Anti-infective nanodisc

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Influenza virus, a significant public health concern, continuously mutates, leading to challenges in vaccine and antiviral drug development. While clinical antiviral drugs primarily target specific viral proteins, nanodiscs have emerged as a novel therapeutic approach. Nanodiscs, composed of phospholipid bilayers and amphipathic proteins, mimic cell membranes, providing a controlled, biologically relevant environment for the study of membrane proteins. Their ability to disrupt viral infection in several ways has been studied. By incorporating nanodiscs containing host cell receptors, they can perforate the viral envelope, rendering the virus unable to replicate. Another inhibitory mechanism involves nanodiscs with antibodies, blocking their viral receptors. Nanodiscs offer advantages including improved stability, specific targeting, and broad biocompatibility. Nanodiscs represent a promising avenue for combating influenza and other enveloped viral infections.

Layman summary

The flu is caused by a virus, called the influenza A virus. The virus continuously changes making it hard to develop vaccines or antiviral drugs. This study is about strategies using nanodiscs, which are tiny structures made of lipids and proteins to combat the virus. Nanodiscs have the unique ability to consist out of the same matter as human cells. Therefore some human proteins can be more easily studied outside their natural environment as the nanodisc mimics this. Nanodiscs are studied before for their drug delivery potential. Inside the disc, water repelling molecules can be easily inserted, as the middle of the disc has the same water repelling characteristics. Some molecules that normally bind to the influenza virus can be inserted inside a nanodisc based on these characteristics. The nanodiscs provides a stable environment, possibly creating a stronger binding of the molecules to the virus. When there is binding of the nanodisc to the virus, there are less spots available for cells to bind to the virus. This blocks the entry of virus particles into cells, so they can not replicate and spread through the body. It is also possible that the virus fuses together with the nanodisc into the cell. During this process, the environment becomes more acidic which normally initiates the virus to infect the cell. However, when the nanodisc is present, the nanodisc blocks the fusion of the virus with the cell. The virus is trapped inside a bubble in the cell where is will be broken down. So, also in this case the viral spreading is blocked. By changing the molecules inside the nanodisc, the function and effectiveness of the nanodisc can be altered. It can be specific for another type of virus for instance. Further research should be done to find out all of the possibilities of the nanodisc to block viral infection. This review talks about using different nanodiscs to target various types of influenza. This could be a great way to develop new treatments for viral infections.

Influenza virus

Influenza, commonly known as the flu, is an infectious respiratory illness caused by influenza viruses that can infect the nose, throat, and in some cases even the lungs [1]. It can manifest with varying degrees of severity and, in some instances, can lead to fatal outcomes [2,3]. Influenza viruses are categorized into four types: A, B, C, and D. The natural host of influenza A viruses (H1–H16) is wild waterfowl, but they can also infect a wide range of poultry and mammals [4]. Influenza A and B viruses are responsible for the seasonal outbreaks of the disease in humans, commonly referred to as “flu”.

Until now Influenza A viruses have been responsible for four major human influenza pandemics [5]. A pandemic can occur when a novel and distinct influenza A virus emerges, capable of efficiently infecting humans and spreading among humans who have limited or no immunity against it.

Influenza returns annually due to the constant changes in the surface proteins of the viruses, hemagglutinin (HA) and neuraminidase (NA) [6]. These mutations occur through two mechanisms: antigenic drift and antigenic shift. Antigenic drift involves small changes in the genes of the influenza virus, leading to structural changes in its surface proteins. These surface proteins are antigens, meaning that they can trigger an immune response including the production of antibodies. These changes occur during the virus's replication process over time, as the polymerase control mechanism is not entirely precise, resulting in substitutions within the gene. The minor genetic changes give rise to viruses that are closely related. However, the cumulative effect of these small changes in HA and NA can eventually lead to antigenically distinct viruses. This means that a person's antibodies may no longer effectively bind to the virus or may not recognize it at all, resulting in reduced protection against that specific influenza strain [7].

The other type of change, called antigenic shift, is a major change in a flu virus [8]. Shift occurs when two or more different strains or types of the influenza virus merge, giving rise to a new subtype that has a mixture of the surface antigens of the two or more original strains. These viruses may carry HA/NA combinations that differ significantly from those in human viruses, leaving most people without immunity to the new virus. Such a shift occurred in the spring of 2009 when an H1N1 virus, processing genetic elements from viruses originating in swine, humans, and birds, emerged to infect humans and rapidly spread ultimately causing a pandemic[1]. During such a shift, most individuals have limited to no immunity against the newly formed virus. Type A viruses undergo both antigenic drift and shift and are the only flu viruses known to cause pandemics, while flu type B viruses change only by the more gradual process of antigenic drift. These constant changes result in significant challenge in battling the virus [9]. Influenza A has been associated with more significant morbidity and mortality, making it a greater public health concern[3]. Therefore, the focus of this review is mainly on Influenza A.

Route of infection

Influenza viruses employ a series of steps to infect host cells, involving both their attachment and internalization processes. The initial step of infection is the binding of viral hemagglutinin (HA) to host cell surface glycoconjugates that contain terminal sialic acid residues. Then, by using the sialidase function of NA the surface is scanned for the proper sialylated "receptor". NA is a receptor destroying enzyme, it cleaves sialic acid, allowing the virus to be released after binding to sialic acid containing molecules that do not lead to viral infection. After succesful attachment of the virus to the host cell surface, the virus enters the cell through receptor mediated endocytosis. It is then transported along the endocytic pathway to late endosomes. Within these late endosomes, the low pH triggers a fusion event between the viral and endosomal membrane, resulting in the release of viral ribonucleoproteins (vRNPs) [10]. These vRNPs are imported into the host cell's nucleus, facilitating viral gene expression and replication (Figure 1) [11,12].

