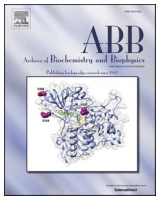
Archives of Biochemistry and Biophysics 755 (2024) 109939

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi



Insights into membrane interactions and their therapeutic potential

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ARTICLE INFO

Handling Editor: Jose L. Neira

Keywords:
Membrane interactions
Biotechnology
Bacteriophage T4
Adenovirus
Photorhabdus virulence cassette

ABSTRACT

Recent research into membrane interactions has uncovered a diverse range of therapeutic opportunities through the bioengineering of human and non-human macromolecules.

Although the majority of this research is focussed on fundamental developments, emerging studies are showcasing promising new technologies to combat conditions such as cancer, Alzheimer's and inflammatory and immune-based disease, utilising the alteration of bacteriophage, adenovirus, bacterial toxins, type 6 secretion systems, annexins, mitochondrial antiviral signalling proteins and bacterial nano-syringes. To advance the field further, each of these opportunities need to be better understood, and the therapeutic models need to be further optimised.

Here, we summarise the knowledge and insights into several membrane interactions and detail their current and potential uses therapeutically.

Abbreviations

Abbreviation	Meaning
AdVs	Adenoviruses
AI	Artificial Intelligence
ALS	Amyotrophic Lateral Sclerosis
AnxA1	Annexin A1
AnxA10	Annexin A10
AnxA11	Annexin A11
AnxA13	Annexin A13
AnxA2	Annexin A2
AnxA3	Annexin A3
AnxA4	Annexin A4
AnxA5	Annexin A5
AnxA6	Annexin A6
AnxA7	Annexin A7
AnxA8	Annexin A8
AnxA9	Annexin A9
API	Active Pharmaceutical Ingredient
Au NPs	Gold Nanoparticles
α-PFT	Alpha-Pore-Forming Toxin
CAR	Coxsackievirus and AdV Receptor
CDC	Cholesterol-Dependent Cytolysin
CDC	Circular Dichroism
COPD	Chronic Obstructive Pulmonary Disorder
CT	Cholera Toxin
DIBMA	Di-Isobutylene Maleic Acid
DT	Diphtheria Toxin
DTR	DT-receptor
EGF	Epidermal Growth Factor
ESCRT	Endosomal Sorting Complex Required for Transport
EVs	Extracellular Vesicles
FDA	Food and Drug Administration
FRET	Fluorescence Resonance Energy Transfer
FTD	Frontotemporal Dementia
GPVI	Glycoprotein 6
HVJ-E	Hemagglutinating virus of Japan envelope
IRF3	Interferon Regulatory Factor 3
ITC	Isothermal Titration Calorimetry
LFT	Long Tail Fibre
LPS	Lipopolysaccharide
LUBAC	Linear Ubiquitin Chain Assembly Complex
M2e	Influenza Matrix Protein 2
MAM	Mitochondrial Associated Endoplasmic Reticulum Membrane
MAPK	Mitogen-Activated Protein Kinase
MAVS	Mitochondrial Antiviral Signalling Protein
MDA5	Melanoma Differentiation-Associated Protein 5
MFN2	Mitofusin 2
MSP	Multisystem Proteinopathy
MWCNTs	Multi-walled Carbon Nanotubes
NF-kB	Nuclear Factor Kappa-Light-Chain Enhancer of Activated B Cells
OmpC	Outer Membrane Protein C

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https://doi.org/10.1016/j.abb.2024.109939
Received 1 November 2023; Received in revised form 31 January 2024; Accepted 19 February 2024
Available online 21 February 2024

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Abbreviation	Meaning
PE	Phosphotidylethanolamine
PFO	Perfringolysis O
PFT	Pore-Forming Toxin
PS	Phosphotidylserine
PSP	Progressive Supranuclear Palsy
Pvc	Photorhabdus Virulence Cassette
Pvc13	Photorhabdus Virulence Cassette 13
RGD	Arginine-Glycine-Aspartic Acid
RIG-I	Retinoic Acid-Inducible Gene I
SA	Sialic Acid
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SMA	Styrene Maleic Acid
SNAREs	Soluble N-ethylmaleimide Sensitive Factor Attachment Protein Receptors
SPR	Surface Plasmon Resonance
STF	Short Tail Fibre
T6SS	Type 6 Secretion System
TRAF	Tumour Necrosis Factor Receptor-Associated Factor
WO3	Tungsten Oxide Nanostructures
β-PFT	Beta-Pore-Forming Toxin

1. Introduction

The cell membrane is a dynamic and complex structure crucial for maintaining cellular integrity, regulating molecular passage, and facilitating numerous vital processes across the domains of life. The cell membrane serves as a crucial boundary between a cell's interior and the external environment. Within eukaryotic and bacterial cells, it consists primarily of a lipid bilayer including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin, along with other molecules like cholesterol, glycolipids, sterols, carbohydrates, and glycoproteins, whilst archaeal membranes feature stable monolayers with unique structures, such as pseudopeptidoglycan and surface-layer proteins for structural support and stability [1–3]. Some cells may contain external structures, such as cell walls made of peptidoglycan (bacteria), or cellulose/chitin (some eukaryotes) for additional support and protection [4,5]. This intricate composition imparts various properties to the membrane, including fluidity, stability, and functionality [1,6–8]. Organelles within cells possess their own membranes, each enabling specific functions, such as energy production in mitochondria [9].

The cell membrane has multifaceted roles, such as selective permeability, controlling the passage of molecules, regulating osmosis and ion transport, and facilitating endocytosis and exocytosis [1,10–12]. It also contains receptors that receive external signals, triggering cellular responses like cell division, gene transcription, and immune recognition [13]. It also contributes to a variety of essential cellular functions. It enables the immune response by recognising and responding to foreign substances or pathogens [14,15]. It maintains essential biophysical properties, including fluidity, electrical properties, flexibility, and mechanical tension, all crucial for various cellular processes, including cells-to-cell communication and adhesion through specialized structures, including gap junctions and cell adhesion molecules [7,16–21].

A key factor in many protein-protein interactions is the concept of entropy reduction upon shifting 3D to 2D systems associated with the membrane and the spatial constraints that membranes impose on proteins. Entropy, a measure of disorder or randomness in a system, tends to decrease when a system's dimensionality is reduced because there are fewer ways to arrange the system's components in space. In a 3D environment, proteins can move in three dimensions, which allows for a high degree of freedom and, consequently, high entropy. When proteins are localised to a 2D membrane, their movement is restricted to two dimensions, thus reducing the number of possible configurations, and reducing the entropy of the system [22]. This reduction in entropy upon membrane localisation is significant for protein-protein interactions because it increases the local concentration of proteins on the membrane surface. When proteins are confined to a 2D plane, they are more likely to encounter each other, which can promote interactions that are less probable in a 3D space, due to the larger search volume [23]. Membrane localisation can be mediated by specific domains within proteins, such as the PM domain, which binds acidic phospholipid membranes. This interaction targets the protein to the membrane and synergises other weak interactions to enhance the recruitment and stabilisation of protein complexes at the membrane [24].

Studying the cell membrane and its interactions is important for both basic and applied research, offering significant value for all branches of life and health science, through the understanding of cellular and protein function, modelling biological systems and drug development. Membrane interaction refers to the binding between a biological membrane and a specific molecule or protein. This binding can be crucial for various cellular processes, including cell signalling, membrane trafficking and protein localisation. Membrane binding can be mediated by several different mechanisms, such as specific headgroup interactions, hydrophobic membrane penetration, electrostatic surface interactions, and shape complementarity [25]. Membrane interactions can also be exploited therapeutically.

For fundamental research, proteins that directly bind to membranes can modulate the shape and dynamics of the plasma membrane [26]. Studying membrane binding and dynamics provides insight into protein interactions with membranes and their influence on structure, protein function and cellular function. Additionally, membrane binding plays a crucial role in the stabilisation of proteins [27]. Dimerisation, which is often facilitated by membrane binding, is important for protein insertion into the membrane and maintenance of protein structure. Moreover, the membrane plays a key role in the assembly and activation of several proteins and macromolecules. For example, many phage and viral capsids require the inner leaflet of the membrane for assembly into higher structures, such as the integral membrane proteins P20 and P22. These proteins form a transmembrane pore in the newly assembled viral membrane, which provides a nucleating site for the recruitment of other capsid-associated proteins. Various capsid-associated proteins then assemble along the viral membrane to form the viral capsid, while P6/P9 assembles onto the transmembrane pore (P20/P22) and forms the unique packaging complex, which completes the assembly of the procapsid [28]. By studying membrane interaction, researchers can uncover the mechanisms of interaction and how these interactions affect protein stability and function [27]. Many membrane-binding proteins are involved in cellular signalling processes, facilitating the transmission of signals and communication into and out of cells. Understanding how proteins bind to membranes is essential for deciphering these intricate signalling networks within cells [29].

Furthermore, surface proteomics can help identify potential biomarkers for diseases, such as cancer [30]. Changes in the expression or modification of cell surface proteins can be indicative of disease states, and studying the surface proteome can aid in the discovery of novel biomarkers for early detection, diagnosis, and monitoring of diseases [31,32]. Many proteins are overexpressed or aberrantly regulated in diseases, and the knowledge of these changes can guide the development of targeted therapies that selectively interact with these proteins or biomarkers, treating the disease [33]. The study of membrane interactions plays an important role in personalised medicine. Within applied research, tailored treatment strategies will be based on the unique characteristics of a patient's membrane proteome, improving the efficacy and precision of therapeutic interventions [34].

The study of membrane interactions plays a vital role therapeutically. A greater understanding of cell-cell recognition and adhesion administers comprehension of cell communication, tissue development, and immune responses further, increasing insights into the mechanisms underlying these processes, and an understanding of what may happen if they go wrong, with potential solutions when they do [34]. In addition, protein and lipid interaction with the membrane are of considerable

interest to human health [27]. Dysregulation of membrane binding processes can lead to diseases and disorders, and understanding the molecular mechanisms of membrane binding can provide valuable insights for the development of therapeutic interventions and drug design [27].

Studying membrane interactions is vital to the development of therapeutic interventions for most conditions. Whilst membrane proteins make up ~23 % of the human proteome, they constitute more than 60 % of current drug targets [34]. Systematic investigations of drug-membrane interactions are hindered by the complexity of natural membranes, but model membrane systems can provide a useful alternative. Insights can be gained into how drugs interact with membranes and more effective drugs can be developed that target specific membrane proteins. Additionally, anticancer drug-membrane interactions are a powerful strategy to improve cancer therapy. Biophysical techniques can be used to study these interactions and develop more effective cancer treatments.

