyxin B by amide linkages.
- 221. The method of claim 205, wherein the ratio of antibiotic to major coat protein is less than or equal to 3:1.
- 222. The method of claim 205, wherein the minimal inhibitory concentration of the filamentous phage is at least 2X, 5X, 10X, 20X, 50X or 100X lower than the minimal inhibitory concentration of the antibiotic alone.
- 223. The method of claim 204, wherein the anticancer agent is a cytotoxic agent, immune checkpoint inhibitor, ligand or receptor inhibitor, radioligand conjugate or enzyme.

- 224. The method of claim 204, wherein the cytotoxic agent is an antibiotic, antimicrobial, antifungal, antiparasitic, immunostimulatory agent, immunomodulatory agent, immunosuppressive agent or anticancer agent.
- 225. The method of claim 190, wherein the binding moiety binds to a cancer cell.
- 226. A pIII-anti-LPS fusion protein comprising SEQ ID NO:2 or SEQ ID NO:4.
- 227. A pIII-anti-LPS fusion protein consisting of SEQ ID NO:2 or SEQ ID NO:4.