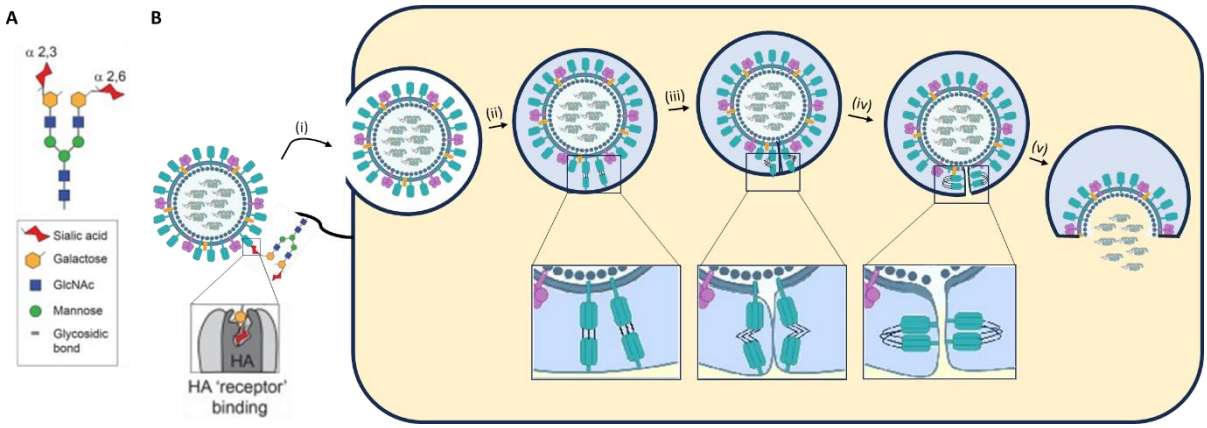

Figure 1: Receptor-mediated cell entry of IAVs [13]*. (A) Diagram of a bi-antennary N-linked glycan. The terminal sialic acid residues are displayed with an α-2,3 linkage, as well as an α-2,6 linkage, to illustrate the "linear" and "bent" presentations. (B) Illustration of IAV cell entry. (i) IAVs initiate cell entry by using the HA receptor-binding domain to associate with sialylated glycoconjugates on a host "receptor." Binding to the "receptor" triggers endocytosis. (ii) The virus then traffics to the endosome where the lower pH facilitates a conformational change in HA, exposing the fusion peptide for insertion into the endosomal membrane. (iii) The HA pre-hairpin conformation begins to collapse, forming a six-helix bundle that promotes hemifusion of the viral envelop with the endosomal membrane. (iv) A pore is created which connects the viral particle's inside with the cytoplasm (v) resulting in a release of the vRNPs inside the host cell.*

To be successful, respiratory viruses must efficiently infect their hosts via the respiratory mucosa, replicate within the appropriate areas of the respiratory tract, and eventually release highly infectious transmissible material that infects a new host. The interaction between the viral attachment protein and its cellular receptor plays a central role within virus infection, replication, and transmission. Thus, the presence or absence of cellular receptors in specific hosts and tissues is one of the key factors that influences the host's susceptibility to viral infection.

Sialic acid receptors

All enveloped viruses rely on glycosylation to modify their membrane proteins, crucial for attaching to target cells and facilitating membrane fusion [14]. Glycosylation involves the addition of carbohydrate molecules, known as glycans, to various biomolecules, including proteins and lipids. This critical process occurs in two distinct subcellular organelles: the endoplasmic reticulum (ER) and the Golgi apparatus. Within eukaryotic cells, glycosylation is among the most prevalent post-translational modifications, playing a central role in protein folding, stability, and cell signalling[14].

Host cells are covered with a dense layer of glycans, crucial for their activity and native functions[15]. These glycans play an important role in facilitating the attachment of influenza viruses to the host cells. Glycans can be categorized into three primary types: N-, O-linked glycosylation and glycolipids. N-linked glycosylation, where the glycan is attached to the nitrogen atom of the amino acid asparagine (N), stands out as the most abundant form of glycosylation in eukaryotic cells, accounting for over 90% of all glycosylation events[16,17]. This process begins with the transfer of a preformed oligosaccharide from a lipid carrier to the nascent protein, primarily carried out in the ER and Golgi apparatus. On the other hand, O-linked glycosylation involves the attachment of the glycan to the oxygen atom of the amino acid serine or threonine (O) and predominantly occurs within the Golgi apparatus. This process begins with the addition of a single sugar residue to the serine or threonine residue of the developing protein, resulting in a diverse array of glycosylated biomolecules [18]. Lastly, glycolipids are synthesized in the Golgi apparatus, comprising lipids covalently linked to carbohydrates, imparting them with an amphipathic nature. This class of compounds primarily includes glycosphingolipids and

glycoglycerolipids. These three primary glycan structures can serve as receptors for Influenza A viruses, making them important during viral infection within a host.

The initial stage of influenza virus infection depends on the binding of the viral HA protein to sialic acid (SA) that are exposed on the surface of epithelial cells within the host. SAs are commonly part of glycoproteins, glycolipids or gangliosides, where they decorate the end of sugar chains at the surface of cells or soluble proteins. SA comes in various forms, with N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc), being among the most prevalent types on the host cell surface. The position of the C2 carbon atom of sialic acids can form distinct glycoside bonds with the C3- or C6-position carbogen of the secondary terminal galactose. These bonds are created by α-2,3 sialyltransferase (ST3Gal) or α-2,6 sialyltransferase (ST6Gal), and the nature of these glycosidic bond significantly influences the binding properties of influenza virus receptors (Figure 1A). This has a major impact on the cross-species transmission of influenza viruses. Human influenza viruses tend to favor α-2,6 sialic acids, while avian influenza viruses show a preference for α-2,3 sialic acids [19–22].