In this review, several important membrane interactions, in addition to their therapeutic potential are discussed, including bacteriophage T4, bacterial toxins, annexins, mitochondrial antiviral signalling proteins, type six secretion systems, and adenoviruses. Additionally, the future of membrane interactions for medicine is hypothesised and experimental directions and approaches for studying membrane interactions are suggested. An overview of this can be seen in Fig. 1.

2. Types of interactions

2.1. Bacteriophage T4

The bacteriophage T4 is one of the most well-studied and fascinating viral examples [35]. T4 infects limited strains of *Escherichia coli* and *Shigella* bacteria and is comprised of three main parts, the multi-protein icosahedral capsid containing the viral genome, the neck region surrounded by a contractile protein sheath, and a hexagonal baseplate with six long tail fibres (LTFs) attached to the baseplate's periphery [36–38]. Each component is assembled independently and merged to form a mature bacteriophage [36]. Bacteriophages have strict host specificity, and the step of adsorption is one of the key factors for determining host specificity [39]. When interacting with *E. coli*, LTFs recognise receptor molecules on the membrane of the host, and upon recognition, six short tail fibres (STFs), located underneath the baseplate, unfold to bind to the host membrane irreversibly, increasing the infection efficiency, which can be seen in Fig. 2 [36,40]. LTFs contain dual binding capabilities, reversibly binding onto the glycolipid, lipopolysaccharide (LPS), composed of three structural domains: Lipid A, the oligosaccharide core, and the distal polysaccharide, or the outer membrane protein C (OmpC) of *E. coli*, which naturally function as a general porin for the non-specific diffusion of small solutes including sugars, ions and amino acids [41,42]. The STFs then undergo a conformational change, rotating downwards and irreversibly interacting with the heptose moiety of the lipid-A region of the host's LPS [43,44]. The binding of STF initiates alteration of the shape of the baseplate from a hexagon to a six-pointed, star-like structure, triggering sheath contraction and penetration of the cell wall, injection of the genomic DNA and ultimately viral infection [45,46]. The top surface of the distal tip head domain of the LTFs interacts with LPS, whilst the lateral surface interacts with OmpC [39]. Infectivity can be increased through the adsorption of additional LTFs, with only three fibres, out of six, being shown to have a low probability of infection [47].

Differences in the interaction of LTFs to the *E. coli* outer membrane are seen depending on the presence of OmpC and the stage of LPS synthesis. In the presence of OmpC, the essential region of LPS for receptor activity was the core-lipid A region, including the heptose moiety that STFs bind, whereas the glucose region of LPS is not required for receptor function. Uniquely, OmpC is not required at all when the distal end of LPS is removed, exposing the glucose residue. Cells lacking both OmpC and the LPS glucose region become resistant to T4 [48]. Thus, the removal of sugars in the outer core of LPS still supports the adsorption of T4 in the presence of OmpC, and T4 can still be adsorbed in the absence of OmpC when LPS has a terminal glucose in the outer core. The LTFs of T4 may therefore exhibit two different modes of interaction with the *E. coli* membrane, with the common mechanism being the binding of LPS, but differing LPS structures between strains may result in the requirement of OmpC as a cofactor.

An exciting therapeutic use of bacteriophage T4 is in phage therapy, the use of bacterial viruses to treat bacterial infections. Such approaches have been used for over a century, with the first documented use being

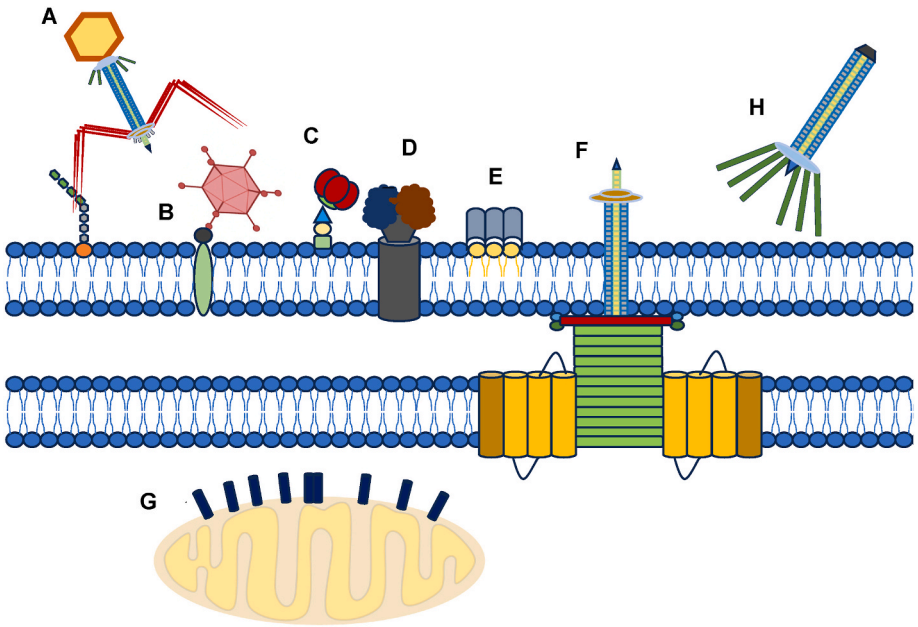

Fig. 1. A summary of membrane interactions.
A) The bacteriophage T4 binding to lipopolysaccharide. B) Adenovirus binding to coxsackievirus and AdV receptor. C) Cholera toxin binding to a ganglioside GM1 receptor. D) Diphtheria toxin binding to human membrane-anchored heparin-binding EGF-like growth factor. E) Annexin A5 binding phosphatidylserine. F) A type 6 secretion system. G) A mitochondrion containing mitochondrial antiviral signalling protein. H) a *Photorhabdus* virulence cassette.

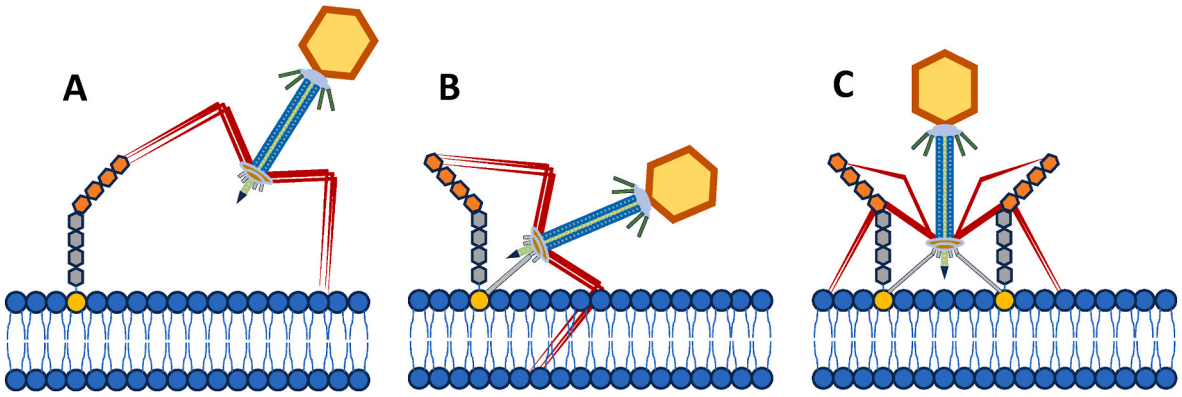

Fig. 2. Bacteriophage T4 binding on the cell membrane.
A) The long tail fibre on the bacteriophage binds to lipopolysaccharide. **B)** Upon binding of the long tail fibre, the short tail fibre, located on the bacteriophage baseplate, unfolds, and binds to the lipid-A region of the lipopolysaccharide, located at the cell membrane. **C)** The binding of both short and long tail fibres to multiple lipopolysaccharides increases the chance of a successful bacteriophage injection.

the treatment of *Shigella dysenteriae* as early as 1919 [49]. Phage therapy did not take off in the same way as antibiotics, due to poor documentation and variable success, but is now widely being considered as an alternative to combat multi-drug resistance. Biotechnological advances have further expanded the repertoire for phage therapeutics, including novel strategies using bioengineered bacteriophage and purified phage lytic proteins. Much is still to be discovered about the interactions between phage bacteria, and the human host, but further research and development will advance the efficacy of phage therapy as a viable alternative to antibiotics [50].

In addition to direct cell killing, bacteriophage T4 has shown potential for use in drug delivery, particularly in the field of nanomedicine and vaccine development. T4 has been used as a platform to generate nanoparticle vaccines, which can stimulate both the innate and adaptive immune systems, eliciting humoral and cellular immune responses [51,52]. Vaccines containing one or more target antigens from pathogenic organisms represent safer alternatives to the whole pathogen vaccines. Assembly of these antigens into virus-like nanoparticles is an effective approach as it allows the presentation of the epitopes in a more native context with a repetitive, symmetrical, and high-density display of antigens, making the antigens better presented to stimulate the host's adaptive immune system. Bacteriophage T4 provides an excellent platform to generate nanoparticle vaccines. The large surface area and native peptide presentation on the T4 capsid allow for a high-density array of antigen epitopes, ranging from small peptides up to multi-subunit complexes. Co-delivery of DNA, targeting molecules and molecular adjuvants provides additional advantages [51,52]. For example, the bacteriophage T4 virus-like particle platform displaying the extracellular domain of influenza matrix protein 2 (M2e) showed high immunogenicity, stimulating robust humoral and cellular immune responses, and conferring complete protection against lethal influenza [52]. M2e is under investigation as a universal influenza vaccine, and combined with the bacteriophage T4 delivery platform, could provide a highly potent solution to the risks of influenza viral infection [53]. Furthermore, a bacteriophage T4 nanoparticle dual vaccine has been developed against *Bacillus anthracis* and *Yersinia pestis*, the causative agents of anthrax and the plague. Antigens for the two were fused to the outer capsid of the bacteriophage T4, providing robust immune response and complete protection against the inhalation of anthrax and the pneumonic plague in three animal models, mice, rats and rabbits [54]. Recent studies have demonstrated the efficacy of the bacteriophage T4 as a delivery mechanism for the SARS-CoV-2 vaccine, expressing spike epitopes on the phage capsid [55].

Additionally, T4 has been explored as a nano-vehicle for the delivery of genes and therapeutics into human cells. Adeno-associated viruses and lentiviruses have been extensively used as delivery vehicles, but their capacity is limited to one or two genes. The large bacteriophage capsid allows the engineering of both its surface and interior to incorporate combinations of DNA, RNA, proteins, and complexes. The large capacity, cell-targeting capability, safety, and inexpensive manufacturing could open new possibilities for cancer, gene, and stem cell therapies. However, they are limited by inefficient entry into human cells and inefficient intracellular trafficking. These must be overcome by evolution and gene engineering to translate phage-delivery technology [56].

Furthermore, T4 has been utilized in nanotechnology applications, such as sensor probes and nanoprobes for cancer diagnostics. Bacteriophage T4 possesses characteristics that make them perfect candidates as materials for sensor probes. The surface can be modified through genetic engineering or direct conjugation, allowing the display of functional moieties, such as antibodies or proteins that recognise a specific target [57]. For example, T4 has been employed in the development of sensors for the detection of bacteria, namely *E.coli* 0157:H7, a common foodborne pathogen that affects millions of people annually [58]. Traditional microbiological methods for the detection of bacteria are time-consuming, involving selective enrichment, serological confirmation, and biochemical screening. Bacteriophage-based biosensors are an attractive alternative, offering high selectivity, sensitivity, accuracy and speed, with naturally high specificity for their host bacterial cell. To produce the biosensor, the active phage is covalently immobilised on the sensor's surface, composed of gold nanoparticles (Au NPs), multi-walled carbon nanotubes (MWCNTs), and tungsten oxide nanostructures (WO_3), with each material having been selected carefully to support the bacteriophage immobilisation and enhance the reproducibility of the impedimetric signal and electrochemical processes. The sensor then allows for measurable and quantifiable impedimetric signals upon binding of the immobilised phage and target host. This technology now allows for the fast and accurate analysis of food samples, with positive results observed in the detection of *E.coli* O157:H7 in beef, white cheese, tomato juice, tap water and luncheon beef [59]. A representation of this can be seen in Fig. 3. The above method has also been adapted for use in cancer diagnostics, with the phage adapted for the binding of tumour antigens through antibody-based phage display [60,61]. Phage display, first described by George P. Smith in 1985, uses bacteriophage to connect protein-protein, protein-peptide and protein-DNA interactions for the study of these interactions. Peptides are displayed on the phage's viral capsid protein by the fusion of the peptide to the capsid [62]. This technology has since been taken further, with antibodies for therapeutic protein engineering being displayed. This technique involves the genetic manipulation of the bacteriophage so that the antibodies can be expressed on their surface [63]. Phage display technology can be utilizsed to discover high-affinity antibodies specific to a wide variety of

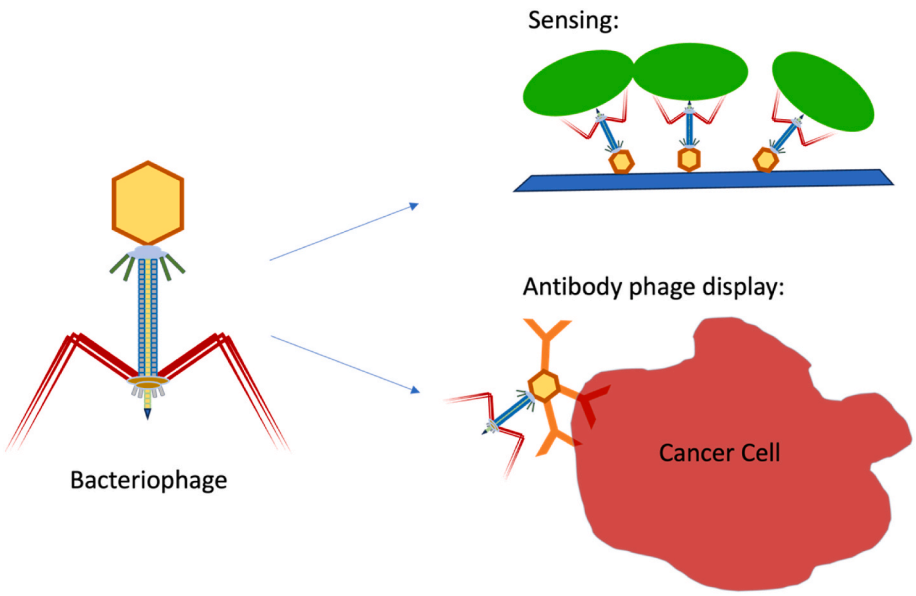

Fig. 3. Biotechnological applications of the bacteriophage.
The bacteriophage can be immobilised onto biosensors for the detection of *E.coli* in food samples. It may also be used in antibody phage display to detect cancer cells.

antigens, as demonstrated in Fig. 3 [64]. Subsequently, phage display-derived antibodies were developed for the specific binding of SARS-CoV-2, allowing the rapid development of antibodies to treat COVID-19 [65].

2.2. Adenovirus

Adenoviruses (AdVs) are nonenveloped, double-stranded DNA viruses of vertebrates. Currently, there are 110 AdVs known to infect humans, with infection symptoms traditionally being cold- or flu-like, including fever, cough, and sore throat. They are known to infect respiratory organs, the eyes, the kidneys, the gastrointestinal tract, and blood cells. AdVs share many aspects of their structure with the phages of Eubacteria and Archaea, being part of the evolutionary lineage comprising the PRD1 phage [66–69]. Both AdVs and PRD1 contain an icosahedral capsid organisation, like most viruses [70].

AdVs bind to specific receptors on the cell membrane to enter the host cell. The receptor binding activity is associated with the viral fibres, trimeric spike proteins that protrude radially from the vertices of the icosahedral capsid. At the foot of each fibre is a pentameric penton base [71,72]. The primary receptor for most AdV serotypes is the coxsackievirus and AdV receptor (CAR), a transmembrane protein that is expressed in many tissues [73,74]. The primary interaction between the AdV and CAR occurs at the terminal knob of the adenovirus fibre protein and the D1 domain of CAR, demonstrated in Fig. 4 [75]. Upon binding to CAR, RGD peptides within the penton base bind to cellular integrins located on the extracellular matrix between cells. The interaction with these integrins by the penton base activates PI3 kinase, $p130^{CAS}$ and Rho GTPases, which signal for rearrangements in the actin cytoskeleton for the initiation of virus internalisation [72,76–78]. Virus internalisation can be seen in Fig. 5. The integrins known to have binding motifs for RGD ligands include αvβ3, αvβ5, αvβ1, α3β1, and α5β1 [79–82]. Within the CAR D1 domain, AdV fibre head has been found to bind to the GFCC'C″ surface, which is an existing and conserved interaction surface for CAR to dimerise [83].

However, some AdV serotypes use other receptors, such as CD46, desmoglein-2, and sialic acid [84]. Crystallographic studies demonstrate that sialic acid (SA) binds to a site at the very top of the fibre knob, and residues potentially involved in SA interaction are located on the top surface of the knob [75]. AdVs first bind to a primary attachment receptor via high-affinity interactions between the virus fibre protein and

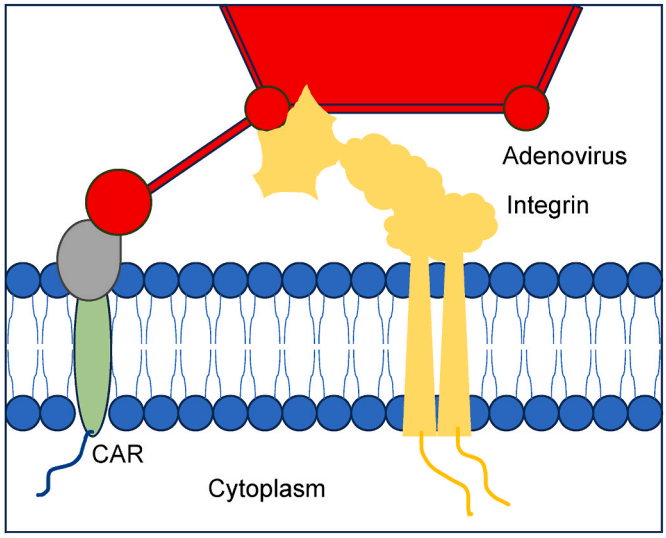

Fig. 4. Adenovirus binding to the cell membrane.
The terminal knob of the adenovirus fibre protein binds to the D1 domain of CAR. The penton base of the adenovirus then binds to integrin. This signals for rearrangement of the actin cytoskeleton for initiation of virus internalisation.

the cell [85]. The globular nature of the AdV knob domain presents large surfaces for binding the receptor laterally and apically [72]. The effect of this architecture is to increase the probability of virus-receptor interactions [86]. The specific receptor used by an AdV depends on the serotype, and different serotypes can use different receptors.

The fibre and the penton base attach and internalise the receptor-binding proteins. Attachment of most AdVs is mediated by CAR, which maintains the integrity of tight junctions in polarised epithelial cells and is normally sequestered on the basolateral surface of these cells [87]. After attachment, the virus is internalised into the host cell by receptor-mediated endocytosis [88]. AdVs may enter the cell via dynamin-dependent or dynamin-independent endocytosis [89]. The virus is then transported to the endosome, where it undergoes conformational changes that allow it to escape the endosome and enter the cytoplasm [90]. In one study, flow cytometric analysis indicates a

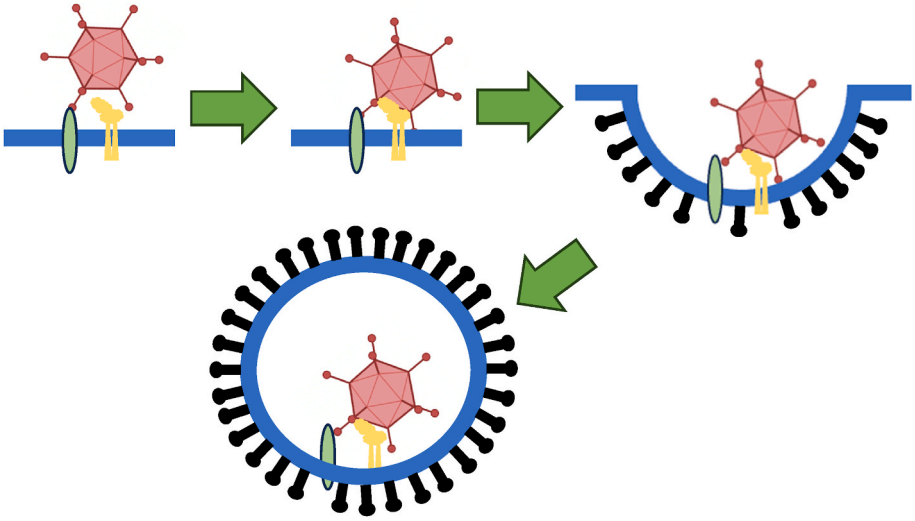

Fig. 5. Schematic representation of the internalisation of the adenovirus.
Adenovirus binds to CAR at the terminal knob of the fibre protein. Integrin then binds to the RGB peptide on the penton base of the adenovirus. This signals for rearrangement of the actin cytoskeleton for internalisation. These changes activate a clathrin-mediated endocytosis.

decrease in the number of endosomes obtained from cells infected with adenovirus, suggesting that the adenovirus rupture the endosome to gain entry to the cytoplasm [91].