When designing antivirals the structure of glycans and SA should be taken in account as these components affect the specificity of the virus to the host cell. Parts of these host-cell glycans can possibly be used as inhibitors by shielding the HA binding site. Understanding the interactions between viral components and these glycoconjugates is essential for designing targeted antiviral strategies that can disrupt or inhibit the crucial steps involved in viral entry and infection [23,24].

Influenza viruses present a substantial public health concern due to their pronounced variability and recombination. They are characterized by their continuous mutation, where genetic segments frequently undergo reassortment, leading to the emergence of new virus subtypes. The constant changing of the viruses poses challenges in the development of vaccines and antiviral medications. Nevertheless, a lot of research is conducted, and this has let to some therapeutic options.

Antivirals

Antiviral agents are categorized by their ability to target specific stages within the viral life cycle, hindering virus development and proliferation. While vaccination remains the most effective strategy for preventing infection, available vaccines have limitations as they are primarily effective against a restricted range of viral strains. This limitation is attributed to mutations in viral RNA (vRNA) segments, the exchange of vRNA segments between human and avian influenza strains, or the emergence of new HA subtypes.

NA inhibitors function by targeting the NA activity in influenza viruses, impeding the release of viral particles from infected cells and effectively suppressing viral replication. Prominent examples of NA inhibitors include oseltamivir, peramivir, and zanamivir. In contrast, M2 ion channel blockers primarily operate by obstructing the hydrogen ion channel activity of the M2 protein in influenza viruses, thus inhibiting viral replication. Representative drugs in this category encompass amantadine and rimantadine.

In the Table below, the antiviral drugs that are approved are listed.

Table 1: Overview of approved antiviral therapeutics.

Drug	Target	Activity against	Administration
Oseltamivir	NA inhibitor	Influenza A and B	Oral
Zanamivir	NA inhibitor	Influenza A and B	Inhaled
Peramivir	NA inhibitor	Influenza A and B[d]	Intravenous
Amantadine	M2 inhibitor	Influenza A	Oral

Rimantadine	M2 inhibitor	Influenza A	Oral
Baloxavir marboxil	Polymerase acidic endonuclease inhibitor	Influenza A and B[f]	Oral

The approved anti-influenza drugs, M2 ion channel inhibitors (amantadine and rimantadine), NA inhibitors (oseltamivir and zanamivir), and a cap-dependent endonuclease inhibitor targeting the viral polymerase acidic (PA) protein, (baloxavir marboxil), currently serve as the primary treatments for individuals infected with influenza, especially during influenza pandemics when effective vaccines are not available [25,26]. However, the Centers for Disease Control and Prevention (CDC) no longer recommends the use of M2 inhibitors in clinical settings due to the prevalence of drug resistance [27]. Additionally, during the 2007–2008 influenza season, the circulating H1N1 strain developed nearly complete resistance to oseltamivir [28]. This underscores the rapid evolution of new influenza strains, their ability to cross species boundaries, and their capacity to develop resistance to existing drugs. Consequently, there is a pressing need for the development of new antiviral medications and combination therapies that target various viral components, with different mechanisms of action, to address a broad spectrum of influenza viruses. This is particularly important in dealing with recurrent drug-resistant strains and unexpected influenza pandemics [29–31].

Experimental therapeutic options based on glycan receptors

Glycans, covalently attached to acceptor molecules such as proteins and lipids, have emerged as promising candidates in therapeutic interventions[32]. The structural remodelling of protein-linked glycans offers several advantages, including the extension of activity and stability both in vitro and in vivo[33]. By binding to viral HA, the glycans can cover the surface area of HA blocking the ligand binding needed for cell entry [34]. The glycans can access a large volume, allowing for a considerable shielding potential. Also, molecules that mimic the structure of sialic acid can bind to viral HA, thereby inhibiting virus attachment to host cell [35,36]. By combining the molecules into a trimer, an even stronger inhibitory effect can be achieved [37] .

In earlier research is the effective inhibition of influenza A virus infection by multivalent 6'-sialyllactose-polyamidoamine (6SL–PAMAM) conjugates demonstrated. The design of these conjugates, with precisely defined ligand valencies and spacings, shows their potency as multivalent inhibitors of influenza viruses. The structural optimization of nanoscale architectures enables simultaneous interactions between 6SL ligands and the three 6SL binding sites on an influenza A virus HA trimer. Notably, the findings emphasize the critical role of manipulating interligand spacing, rather than ligand number (valency), in spherical G4 and G5 scaffolds for binding to the target HA trimer on IAV surface proteins, thereby effectively inhibiting infection [38].

However, achieving a quantitative preparation of glycans and glycoconjugates with well-defined structures remains a challenging task from a technical standpoint [39].

Antibodies

A number of human monoclonal antibodies have been described that can bind and neutralize a broad range of influenza A and B viruses [40,41]. Monoclonal antibodies targeting specific regions on hemagglutinin can block viral attachment and entry. These antibodies are being investigated for their potential as therapeutic agents, for example, broadly neutralizing antibodies directed against the conserved HA stalk domain can prevent membrane fusion but also engage antiviral effector functions by natural killer cells or macrophages [42]. However, due to the continuous changing of the envelop proteins it is hard to develop vaccines with durable immune protection. Thus, studies targeting

conserved internal nucleoprotein (NP) or matrix protein 1 (M1) are performed [43] are of particular interest.

Only a few therapeutic antibodies have been approved as antiviral agents, except for those urgently needed against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), whereas many therapeutic have been marketed, mostly as oncology, autoimmune, and inflammatory therapeutics[44].

Nanostructures

Utilizing natural or biomimetic materials such as lipids as carriers, nanomedicines facilitate the delivery of both hydrophobic and hydrophilic drugs, directing them specifically to diseased areas to enhance their bioavailability. Antiviral nanostructures have been mostly developed as decoys. When decoy molecules that target viral proteins are contained in nanostructures of a dendrimer or a liposome, it interferes better with the virus-host interaction compared with their monomeric forms through multivalent interactions. While liposome-based drug delivery systems have already seen the release of several medications [45], they do come with limitations. These include short circulation half-lives, limited drug encapsulation capacity, and instability in biological fluids. Here, nanodiscs step in to mitigate some of these lipid-related disadvantages [46].

In earlier research, liposomes and glycans are combined creating a multivalent system. A streamlined and effective synthesis method was developed for phospholipid conjugation of S-Neu5Acα2-6Galβ1-4GluNAcβ1-3 and its 6-sulphate analogue. Through the acyl chains of the phospholipids, the structures can easily self-assemble into micelles and liposomes in solution. These self-assembled trisaccharides demonstrated inhibitory effects on the entry of the H1N1 influenza virus into MDCK cells. Particularly, the compound bearing both a liposome and a micelle exhibited inhibitory activity compared to its 6-sulfate counterpart in both the virus neutralization assay and the hemagglutination inhibition assay [47].

Compared with the reconstruction in micelle and liposome, integrating the membrane protein into the nanodisc can maintain its structure in the natural state to the maximum extent because the nanodisc is flat and produces no tension that affects the membrane protein conformation. The surface of the micelle and liposome is curved or spherical, which will inevitably affect the conformation of membrane proteins. The size of a nanodisc is also optimal to perform many biochemical, biophysical, and functional experiments. Nanodiscs show promising antiviral effects [48,49].

What is a nanodisc?

Nanodiscs are primarily composed of phospholipid bilayers and an amphipathic protein, such as membrane scaffold proteins (MSP) or synthetic polymers, which forms a belt-like enclosure around the acyl chains of the phospholipids. Within this structure, the non-polar residues of the encapsulating molecule or protein face inwards toward the hydrophobic core, while the polar or charged residues are oriented outward, interacting with the surrounding aqueous environment [50,51].

The nanodiscs mimic the structure and properties of the cell membrane, creating a controlled and biologically relevant setting for the study of membrane proteins. This unique characteristic enables researchers to investigate these proteins in a native-like environment, providing valuable insights into their behaviour, interactions, and functions. Nanodiscs are a valuable tool for studying the structure, function, and interaction of membrane proteins as well as for drug discovery and development.

There are two types of nanodiscs, MSP nanodiscs and synthetic nanodiscs. They differ between the amphipathic molecule shielding the acyl chains.

Table 2: Differences between MSP and synthetic polymers.

	MSP nanodisc	**Synthetic nanodisc**
Stabilizer	MSP	Synthetic polymer (SMA, DIBMA)
Phospholipid source	Artificial phospholipids	Native cell membrane
Preparation method	Membrane proteins with detergent self-assemble into nanodiscs or nanodiscs combination with cell-free expression system	Synthetic polymers dissolve the cell membrane
Need for detergent	Yes or no	No

How are nanodiscs prepared?

MSPs closely wrap around the artificial phospholipids, resulting in the formation of MSP nanodisc. Membrane scaffold protein 1D1 (MSP1D1) is a synthetic derivate of apolipoprotein A-I (apo A-1), which is the major protein element of human high-density lipoproteins (HLD). Apo A-1 plays a key role in facilitating the transport of specific lipids, including cholesterol ester and other small molecule metabolites, as it helps in the removal of cholesterol from white blood cells located within artery walls. Each MSP protein contains 200 amino acid residues and is soft and malleable. MSP provides a hydrophobic surface facing the hydrophobic tail of the lipids, and a hydrophilic surface on the outside. This setup makes nanodiscs highly soluble in aqueous solutions. Once assembled into nanodiscs, membrane proteins can be kept in solution without detergents [52].

Before assembling the nanodisc, the MSP protein is cloned in pET-28a vector and expressed using *e. coli.* Afterwards, the proteins are purified by immobilized metal affinity chromatography (IMAC) through the attached His-tag. To create a nanodisc, a mixture of lipids and gangliosides is combined with detergent in an 80:20 ratio. The MSP stock is introduced into this lipid-detergent mixture, facilitating the spontaneous orientation around the lipids. Following this assembly process, the detergents are eliminated using hydrophobic adsorbents, resulting in the spontaneous formation of nanodiscs. The resulting nanodiscs can be purified through size exclusion chromatography. This process can easily be adapted based on the inserted protein or glycan of interest (Figure 2) [53].