AdVs have shown potential for therapeutic applications, particularly in the fields of gene therapy, vaccination, and cancer therapy. AdV vectors are commonly used in gene therapy to deliver therapeutic genes to target cells. Adenovirus vectors can be replication-defective, meaning that certain essential viral genes are deleted and replaced by a cassette that expresses a foreign therapeutic gene. As such, these vectors can be used for gene therapy, vaccines, and for cancer therapy. Replication-competent (oncolytic) vectors can be employed for cancer gene therapy as well, as displayed in Fig. 6 [92–95]. AdV vectors are also used as vaccines to express foreign antigens. AdV vectors can be engineered to express antigens from pathogens, such as influenza virus, to stimulate an immune response and provide protection against infection [96]. AdVs showcase a promising platform for selective interferon expression in cancer tissues. Interferons are a group of cytokines that have a strong antitumour effect, often used in the treatment of pancreatic cancer. AdVs loaded with interferon cytokines, oncolytic adenovirus Ad5/Ad3-Cox2-ΔE3-ADP-IFN, have been shown to selectively replicate inside pancreatic cancer cells expressing cyclooxygenase 2, reducing the limitations of traditional interferon-based chemotherapy, such as dose-limiting systemic toxicity and low intratumoural concentration of interferon because of its short half-life in the bloodstream [97–99].

2.3. *Bacterial toxins*

Pathogenic bacteria have evolved a collection of virulence factors to allow them to colonizse, invade and replicate with a competent host. Bacterial toxins play an essential role in this pathogenicity, having the capability to manipulate host cell functions and control vital processes to favour infection [100,101]. Bacterial toxins interact with a variety of components on the cell membrane to exert their effects, including lipids and proteins. The binding site depends on the specific toxin involved. For example, cholera toxin (CT) binds to the ganglioside GM1 receptor on the plasma membrane, while diphtheria toxin (DT) binds to the epidermal growth factor (EGF)-like domain of human membrane-anchored heparin-binding EGF-like growth factor [102,

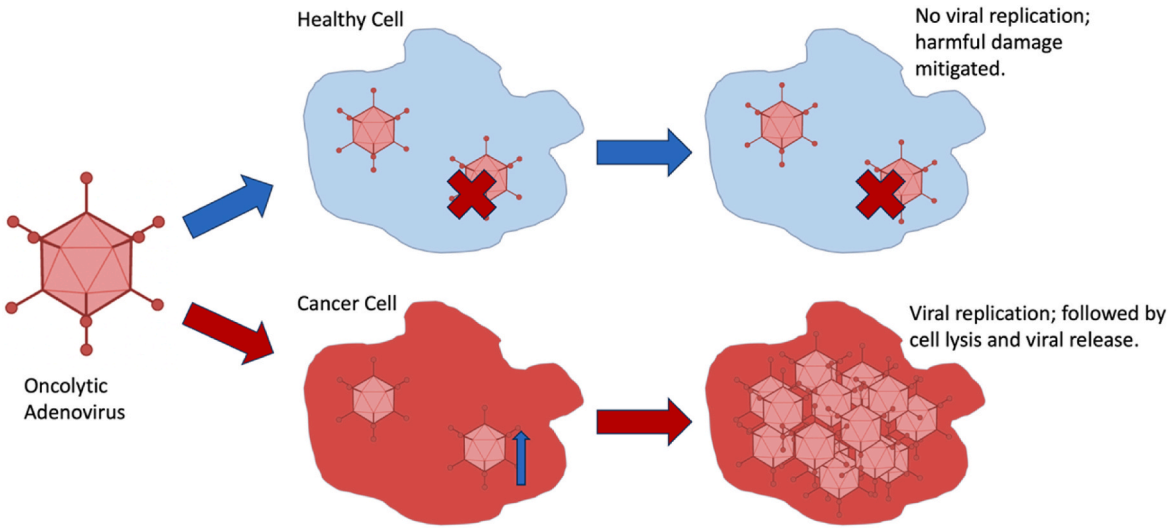

Fig. 6. Adenovirus's role in cancer therapy.
The oncolytic adenovirus has the capacity to replicate in cancer cells, but not in healthy cells, eventually leading to cell lysis and viral release in the cancer cell.

103].

CT can enter the cell by numerous modes of endocytosis [104–107]. Early studies discovered caveolae to be important in CT endocytosis, and the ganglioside GM1 receptor is found abundantly in caveolae [104, 108–110]. However, later studies found that CT can enter cells by both clathrin-dependent (noncaveolar) and clathrin-independent mechanisms, with the pathway for entry being dependent on cell type and membrane composition [105,111–113].

Only the entry of one of the three distinct domains of DT, the catalytic domain, is required for the cytotoxic action of DT [114]. The receptor binding domain binds to the cell surface and is internalised by receptor-mediated endocytosis. A conformational change occurs in DT, resulting in the insertion of DT into the endosome membrane through regions of the transmembrane domain before the cytotoxic domain is translocated to the cytosol [115–118]. The DT-receptor (DTR), located on the receptor-binding domain, is the primary protein involved in binding to the membrane [119]. DTR is identical to the precursor form of heparin-binding EGF-like growth factor, a heparin-binding member of the epidermal growth factor family [120–123]. Phe^{115}, Leu^{127} and Glu^{141} are critical amino acid residues on the EGF-like domain for DT binding, with significant reductions in binding activity being observed in mutations of the three [103]. These interactions allow the toxins to enter the cell and cause damage, leading to the symptoms associated with the respective bacterial infections.

Another form of bacterial toxins, pore-forming toxins (PFTs) represent over a third of all bacterial toxins, and many bacteria will secrete PFTs to alter the membrane of the host cell and trigger a release of nutrients into the extracellular environment, providing additional factors for the growth of these bacteria. PFTs adopt two central mechanisms for pore formation: insertion of amphipathic α-helices or insertion of amphipathic β-hairpins organised in a β-barrel. The former is represented by a group of bacterial exotoxins known as α-PFTs. The monomeric forms fold into the lipid membrane upon binding with specific receptors, unique to the PFT, before oligomerising to form transmembrane pores with refined architecture. 19-residue long α-helices, which initially lie parallel to the bilayer, between the polar heads and acyl chains of the phospholipids, associate in bundles to form the hydrophobic channel [124–127].

In contrast, most bacterial PFTs belong to the β-PFT family, which are hydrophilic proteins known to bind to the cell surface and oligomerize into β-barrels before being inserted into the lipid bilayer to create a channel. Whilst all β-PFTs share the common mechanism of producing a β-barrel, different types can be distinguished by their receptor, monomer number to form pores, pore size and functionality. Most bacterial β-PFTs associate into heptameric structures leading to relatively small pores (15–30 Å), however, some form oligomeric structures containing dozens of monomers with significantly larger pores (350–500 Å) [128].

One example of a β-PFT is cholesterol-dependent cytolysin (CDC), produced by *Clostridium perfringens* perfringolysin O (PFO), which recognises cholesterol as a receptor, and is unique in that it can concentrate into lipid raft microdomains, ultimately promoting the oligomerizsation of the toxin monomers to produce the pore [129]. PFO is a prototype of PFT from the CDC family, and contains an unusual elongate rod shape, rich in β-sheets, with a mostly hydrophilic surface [130]. The specific interaction between PFO and cholesterol is unknown, however a cysteine residue found in a conserved 11-residue sequence (ECTGLAWEWWR), located near the C-terminus, is found to be involved [131]. The binding of cholesterol to this region induces the displacement of a tryptophan-rich loop, assisting in the oligomerization and membrane insertion of the molecule into organised arcs [130]. Oligomers consist of 40–50 monomers, which form arcs and rings on the membrane surface. Upon reorganisation into oligomers, conformational shifts in the monomers promote the exposure of hydrophobic residues to the surface of the PFO rings, forcing the insertion of the 300 Å to 450 Å β-barrel into the membrane [128,132]. Cooperation between PFO monomers is required to drive the β-barrel into the membrane [133]. The hydrophilic face forms the inside of the pore, whilst the hydrophobic outer face is protected from the lipid bilayer by the cholesterol molecules, indicating that cholesterol plays a more significant role in the efficacy of the PFO than just as simply a binding partner [130].

Bacterial toxins have a wide scope for use therapeutically, most notably as anticancer agents. Some bacterial toxins, such as *Diphtheria* toxin, *Pseudomonas* exotoxin A and *Clostridium perfringens* enterotoxin, have been shown to specifically target cancer cells, inducing cell death or disrupting cellular processes involved in cancer progression [134]. Cancer cells often have a high number of tumour-specific antigens on the cell surface, and bacterial toxins can be adapted to bind to these antigens. Tumour-selective toxins can bind to a receptor on the target cell, before being internalised, resulting in cell death [135]. For example, LMB2, a Fv fragment of an antibody, fused to a truncated *Pseudomonas* exotoxin, has shown clinical activity in hair cell leukaemia and T cell neoplasms. Alternatively, *Clostridium perfrigens* enterotoxin binds directly to CLDN3 and CLDN4 receptors, which are upregulated in tumour cells, showing significantly inhibited tumour development [136]. DT, binding to epidermal growth factor (EGF)-like domain of human membrane-anchored heparin-binding EGF-like growth factor, displays naturally high toxicity, and has been adapted to reduce angiogenesis and regress tumour masses, in addition to inhibiting subcutaneous growth of Lewis lung carcinomas [136]. A recombinant DT variant containing an antibody to target the IL-2 receptor, which is highly expressed in malignant T cells, called Denileukin diftitox DAB389, became the first bacterial toxin to be approved for therapeutic use by the Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma. However, in 2006, the FDA added a black box warning to the treatment due to severe side effects [137,138].