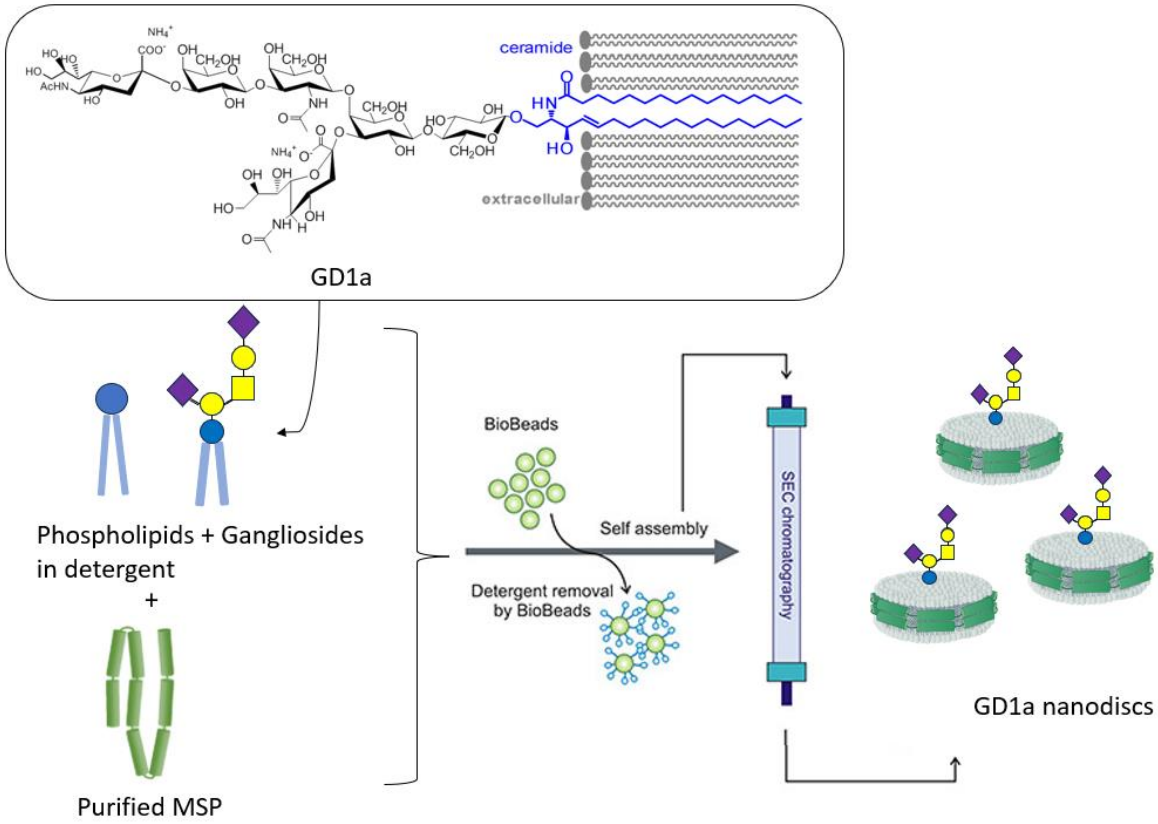

Figure 2: Overview of the production of nanodiscs with a ganglioside inserted in the lipid membrane. First, the pure components are added together in the right ratio with detergent. The assembly process is initiated by removal of the detergent. The nanodiscs containing the gangliosides can be selected using SEC chromatography.

Application of nanodiscs

Nanodiscs can provide a membrane-like environment with strong stability, high solubility, and precise targeting advantages, and have been widely used in proteomics and biomedicine fields, including the production and purification of membrane proteins, receptor- and ligand-binding drug target research, and structural biology [54]. Nanodiscs are versatile tools that bridge the gap between the complex native cellular environment and the need for controlled, reproducible conditions in various research and applications[51]. Their ability to mimic cell membranes and solubilize membrane proteins makes them invaluable in a wide range of scientific and medical fields.

Membrane protein studies

Membrane proteins are defined as proteins being associated or attached to the membrane of a cell or an organelle inside the cell[55]. They are divided into peripheral and integral proteins. Together with hydrophilic extra- and intracellular domains, the majority of membrane proteins show an amphipathic character. The amphipathic character additionally produces a signature by which integral membrane proteins can often be identified. This is due to their primary structures containing 19-23 hydrophobic amino acids in their linear sequences, required to span the hydrophobic interior of a membrane. β-barrels with hydrophobic residues pointing to the outside of the barrel can also act as a good indicator of a membrane protein [55].

Cell membranes act as the final gatekeepers for many substrates and drugs. Highly specialized membrane proteins, therefore, interact in a fraction of a second with a plethora of substances to determine which of them are allowed through and which are not. About half of all currently approved

drugs act on this protein type as a therapeutic target structure to cause signal transductions, trigger cascades, or catalyze reactions to combat various clinical conditions [56].

In spite of this, little is still known about many membrane protein structures as they are difficult to study for a number of reasons [57]. Their surface is relatively hydrophobic, and they can only be extracted from the cell membrane using detergents, which cover the hydrophobic surface of the protein. Next to this, they are flexible and highly unstable resulting in a structural change compared to their native environment. Solubilization of the membrane protein via a nanodisc can overcome these limitations[54].

Drug development

Nanodiscs provide a native-like environment for the incorporation of membrane proteins, aiding in the identification of drug targets. The bacterial outer membrane-derived nanodiscs serve as a valuable platform for studying bacterial membrane proteins, contributing to the discovery of targets for antibacterial drugs [58].

Therapeutic delivery agent

Nanodiscs have demonstrated success as therapeutic delivery agents, particularly in the targeted delivery of anticancer drugs [59]. They have been utilized for the delivery of various agents, including anticancer drugs, peptides, short interfering RNAs (siRNAs), and neoantigens. This targeted delivery approach enhances drug efficacy while minimizing off-target effects [54,60,61].

How can a nanodisc be used against viral infection?

Nanodiscs have emerged as a tool in the battle against viral infections. These versatile structures offer a unique approach to intercept and neutralize viruses at the early stages of infection. The different ways in which nanodiscs can be used to combat viral infections are discussed below.

Vaccination

Various vaccine formulations, including those containing soluble HA, have been developed to combat viral infections as vaccines [62–64]. Recombinant glycoproteins, as previously discussed, offer distinct advantages. In experimental settings, both recombinant HA (rHA) and recombinant NA have demonstrated the ability to protect mice against lethal challenges [65,66]. Some rHA-based vaccines elicit higher titers of anti-HA antibodies. However, these antibodies may not effectively interact with the "native epitopes" due to differences in structure between the soluble rHA used in the vaccine and native HA, which prefers to bind as a trimer [67]. A way to neutralize the virus is by using more native like HA proteins, which can be achieved by using nanodiscs. The nanodisc provides a native membrane environment, facilitating proper folding and enhanced binding of HA, thereby contributing to a more effective neutralization of the virus.

rHA from influenza virus A (A/New Caledonia/20/99 [H1N1]) underwent solubilization using β-OG and was subsequently incubated with POPC (lipids) and MSP1T2 (membrane scaffolding protein). The removal of detergent facilitated the self-assembly process, resulting in the formation of nanodiscs containing HA (HA-ND). It's noteworthy that ELISA analysis, utilizing anti-HA and anti-MSP antibodies, has revealed that the HA protein incorporated into these nanodiscs has the ability to form trimers or higher-order multimers. While rHA alone can initiate an immune response, the introduction of HA-ND was anticipated to amplify its immunogenicity. To assess this, antibody responses were monitored in mouse sera following exposure to TBS, soluble HA, and HA-ND at days 0, 7, 14, and 21. Strikingly, by day 14, the HA-ND elicited significantly higher levels of anti-HA IgG antibodies compared to HA alone [68].