Another example of the functionalised use of bacterial toxins is botulinum toxin, or Botox, a drug made from the toxin produced by the bacterium Clostridium botulinum. Botulinum is a neurotoxic protein that prevents the release of the neurotransmitter acetylcholine from axon endings at the neuromuscular junction, leading to muscle paralysis [139]. Botox injections work by weakening or paralysing certain muscles or by blocking certain nerves. Botox binds to the cell membranes at two main sites, both of which are located on the heavy chain domain of the toxin. The C-terminal of the heavy chain domain is the primary binding site, attaching to the protein receptor Synaptotagmin II, with the N-terminal being the secondary binding site, interacting with ganglioside receptor GD1a. There is no direct contact between the two receptors and the binding affinity to Synaptotagmin II is not influenced by the GD1a. Furthermore, the N-terminal domain of the heavy chain of botulinum neurotoxin B is found to bind with the sialic acid 5 moiety of GD1a [140]. Additionally, the C-terminal receptor-binding domain of botulinum neurotoxin B contains an extended loop, known as the lipid-binding look, which penetrates lipid membranes and enhances the toxin's ability to bind to nerve cells [141]. This unique binding ability allows for high affinity and specificity for neurons. Once bound, the toxin is uptaken into the neuron via receptor-mediated endocytosis [142]. Once inside the neuron, the disulphide bond linking the heavy chain to the light chain is reduced, releasing the light chain intracellular protease, the active part of the toxin [143]. The light chain targets a group of proteins called soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs), which are essential for the fusion of vesicles with the plasma membrane, a process necessary for the release of neurotransmitters. By cleaving these proteins, Botox prevents the fusion of synaptic vesicles with the neuronal membrane, thereby blocking the release of acetylcholine into the synaptic cleft [144].

In addition, bacterial toxins can be used as sensitising agents in combination with chemotherapeutic treatments, sensitising the cancer cell to the effects of the chemotherapy, and enhancing the treatment [145]. Bacterial toxins can be used in combination with conventional chemotherapeutic agents to overcome the limitations of chemotherapy, such as lack of specificity and drug resistance. Often, the tumour

microenvironment escapes the effects of the host immune system due to its lower immunogenicity, leading to their acceptance as self-antigens of the body. Bacterial toxins can act as potent immune-stimulating agents in tumour microenvironments [146]. For example, attenuated strains of *C. novyi,* proliferate well across the necrotic areas of the tumour, initiating inflammatory reactions, and leading to tumour destruction [147]. The use of the bacterial toxin can then be controlled through antibiotics when they are no longer required, allowing for a more precise and effective treatment approach.

The use of bacterial toxins in cancer therapy is still an active area of research and development, and further studies aim to increase understanding of the mechanistic action of the bacterial toxin, optimisation of their delivery and improvement in efficacy and safety profiles.

Bacterial toxins can be used for drug delivery by exploiting their natural ability to target specific cell subtypes and efficiently penetrate cell membranes. By harnessing these properties, it is possible to develop targeted drug delivery systems for cancer therapy and other clinical applications. One example is the use of anthrax toxin and Pseudomonas exotoxin-based drug delivery systems for cancer therapy. These toxins have been modified to target cancer cells and deliver therapeutic agents, resulting in the selective killing of cancer cells while sparing healthy cells [148]. Another approach involves using bacterial toxins to trigger drug release from nanoparticle-stabilized liposomes, which can selectively deliver antimicrobials to the sites of bacterial infections [149]. However, some challenges and limitations need to be addressed before bacterial toxin-based drug delivery systems can become a viable new generation of drug delivery approaches in clinical translation. These challenges include immunogenicity, solubility, and stability of therapeutic fusion proteins [150]. Despite these challenges, bacterial toxins hold promise as a novel drug delivery platform and ongoing research aims to overcome these limitations and develop effective therapies using bacterial toxins for drug delivery.

2.4. Bacterial nano-syringes

Another membrane-interacting therapeutic is nano-syringes, adapted from *Photorhabdus* virulence cassette (PVC). PVC is a type of extracellular contractile injection system, a macromolecular injection device found in several bacteria, most notably the *Photorhabdus* genus [151]. Their structure is homologous to the contractile tails of bacteriophages, with whom they share ancestry [152,153]. Nano-syringes consist of a rigid tube inside a sheath. The sheath contracts upon binding of the nano-syringe tail fibres onto the host target, driving the tube forward, puncturing the cell membrane and allowing injection of the toxin into the target cells [153–155]. Nano-syringes are involved in toxin delivery, producing a pathogenic effect on insects, whilst symbiotically assisting the nematode worm, the gut of whom *Photorhabdus* naturally inhabits [156]. The programmable nature of nano-syringes has led to their exploration in various applications, including for the site-specific delivery of therapeutic drugs, providing a safe and efficient way to deliver different therapies. Two key adaptations need to be applied to nano-syringes to facilitate their use therapeutically. Firstly, PVC naturally contains the toxins Pdp1 and Pnf [157]. Swapping the toxins for an active pharmaceutical ingredient (API) encapsulates the API to control release and provide protection within the body. Secondly, the tail binding fibres, or Pvc13, of the nano-syringe, need to be engineered to bind to the intended cellular target for the delivery of the drug. Pvc13 is the 'legs' of the nano-syringe, responsible for the binding of PVC to the cell membrane. Genetic manipulation of Pvc13 will affect its natural binding, allowing for the targeting of different disease biomarkers and the delivery of the API in a site-specific manner. This will minimise the side effects on healthy tissues. Recent studies have demonstrated that PVC can be reprogrammed to target cells not natively targeted by this system [155]. Whilst the native membrane binding site for Pvc13 is unknown, current research is taking steps to elucidate the molecular targets of these bacterial nano-syringes.

One such approach to identifying the molecular targets of Pvc13, is the use of native membrane nanodiscs. The location of membrane protein and membrane-bound targets within the lipid bilayer poses a challenge to investigation into structure and function. Traditional solutions involve the use of detergents, which disrupt the lipids and form micelles around the hydrophobic membrane targets [158]. This approach presents several weaknesses. Firstly, the detergent micelle does not fully mimic the environment of the lipid bilayer. Additionally, the detergent may only isolate hydrophobic regions of the membrane, such as surrounding membrane proteins, meaning the study of non-protein-based membrane targets is nearly impossible using detergent [159]. Alternative systems, such as the use of amphipathic polymers, are required to maintain the native membrane environment [160]. Amphipathic polymers insert into biological membranes to create nanoparticles containing membranes and lipids [161]. Examples of amphipathic polymers include styrene-maleic acid (SMA), a copolymer composed of hydrophobic styrene and a hydrophilic maleic acid monomer, and di-isobutylene maleic acid (DIBMA), composed of di-isobutylene and maleic acid [162]. Utilising pull-down assays, such as co-immunoprecipitation, can isolate nanodiscs, taken from insect cell membranes, such as Sf9, containing the binding partner for Pvc13. Consequently, various mass spectroscopy techniques can be performed on these pulled-down nanodiscs to identify the collection of membrane components. The binding partner for Pvc13 can be classified based on the enrichment of certain membrane components compared to the contents of a native nanodisc, or one that has not been bound to Pvc13 through a co-immunoprecipitation pull-down assay. A flow diagram of this methodology can be seen in Fig. 7.

2.5. Annexins

Annexins are calcium-dependent phospholipid-binding proteins that have a diverse set of functions, including membrane binding and scaffold roles [163]. They exhibit potent anti-inflammatory, anticoagulant and fibrinolytic properties, playing an important role in the context of extracellular vesicles (EVs) [164]. EVs are vesicles secreted by cells into the extracellular space, ranging in functions from the transfer of proteins, lipids and genetic material, immune response modulation, coagulation, tumour progression and mineral nucleation [165–169]. In EVs, annexins are responsible for mediating EV tethering and aggregation, mineral nucleation and facilitating cellular uptake of EVs [170,171]. More than 100 annexins have been identified, with 12 confirmed in vertebrates [172]. These annexins are conventionally referred to as AnxA1-13, with AnxA12 being unassigned, as referred to in Table 1 [173].

Annexin A5 (AnxA5) plays an important role in the facilitation of cellular uptake of EVs, having been found to bind to phosphatidylserine (PS), before self-assembling to form a 2D-lattice on the cell surface in a calcium-dependent manner [170,214]. This binding to PS facilitates AnxA5 for use as a diagnostic tool for the detection of apoptotic cells and EVs [215–217]. PS is a phospholipid that is normally located on the inner leaflet of the plasma membrane but becomes exposed on the outer leaflet during apoptosis. During early apoptosis, membrane asymmetry is lost, and PS translocates to the outer leaflet, serving as an 'eat-me' signal for the recognition of phagocytes and other immune responses [218,219]. AnxA5 binds in a calcium-dependent manner to PS, with ellipsometric measurements showing that PS-binding of extracellular AnxA5 occurs when the membrane contains a low mole fraction of PS [220,221]. AnxA5-PS binding has also been shown to be influenced by the membrane environment around PS, with the minimal mole fraction of PS necessary for AnxA5 binding decreasing in the presence of phosphatidylethanolamine (PE), indicating that AnxA5 binding to PS depends on calcium concentration and the cellular environment around PS [222].