Moreover, for specific virus strains, sera from HA-ND demonstrated increased hemagglutination inhibition (HI) titers, indicating a broader and more robust reactivity. These results collectively suggest that the incorporation of rHA into nanodiscs enhances its protective efficacy, making it a promising avenue for improving vaccine development and effectiveness against diverse influenza virus strains [68].

Perforating viral envelope

Targeting the viral membrane presents a strategy to overcome resistance, as the envelope is derived from the host cell membrane, which is not under direct control of the virus [69,70]. However, a significant drawback of membrane targeting is its lack of specificity to the virus, potentially resulting in cytotoxicity. To address this challenge, nanodiscs containing a virus-specific receptor offer a targeted approach to interact with the viral membrane.

In this context, a total ganglioside (TG) extract serves as the receptor for binding with HA. This extract, comprising glycosphingolipids containing sialic acid (SA) residues in the glycan chain, can be easily inserted into the nanodisc through their acyl chain. The efficacy of nanodiscs with the ganglioside receptor (NDTG) against mouse-adapted influenza virus A/PR/8/34 (H1N1) infection was evaluated using cytopathic effect (CPE) reduction and plaque reduction assays. Remarkably, NDTG significantly reduced plaque size compared to lysosomes containing TG, exhibiting a twofold reduction. This suggests that the nanodisc's shape plays a crucial role, indicating it functions not merely as a decoy but actively influences viral infectivity. Analysis with RT-PCR and EM followed by immunogold labelling showed that at low pH levels (pH 5), NDTG induced the release of viral ribonucleoproteins (vRNPs), contrasting with high pH conditions (pH 7) or in the absence of NDTG. TG liposomes showed minimal vRNP release [71]. Next to this, lipid-mixing assays using FRET showed perforation of the viral membrane inside the endosome which was confirmed by a co-localisation assay with the confocal microscope. This underscores the crucial role of NDTG in entrapping the virus inside the endosomes.

Considering that a virion of influenza carries an estimated ~400 HA trimers[72], an effective binding inhibitor needs to block a substantial number of HA proteins. Nanodiscs leverage these HAs as decoys, increasing the likelihood of viral envelope perforation when a multitude of HA molecules are present. This innovative approach demonstrates the potential of nanodiscs in targeted antiviral strategies, combining specificity and efficacy in membrane-targeted interventions.

Upon influenza virus infection, the adaptive immune system can trigger an antibody response [73]. In the presence of antibodies, the previously described anti-viral nanodiscs will lose its effectiveness as is can not bind to the HA protein that is occupied by the antibody. Therefore, a nanodisc is designed that enable infected cells to self-eliminate viruses invading the host cells, particularly in the presence of neutralizing antibodies.

The focus on combating viral infections has led to the development of Broadly Neutralizing Antibodies (bnAbs), representing a significant advancement. These specialized antibodies target conserved epitopes on viral proteins, particularly within the HA protein. Notably, bnAbs exhibit specificity toward critical regions on HA, including the HA2 stalk domain and the Receptor Binding Domain (RBD). By recognizing these conserved epitopes, bnAbs have the capacity to neutralize a broad spectrum of viral strains, providing a versatile defence mechanism against evolving viruses. An example is MED18852, an bnAb that has shown to be effective to a broad range of influenza virus strains. MED18852 disrupts the conformational change of HA, effectively inhibiting the fusion of HA with the host cell endosome.

In the ND assembly process, the steps remain consistent, with an addition of an Fc-III peptide attached to MSP. This Fc-III, a 13-mer, can bind to human IgG. This peptide was introduced before the MSP units inside the plasmid (Figure 3A), to create a ND with an affinity for MED18852 which was confirmed by

SEC combined with light scattering. Upon expression, the peptide protrudes outside the disc, creating a "anchor" for antibodies as MED18852 (Figure 3) [74]. These nanodiscs, containing the Fc-binding peptide sequence, create an anti-nanodisc complex that can co-endocytose into cells infected with the influenza virus leading to envelope perforation within the endosome confirmed by lipid-mixing assays. In a plaque reduction assay, the antiviral activity of nanodiscs with MEDI8852 exhibited a reduction compared to the activity observed with MEDI8852 alone against H1N1. The binding of the antibody containing nanodisc was confirmed with TEM, showing both co-endocytosis and binding to the viral envelope. Alternatively, an excess of antibodies might prevent the host cell's receptor from binding to HA, resulting in the blockade of endocytosis (Figure 3B).

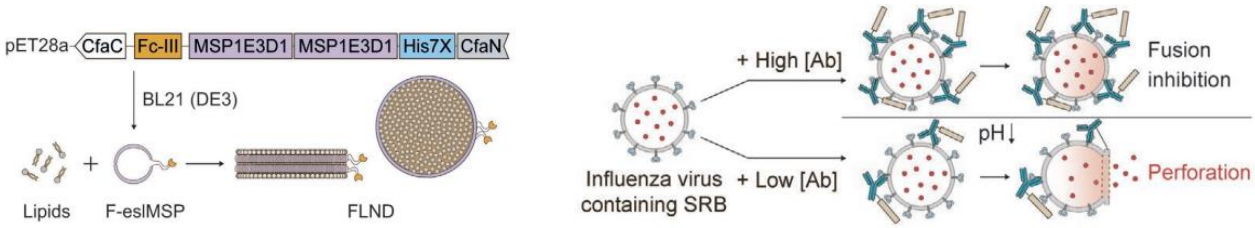

Figure 3: A) Plasmid construction of MSP with Fc-II peptide. B) Switching antiviral mode of action at different antibody concentration. At high antibody concentration, the ND mostly functions as binding inhibitor. While at lower concentrations, the ND perforates the membrane blocking fusion of the viral envelope with the host cell.

Combination nanodiscs

To achieve universal protection, . Depending on the SA receptor inserted inside the ND, it will bind to the virus. While different virus strains favour different types of glycans, as a2,3-linked SA or 6'-sialyllactose. So, another strategy is the utilization of di-disc nanodiscs which introduces an approach to broaden the spectrum of influenza strains effectively targeted. Unlike traditional nanodiscs, di-disc nanodiscs consist of two discs covalently bound via disulfide bonds, each equipped with different receptors.

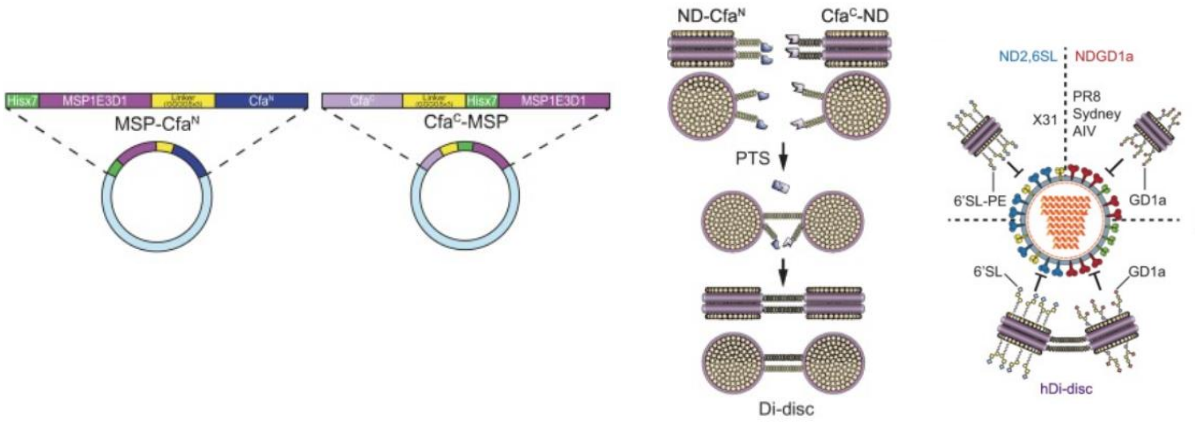

Figure 4: A) Plasmid structure of MSPs. B) Cartoon representation of trans-splicing creating a di-disc. C) Cartoon representation illustrating the synergistic and cooperative effects between individual mono-disc components within the hetero di-disc in their interaction with influenza strain HAs. These effects highlight the advantages of hetero di-discs in binding to influenza viruses.

Several approaches were explored to develop a nanodisc containing two distinct receptors. Simply combining both receptors in one disc proved ineffective, as the abundance of one overpowered the other, rendering only one receptor effective. The mixing of single-labelled NDs also led to interference between the discs. To overcome these challenges, a di-disc was engineered by connecting single-labelled nanodiscs through protein trans-splicing. This process resulted in a covalently bound di-disc via disulfide bonds, with each disc equipped with different receptors (Figure 4)[75].

The Di-disc increased survival rate of H3N2-infeceted mice with improved loss of body weight. Additionally, the CPE inhibition increased from 16% to complete inhibition when transitioning from mono-disc to di-disc. Plaque-forming assays further demonstrated the pronounced antiviral effect of homo di-discs in comparison to mono-discs. While the homo di-disc significantly enhanced antiviral activities, the hetero di-discs demonstrated synergistic effects by optimizing the function of each mono-disc component through their proximity. In essence, the virus-bound component of the hetero di-disc markedly enhanced the potential of the other component, even with a less preferred receptor, to bind more effectively to the viral surface. This proximity effect facilitated the fusion of both components, allowing not only the preferred receptor component but also the less preferred one to interact with the viral membrane. So, the di-discs exhibit heightened antiviral efficacy due to the synergistic effects of proximity and an expanded surface area. This configuration allows multiple hemagglutinin molecules to engage with the planar bilayer, enhancing their collective antiviral activity.

Mode of action/mechanism

Viral membrane perforation

By incorporating host cell receptors as SA within these nanodiscs, they function as fusion and binding inhibitors. During viral infection, influenza viruses initially bind to host cells by attaching to SA receptors on the cell surface. The binding of the other available viral HA to the host cell SA triggers endocytosis. The remarkable feature of nanodiscs lies in their ability to co-engage with the virus by binding SA to HA during the process of endocytosis. As the nanodiscs and the virus travel along the endocytic pathway, they encounter an environment with lower pH levels, such as within late endosomes. In this acidic environment, typical influenza viruses would undergo a membrane fusion event, allowing the release of their genetic material into the host cell, which is essential for viral replication. However, the presence of these specially designed nanodiscs disrupts this process. As the pH decreases within the endosome, the nanodiscs respond to this change and merge with the viral envelope. This merging results in the formation of pores in the viral membrane. Consequently, the viral particles are incapable of further replication, as their genetic material remains trapped within the endosome. The nanodiscs, by acting as fusion inhibitors, provide an effective and innovative means to hinder viral infection and propagation, presenting a promising strategy in the ongoing battle against influenza and other enveloped viruses.