AnxA5 contains four domains, each of which plays a significant role in the binding of AnxA5 to PS. Previous research has shown that AnxA5

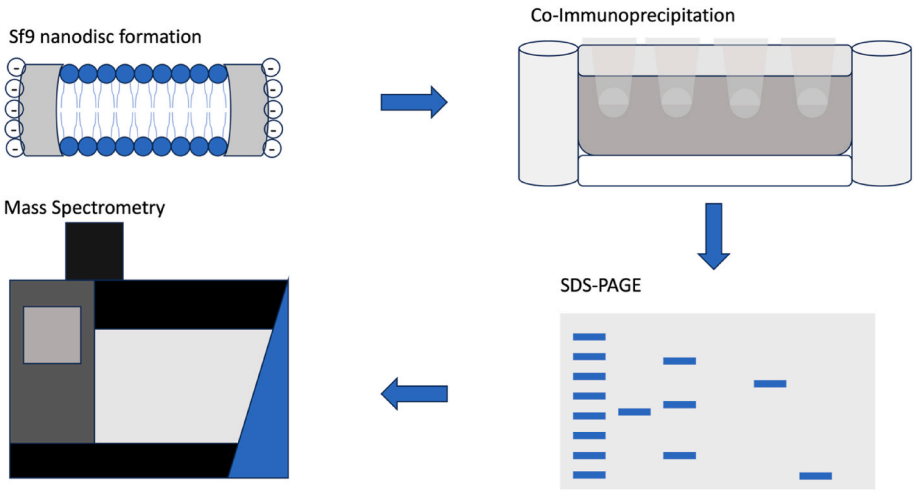

Fig. 7. Elucidating the molecular targets of bacterial nano-syringes.
Firstly, Sf9 nanodiscs are produced using polymer nanoparticles, such as DIBMA or SMA. Co-immunoprecipitation could be used to 'pull-down' Pvc13, bound to Sf9 nanodiscs contains Pvc13's natural binding partner. Co-immunoprecipitation elutions would then be run on a SDS-PAGE gel, and positive hits would be assessed through mass spectrometry for comparison with native Sf9 nanodiscs.

mutants lacking one or more domain(s) have significantly different biological activities to the wild type. Domain I is thought to play the most prominent role in PS-binding, whilst III and IV are considered to have more limited roles. However, all four domains together guarantee the maximum binding of AnxA5 to PS [223–227]. Domain IV contains the calcium-binding region, with a loop located between α-helices D/E of domain IV. Truncation of domain IV destroys AnxA5 calcium-binding ability, impairing affinity for PS, and ultimately impairing the ability of AnxA5 to label apoptotic cells and their ability as an anticoagulant [227].

Annexins have strong potential therapeutic applications for cancer, autophagy-related diseases and kidney and cardiovascular disorders. Their anti-coagulant and anti-inflammatory activity can be exploited for use as a therapeutic agent in conditions where inflammation and coagulation play a large role in the pathophysiology, such as sepsis and COVID-19 [228]. Annexins have been demonstrated to inhibit cancer cell proliferation, induce apoptosis, and enhance the effectiveness of chemotherapeutic agents. Annexin A1 (AnxA1) is effective in both acute and chronic inflammation for use as an immunotoxin incorporated into cancer vaccines [229,230]. Moreover, AnxA1 has been used as a basis for the development of an anti-inflammatory peptide, MC-12, which has shown effectiveness in the treatment of experimental colitis. This suggests that AnxA1-based peptides could be used for the treatment of inflammatory diseases [231]. However, further research is needed to fully evaluate their effectiveness and safety in these contexts.

Annexins can be used for drug delivery by exploiting their natural ability to bind to specific cell membrane components and mediate cellular processes. AnxA5 has shown potential as a therapeutic platform for targeted drug delivery and cell entry to treat various diseases, including cancer and cardiovascular disease. This is due to its specific internalisation properties, which allow it to enter cells and deliver therapeutic agents. AnxA5 binds to PS, before polymerising and forming a two-dimensional network on the surface that causes its internalisation, through the bending of the membrane patch nanomechanically, eliciting budding and endocytic vesicle formation [232]. This opens possibilities for targeting pharmacological compounds to the intracellular environment of PS-expressing cells, such as apoptotic cells and primary and secondary necrotic cells and living cells, such as tumour cells, myoblasts, and stressed neurons [232–234].

One example of annexin-based drug delivery is the development of an innovative 16-mer DNA aptamer-based AnxA1 targeted anti-cancer drug delivery system using doxorubicin-conjugated AnxA1. This novel strategy has the potential to reduce tumour chemoresistance towards doxorubicin, thereby improving its therapeutic efficacy. Aptamers are small, functional single-stranded DNA or RNA oligonucleotides that bind to their targets with high affinity and specificity. A 16-mer aptamer, conjugated with doxorubicin, was designed to bind to AnxA1. AnxA1 is overexpressed in A549, HepG2 and U-87 MG cancer cells. Doxorubicin delivered into these cells via the aptamer-AnxA1 delivery system was able to evade the drug efflux system, indicating the potential of the novel drug delivery system to reduce tumour chemoresistance [235].

2.6. *Mitochondrial antiviral signalling protein*

All membranes contain important and unique binding mechanisms, including those found inside cells. Located on the outer membrane of mitochondria, peroxisomes and mitochondrial-associated endoplasmic reticulum membrane (MAM) are Mitochondrial Antiviral Signalling Proteins (MAVS), which play a major role in the innate immune response against DNA and RNA viruses [236,237]. Upon viral infection, cytosolic proteins detect the presence of the virus and bind to MAVS, therefore activating it. The activation of MAVS drives the secretion of cytokines and the induction of the antiviral immune response. At a resting state, mitofusin 2 (MFN2) interacts with MAVS, preventing MAVS from binding to cytosolic proteins retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), which bind to and are activated by viral RNA [238]. Upon recognition of the virus in the cytosol, MAM and mitochondria become physically tethered by MFN2, creating a complex involving the MAM, mitochondria, and dimeric RIG-I. This complex interacts with TRIM25 ubiquitin ligase and molecular chaperone 14-3-3e to form a translocon [236–241]. The translocon then directs RIG-I redistribution from the cytosol to mitochondrial membranes, calling MAVS to activate other copies of itself. MAVS then aggregate together on the mitochondrial membrane. Upon MAVS aggregation, a collection of E3 ubiquitin ligases, including tumour necrosis factor receptor-associated factor (TRAF) 2, TRAF5, TRAF6 and linear ubiquitin chain assembly complex (LUBAC), relay the antiviral signal by binding to clustered MAVS. MAVS harbour binding motifs for each of the E3 ubiquitin ligases. The motif PVQET (143–147) is known to bind TRAF2 and TRAF5, and perhaps TRAF3, whereas two motifs, PGENSE (153–158) and PEENEY (455–460) bind TRAF6. The second TRAF6-binding motif has also been suggested to bind TRAF3 [242]. These E3 ligases string ubiquitin together in chains called

Table 1
All the types of annexins identified in humans, and their respective roles.

Human Annexin	Activity
AnxA1	AnxA1 is a mediator of the anti-inflammatory effects of glucocorticoids and is involved in the regulation of the inflammatory process. AnxA1 has also been shown to act as a tumour suppressor gene and modulates the proliferative functions of estrogen in breast cancer. It protects against DNA damage induced by heat in breast cancer cells and is associated with treatment resistance [174,175].
AnxA2	AnxA2 is involved in endocytosis, exocytosis, and membrane domain organisation. It plays a role in the regulation of vesicle trafficking and fusion with the plasma membrane [176]. It also has a role in actin remodelling, important for cell motility and shape changes [177]. AnxA2 is involved in signal transduction pathways, although the mechanisms are not fully understood [178]. AnxA2 consists of two heavy chains and two light chains. The light chain subunits initiate a fibrinolytic cascade, whilst the heavy chain subunits stabilise and anchor the light chains to the plasma membrane [179].
AnxA3	AnxA3 is a calcium-dependent phospholipid-binding protein that plays various roles in cellular processes, particularly in the regulation of cellular growth and signal transduction pathways. AnxA3 has been implicated in the development of metastasis of various cancers, including gastric cancer and ovarian serous carcinoma [180]. Its aberrant expression can promote tumour cell proliferation, invasion and metastasis [181]. High expression of AnxA3 has been associated with improved tumour prognosis in ovarian serous carcinoma, potentially due to its role in inducing stronger T-cell-mediated immunity against tumour cells [180]. AnxA3 has also been identified as a potential angiogenic mediator, suggesting its involvement in the formation of new blood vessels [182].
AnxA4	AnxA4 is another calcium-dependent phospholipid-binding protein. It's associated with membrane-related events along exocytotic and endocytotic pathways [183]. It's also shown to play a role in the plasma membrane repair response, enabling cells to quickly cope with life-threatening disruptions. It is recruited to wound edges and provides curvature force during the repair process [184]. It's almost exclusively expressed in epithelial cells, and demonstrates anticoagulant activity through the inhibition of the activation of coagulation factor XII, a plasma serine protease that initiates the intrinsic pathway of blood coagulation upon contact with anionic substances, such as sulphated glycolipid sulfatide [185].
AnxA5	AnxA5 acts as an anticoagulant protein that indirectly inhibits the thromboplastic-specific complex involved in blood coagulation [186]. It also binds to phosphatidylserine externalised by apoptotic cells, thereby hindering their interaction with immune cells, suggesting AnxA5 serves as an immune checkpoint inhibitor [187]. AnxA5 also bind to LPS to reduce the endotoxin activity related to bacterial infections [188].
AnxA6	AnxA6 is involved in the regulation of membrane dynamics-related events, including cholesterol and caveolin distribution within the cell [189]. It may also act as a scaffold or targeting protein for several signalling proteins, implying its involvement in several signalling pathways [190]. AnxA6 has been implicated in membrane repair in muscle, heart, and nerve cells, and has been identified as a genetic modifier of muscle repair and muscular dystrophy [191]. AnxA6 also plays a role in exosome secretion, being required for calcium-dependent fusion of multivesicular bodies with the plasma membrane [192].
AnxA7	AnxA7 has been found to regulate trophoblast proliferation and apoptosis in preeclampsia, a pregnancy complication characterised by high blood pressure and signs of damage to other organ systems [193]. AnxA7 promotes membrane fusion and is involved in exocytosis, as well as facilitating ESCRT III-mediated shedding of the damaged edges of wounded plasma membrane [194]. AnxA7 also regulates oxylipin metabolism and Ca2+-dependent platelet activation downstream of GPVI, a collagen receptor on platelets [195]. There are associations between AnxA7 and cancer prognosis, with tumour progression observed in hepatocellular carcinoma and breast cancer, yet tumour suppression in glioblastoma multiforme, melanoma and prostate cancer [194,196].
AnxA8	AnxA8 has been found to be involved in cell migration, cell adhesion, and vasculature development, all critical processes in tissue development and repair, as well as in the progression of diseases such as cancer [197]. AnxA8 is also involved in the regulation of endosome morphology and function, in addition to P-selectin-dependent leukocyte recruitment, playing important roles in the immune system [198,199].
AnxA9	AnxA9 is primarily involved in the organisation and regulation of membrane/cytoskeleton linkage and interacts with membrane phospholipids in a calcium-dependent manner [200]. It also demonstrates a role in cell migration, cell adhesion and vasculature development, all critical in the tissue development and repair process. However, it has been found to be a prognostic marker in colorectal cancer and gastric cancer, with expression levels being associated with invasion depth and lymphatic metastasis [200,201].
AnxA10	AnxA10 is associated with cell cycle regulation, ribosome biogenesis and DNA replication. This is linked with its role in the Wnt and Hippo signalling pathways, which play crucial roles in development and tissue homeostasis [202]. It also plays a role in tumour suppression, with downregulation of AnxA10 showing poor prognosis in hepatocellular carcinoma and melanoma, whilst its overexpression has been shown to reduce colony formation, cell migration and invasion in hepatocellular carcinoma and melanoma [203]. It is also involved in the induction of Pancreatic Duodenal Homeobox-1, playing a role in gastric cancer development and progression as well [204].
AnxA11	AnxA11 plays a critical role in regulating cell proliferation, migration, and invasion, and is involved in apoptosis through the mitogen-activated protein kinase (MAPK) and P53 pathways [205]. Additionally, AnxA11 is implicated in neurodegenerative disorders, particularly in a novel multisystem proteinopathy (MSP) type 6, a rare genetic condition characterised by a combination of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) [206]. Further studies have identified AnxA11 mutations in patients with ALS, ALS-FTD and atypical ALS with progressive supranuclear palsy (PSP)-like symptoms [206–208]. The exact mechanism behind the role of AnxA11 in neurodegenerative disorders is not fully understood, but is believed to be involved in protein homeostasis, autophagy, and RNA metabolism, which are crucial for maintaining neuronal function [207].
AnxA13	Expression of AnxA13 is limited to the epithelial cells, with the AnxA13a isoform localised to both apical and basolateral membranes, but AnxA13b enriched in the apical region in the lower three-quarters of the microvilli of polarised epithelial cells, with both being implicated in raft-mediated delivery of apical proteins in their respective areas [209–211]. AnxA13 is capable of binding negatively charged phospholipids in a calcium-dependent manner, suggesting involvement in membrane-related cytoskeletal interactions [212]. It has also been shown to promote tumour cell invasion in vitro and is associated with metastasis in colorectal cancer [213].

polyubiquitin, recruiting other innate immune pathway proteins to activate interferon regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), and subsequently, the transcription of genes involved in the innate immune response, such as those that signal for cytokine production, stimulates an inflammatory response and neutralises the viral threat [238,242–246].

MAVS represent a potential therapeutic target for antiviral therapy and the treatment of inflammatory diseases. By enhancing the activity of MAVS, it may be possible to boost the innate immune response to viral infections and reduce viral replication. Targeting MAVS may be particularly relevant in the context of COVID-19, as mitochondrial dysfunction plays a role in inhibiting antiviral responses in infected cells [247–249]. Targeted mitochondrial therapy with over-expressed MAVS protein from mesenchymal stem cells has been proposed as a new therapeutic approach for viral infections. This approach involves using mesenchymal stem cells to deliver MAVS to the mitochondria of infected cells, enhancing the antiviral response and reducing viral replication [247]. MAVS is involved in the regulation of mitochondrial dynamics, which can be exploited by viruses to modulate host antiviral immune signalling. Understanding the mechanisms by which viruses alter mitochondrial dynamics and functions can provide new insights into the

development of antiviral therapies [250]. MAVS have been shown to be required for optimal NLRP3 inflammasome activity. The NLRP3 inflammasome is involved in the regulation of inflammation and immune responses, and targeting MAVS may have implications for the treatment of inflammatory diseases [251].

Recent research has explored the potential of MAVS as drug targets, particularly in the field of cancer therapy. MAVS can be used as a platform for targeted drug delivery to cancer cells. Hemagglutinating virus of Japan envelope (HVJ-E) derived from inactivated replication-defective Sendai virus was found to have antitumour activity dependent on RIG-I/MAVS signalling. HVJ-E activates the RIG-I/MAVS signalling pathway to upregulate the expression of its downstream pro-apoptotic genes TRAIL and Noxa, inducing the selective apoptosis of tumour cells. Interestingly, this does not occur in normal cells. If chemotherapeutic agents can be developed to target the RIG-I/MAVS signalling pathway, apoptosis can be induced in cancer cells [248, 252–254].

2.7. Type six secretion systems

Type 6 secretion systems (T6SS) are membrane-embedded, spear-like nanomachines found in many gram-negative bacteria. Being found in over 25 % of gram-negative bacterial species, they play a crucial role in bacterial competition and virulence. They inject a diverse set of virulence factors, called effectors, into prokaryotic and eukaryotic cells. They share many similarities with the puncturing mechanics of bacteriophages and *Photorhabdus* virulence cassette [255]. All T6SS are composed of two universal complexes: a dynamic bacteriophage-like structure and a cell-envelope-spanning membrane-associated assembly [256]. Activation of the T6SS is dependent on various environmental factors, such as quorum sensing, temperature changes and pH.

The primary function of T6SS is to destroy other bacteria, but it also modulates bacterial interactions with eukaryotic cells [255]. T6SS can deliver toxins to neighbouring pathogens and translocate protein effectors into host cells, providing a survival advantage for bacteria like *Pseudomonas aeruginosa* [257]. In addition to interbacterial competition, T6SS is involved in modifying and manipulating diverse cellular processes in eukaryotic cells, allowing bacteria to colonize, survive, and disseminate [258]. The toxin delivery mechanism of T6SS involves a contractile tail machine comprising a TssB/C sheath and an expelled puncturing device consisting of an Hcp tube topped by a spike complex of VgrG and PAAR proteins. Contraction of the sheath propels the tube out of the bacterial cell into a target cell, leading to the injection of toxic proteins [259–262]. In host-pathogen interactions, T6SS plays a role in bacterial virulence and pathogenesis. It can inject antibacterial effector proteins into rival bacterial cells, modulating polymicrobial communities [257]. T6SS also participates in various physiological processes, including bacterial competition, host infection, and stress response [263].

The T6SS is anchored to the cell membrane through a membrane-associated complex, which comprises three T6SS core components: the TssL and TssM inner membrane proteins and the TssJ outer membrane lipoprotein. In most T6SS, the membrane complex is anchored to the cell wall by the TagL accessory protein. This complex is responsible for providing a channel through which substrates are propelled by the contraction of the phage tail-like tubule [264]. The T6SS assembly is initiated by the formation of a membrane complex that binds a phage-like baseplate with a sharp spike [265]. The baseplate-sheath structure is anchored to the cell *trans*trans-envelope via the membrane-associated complex, presumably making a *trans*-trans-periplasmic channel for passage of the tail tube/spike [264]. The puncturing of the cell membrane in the T6SS is caused by the contraction of a sheath-like structure that propels an inner tube terminated by a membrane-puncturing spike towards the target cells [266]. The T6SS functions as a nano-crossbow, and upon contraction of the sheath, the inner tube is propelled towards the target cell, allowing effector delivery [267]. The physical rupture of the target cell membrane might be enough to push large substrates across without a need for a protein dedicated to engaging the membrane [265].

Bacteria containing virulence factors must be able to protect themselves from the effectors they carry. Protection comes in the form of immune proteins, which often bind to the effector in the cell to render it inactive. These immune proteins can also shield bacteria from toxins released by other cells. If the bacteria have the immune proteins to match an attacker's effectors, they can protect themselves. Bacteria are prevented from killing their kin, whilst eliminating 'non-self' cells [268]. Bacteria containing T6SS are prime examples of bacteria containing immune protection from their own effectors. If the bacterial immune system can be exploited, therapeutics to neutralise the impact of bacterial toxins may be developed.

T6SS have shown potential for therapeutic applications, particularly in the context of antibacterial treatments and microbiome manipulation. T6SS has been engineered to deliver exogenous effectors and Cre recombinase, demonstrating improved efficacy against specific pathogens. Using multiple T6SS fusion proteins, *Aeromonas dhakensis* or attenuated *Vibrio cholerae* donor strains, and a gain-of-function cassette for detecting Cre recombination, successful delivery of active Cre directly into recipient cells was demonstrated. The most efficient transfer was achieved using a truncated version of PAAR2 from *V. cholerae*, resulting in a relatively small (118-amino-acid) delivery tag. This protein delivery system has potential applications in studying T6SS effectors and genetic editing [269]. Moreover, the engineered system can improve T6SS efficacy against specific pathogens, proposing future innovation in the application of microbiome manipulation or as a next-generation antimicrobial.

Furthermore, customizable, modular, and inducible antibacterial toxin delivery platforms have been engineered using a T6SS into *Vibrio natriegens*. This platform could be used for the development of novel antibacterial bio-treatments with inducible and customizable properties. The platform allows for the induction, or 'switching on' of the system upon sensing the pathogen to be targeted, preventing purposeless activation. The target pathogen's quorum-sensing regulation machinery drives the expression of the platform's inducible switch. The platform can be adapted to only intoxicate the desired pathogens whilst remaining benign to other bacteria, avoiding unwanted dysbiosis. The platform can be equipped with effectors that exhibit target-specific toxicity. Finally, the effector and immunity modules can be introduced into the platform to regulate the T6SS on/off switch, so future customisation only requires the replacement of one regulation module. This can be achieved through the control of a promoter from the VpT6SS1 operon, upregulated by VP1407 [270].

T6SS are being explored as a potential mechanism for delivering antibacterial drugs to eliminate *Pseudomonas aeruginosa* [271]. *Pseudomonas aeruginosa* are a type of bacteria that can cause infections in humans, mostly in hospital patients. It is a gram-negative bacillus found widely in nature, soil, and water. *P. aeruginosa* is an opportunistic pathogen, meaning it can cause infections in people with weakened immune systems, such as those with cystic fibrosis, burn wounds, immunodeficiency, chronic obstructive pulmonary disorder (COPD), cancer, and severe infection requiring ventilation, such as COVID-19. It is also the most common pathogen isolated from patients who have been hospitalized longer than 1 week, and it is a frequent cause of nosocomial infections [272]. The hypothesis is that T6SS secretion systems can be manipulated, through adaptation, to deliver antibacterial drugs advantageous to the host and eliminate *P. aeruginosa*. Further research is needed to optimizse these systems and explore their full potential in various therapeutic applications [273].

3. Conclusion

3.1. Experimental direction or approaches

There are many challenges to identifying molecular targets when studying membrane interactions. Biological systems are highly complex, with multiple interacting components and pathways. Identifying specific molecular targets within this complexity can be difficult, especially when the targets are not well-characterised or their functions are not fully understood [274]. Additionally, in many cases, the underlying molecular mechanisms of diseases are not fully understood. This lack of knowledge makes it challenging to identify the specific molecular targets that are involved in the disease processes [275]. Thirdly, the tools and technologies available for target identification are not always sufficient to identify molecular targets accurately and efficiently. This can be due to limitations in sensitivity, specificity, or throughput of the methods used [274]. Some drugs may interact with multiple molecular targets, leading to off-target effects. This can make it difficult to determine the primary target of a drug and its specific mechanism of action, whilst drugs with multi-target effects also pose a challenge [276]. Likewise, the expression levels and patterns of molecular targets can vary among individuals, tissues, and disease states. This variability can make it difficult to identify and validate specific targets for drug development [274]. Once a potential molecular target is identified, it is important to validate its relevance to the disease and its potential as a therapeutic target. This validation process can be time-consuming and resource-intensive [274].

Whilst the study of membrane interactions and the elucidation of molecular targets poses a challenge, several experimental approaches can be employed. Biochemical assays are widely used to study membrane binding of proteins [26]. These assays involve the use of purified proteins and lipid vesicles to examine the binding interactions. Techniques such as co-immunoprecipitation, pull-down assays, and surface plasmon resonance (SPR) can provide valuable information about protein-membrane interactions. Mass spectrometry is a powerful technique for studying membrane binding [277]. It can be used to analyse the binding of lipids to membrane proteins and monitor different modes of lipid binding. Mass spectrometry-based approaches can provide insights into the stoichiometry, affinity, and structural changes associated with membrane binding. Fluorescence-based methods, such as fluorescence spectroscopy and fluorescence resonance energy transfer (FRET), are commonly used to study membrane binding [278]. These techniques involve labelling proteins or lipids with fluorescent probes and monitoring the changes in fluorescence signals upon binding to membranes. They can provide information related to binding kinetics, affinity, and localiszation. Biophysical methods including SPR, isothermal titration calorimetry (ITC), and circular dichroism (CD), are employed to study membrane binding [279,280]. These techniques can provide insights into binding affinities, thermodynamics, and structural changes associated with membrane interactions. Microscopy techniques, such as confocal microscopy and electron microscopy, can be used to visualize membrane binding events [278]. These methods allow for the direct observation of protein-membrane interactions and can provide insights into the spatial organization and dynamics of membrane binding processes. Computational modelling approaches, such as molecular dynamics simulations, can complement experimental methods for studying membrane binding [279]. These simulations provide atomistic details of protein-membrane interactions, including binding sites, conformational changes, and lipid-protein interactions.

These methods, either used individually or in combination, provide valuable insights into the mechanisms, dynamics, and functional implications of membrane binding. They contribute to our understanding of various biological processes and can aid in the development of therapeutic strategies targeting membrane-associated interactions.

3.2. Future of drug targets

Studying membrane interactions is vital to the innovation and progression of drug discovery. Drug discovery is a lengthy and costly process, one which has encountered a decline in productivity over the last twenty years. The future of drug discovery and membrane interaction is paramount to ensuring the ability of humans to adapt to antibiotic resistance and fight declining life expectancies in parts of the world [281].

Emerging approaches in the role of membrane interactions for therapeutic interventions are associated with the encapsulation of pharmaceutical drugs for targeted delivery to the desired site of activation, such as a tumour or infection. This can enhance the drug's efficacy while minimising its side effects on healthy tissues. Additionally, drug encapsulation offers additional protection to drugs that are sensitive to degradation or inactivation in the body, providing a protective barrier and ensuring the drug reaches the target in an active form. Encapsulation offers the capacity for controlled release, providing a sustained therapeutic effect and reducing the frequency of dosing. Moreover, many drugs have poor solubility in water, which can limit their effectiveness. Encapsulation can enhance the solubility of these drugs, improving their bioavailability and therapeutic potential. Also, encapsulation allows for the simultaneous delivery of multiple drugs, enabling combination therapy and potentially enhancing treatment outcomes. Likewise, some drugs can be toxic to healthy tissues at high concentrations. Encapsulation can help to reduce the systemic exposure of these drugs, minimising their toxicity while maintaining their therapeutic effects at the target site. Encapsulated drugs can translocate across the cell membrane through various mechanisms. These include passive diffusion for small, moderately polar molecules, the utilisation of specific membrane transporters, endocytosis where the cell engulfs the drug or its delivery system, and the interaction of liposomes with the cell membrane, which can lead to the release of their contents into the cell. Additionally, the active transport of lipids and the use of cell-penetrating peptides are also involved in facilitating the delivery of encapsulated drugs into cells. The choice of mechanism depends on the properties of the drug, the design of the drug delivery system, and the specific target cell or tissue.

Another avenue for the development of pharmaceutical drugs is the repurposing of those currently used. Many molecules interact with multiple, often unrelated targets, through the nature of polypharmacology [282]. Across drug families, similarities in binding patterns occur for similar ligands, such as the wide binding specificity of kinase inhibitors providing the best-known example of polypharmacology, with their bioactivity being routinely profiled during the drug discovery and development process [283]. Twenty kinase inhibitors have been approved by the FDA since 2011, and a recent example of a kinase inhibitor being repurposed was for the treatment of COVID-19 [283,284]. Kinases are required for viral entry, metabolism, and reproduction, so pose an attractive target for antiviral treatment [284]. Several kinase inhibitors also have the potential to reverse pulmonary insufficiency, resulting from their anti-inflammatory activity, cytokine suppression and antifibrotic activity [284].

Target-based drug discovery is another opportunity within drug development, starting with a well-defined molecular target, such as that of a membrane protein or ligand. The typical process includes target identification, target validation, assay development, hit identification, lead optimiszation and preclinical and clinical development. Machine learning models, or an algorithm that can find patterns, make predictions, or make decisions based on input data, can improve the outcomes of target-based drug discovery through virtual drug screening and de novo drug design, improving the speed and accuracy of drug development. Quantitative structure-activity modelling helps to better identify the relationship between the molecular structure of the pharmacologic, its binding mechanism with the drug target and ultimately its activity [285]. One example of repurposing

membrane-interacting drugs is the use of certain antibiotics, originally designed to target bacterial membranes, for the treatment of cancer. For instance, daptomycin, an antibiotic that interacts with bacterial membranes, has shown potential for disrupting cancer cell membranes and inducing cancer cell death. This repurposing strategy leverages the drug's membrane-interacting properties to target cancer cell membranes [34]. Another example involves the repurposing of the antifungal drug, amphotericin B, which interacts with fungal membranes, for the treatment of the parasitic disease - leishmaniasis. Liposomal encapsulation of amphotericin B has been a major focus of repurposing efforts. Liposomal amphotericin B has been used with increasing frequency to treat visceral leishmaniasis and is considered the treatment of choice for immunocompetent patients. This approach has been associated with improved efficacy and reduced toxicity, making it a valuable option for leishmaniasis treatment [286].

Furthermore, predicting membrane partitioning and identifying genuine molecular interactions are two distinct but interconnected aspects of understanding molecular behaviour in biological systems. Membrane partitioning prediction is a computational approach that estimates how a molecule will distribute between aqueous and lipid phases based on its chemico-physical properties, which is crucial for drug design and distribution within the body [287]. The accuracy of these predictions is crucial for understanding drug distribution within the body and can be used to optimize drug design for therapeutic efficacy and bioavailability. Identifying genuine molecular interactions involves determining the specific interactions between molecules, such as proteins and lipids or other proteins, using experimental and computational methods. These interactions, which are mediated by noncovalent forces, are essential for understanding the functional roles of proteins and other molecules in biological systems. This is more complex than predicting partitioning because it requires understanding the biological context and the functional roles of these interactions. For instance, certain lipids may directly interact with membrane proteins and play critical roles in their function, which can be identified through mass spectrometry and other biophysical methods [288].

One of the most revolutionary opportunities within pharmacology is artificial intelligence (AI). AI-enabled drug discovery holds massive potential to increase the accessibility of viable drugs by speeding up the drug discovery process, improving drug efficacy and safety, enabling personalised medicine, lowering drug development costs, and treating presently incurable conditions through computational modelling. AI systems are being used to design drug molecules, analyse vast amounts of data, and accelerate the drug discovery process. In early 2020, Excientia announced the first-ever AI-designed drug molecule to enter human clinical trials [289]. Taking this one step further, in February 2022, Insilico Medicine started phase I clinical trials for the first-ever AI-discovered molecule based on an AI-discovered novel target [290]. The first step AI may be used for is target identification, using databases like AlphaFold to predict 3D structures of drug targets. AI may also be used to simulate the molecular interactions between the drug and its targets, running a high number of models to demonstrate a significantly higher chance of success before human trials. After molecular simulation, AI can predict the drug properties, including toxicity, bioactivity, and physicochemical properties of the molecules. AI-driven *De novo* drug design can create large libraries of candidate molecules, and candidate drug prioritisation can identify promising 'leads' before synthesis pathways can be generated for these hypothetical drug compounds [291].

In conclusion, the exploration of membrane interactions has revealed a spectrum of therapeutic prospects, fostering advancements in the bioengineering of both human and non-human macromolecules. While the primary focus of current research lies in foundational progress, the emergence of studies highlights innovative technologies poised to address afflictions like cancer and inflammatory diseases. Manipulation of diverse entities such as bacteriophages, adenoviruses, bacterial toxins, type 6 secretion systems, annexins, mitochondrial antiviral signalling proteins, and bacterial nano-syringes, showcases promise in therapeutic applications. To propel the field forward, a deeper understanding of each opportunity is imperative, accompanied by further optimisation of therapeutic models. In this review, the wealth of knowledge and insights garnered from various membrane interactions has been encapsulated, shedding light on their current applications and untapped potential for therapeutic interventions.

CRediT authorship contribution statement

Calum Upton: Conceptualization, Writing – original draft, Writing – review & editing. **Joseph Healey:** Writing – review & editing. **Alice J. Rothnie:** Conceptualization, Writing – original draft, Writing – review & editing. **Alan D. Goddard:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

Joseph Healey is CEO and co-founder of the biotechnology company Nanosyrinx (https://www.nanosyrinx.com), which is developing a novel system based around naturally occurring 'nanosyringes' for targeted drug delivery.

Acknowledgements

CU is part of the MIBTP (Midlands Integrative Biosciences Training Partnership) doctoral training centre funded by the BBSRC (Biotechnology and Biological Sciences Research Council), United Kingdom.

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