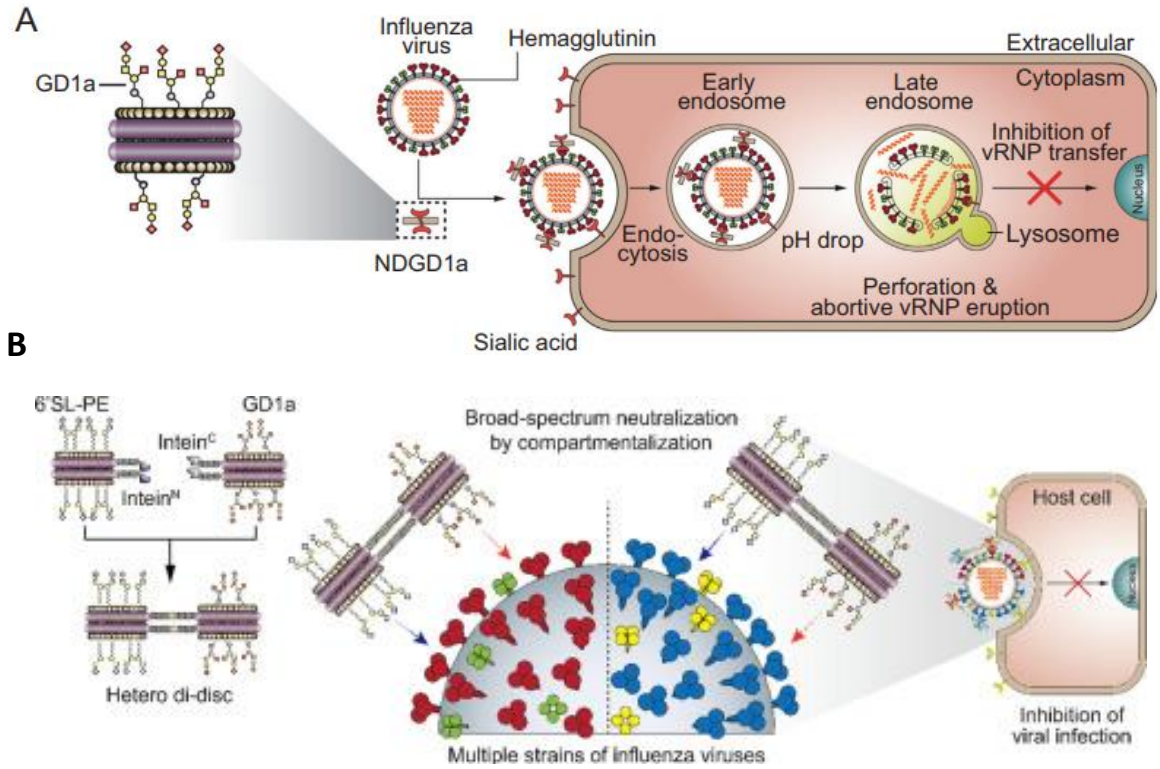

Figure 4: A) Binding of the nanodisc with the inserted sialic acid receptor to the HA of the viral particle. The nanodisc co-endocytosis and initiates perforation of the viral membrane inside the endosomes. B) Cartoon of hetero di-disc binding to two different strains of influenza.

The dual disc has the same inhibiting mechanism as the GD1a ND. However, it distinguishes itself by having the ability to recognize a broader range of influenza strains. Moreover, it exhibits heightened inhibitory activity due to the increased surface area of the disc and the distinct roles played by each component of the disc.

When the nanodisc incorporates an Fc-III region capable of binding to antibodies, the co-endocytosis process described earlier can still occur. The nanodisc will attach to the secreted antibody, and through the antibody, it will establish a connection with the viral particle by binding to the enveloped proteins. As illustrated in Figure 5A, the nanodisc will merge with the viral envelope inside the endosome, preventing fusion with the host cell. In the presence of a substantial quantity of antibodies, fusion becomes hindered as there are insufficient HA receptors available to bind with the host cell's SA. This occupation of the HA binding sites obstructs viral entry, as depicted in Figure 5B.

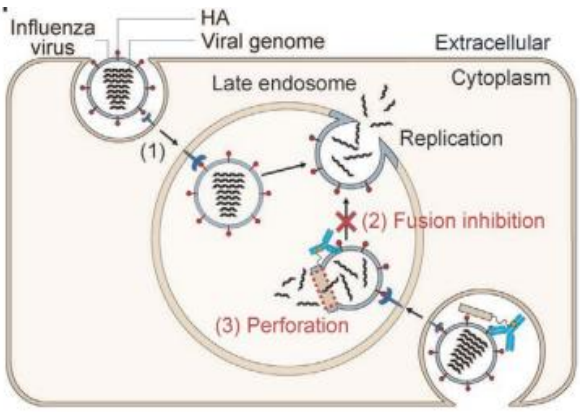

Figure 5: Viral membrane perforation via co-endocytosis of the antibody-nanodisc. The nanodisc will initiate perforation of the viral envelope resulting in inhibition of viral replication

Discussion

Nanodiscs, nanometer-sized lipid bilayers stabilized by membrane scaffold proteins (MSPs), have emerged as versatile tools in the field of biophysics and molecular biology. Their ability to mimic the natural lipid environment makes them ideal candidates for studying the structure and function of membrane proteins. Beyond their research applications, recent developments suggest that nanodiscs with different receptors could hold significant promise as a novel approach for antiviral therapy. In this discussion, we will explore how nanodiscs with various receptors can be harnessed as a potential antiviral strategy.

Relative to soluble HA, the ND-embedded HA molecules mimic the viral envelope better, perhaps as consequence of HA assuming a conformation that is closer to that of the HA on the viral envelope when it is embedded in the NDs. The approach of integrating recombinant HA into nanodiscs not only improves the stability of the viral protein but also promotes specific immune recognition. The induction of IgG1 subclass antibodies suggests a systemic and enduring immune response[68]. IgG1 antibodies are recognized for their capacity to activate diverse immune effector mechanisms, including antibody-dependent cellular cytotoxicity (ADCC) and complement activation, enhancing the effective elimination of viral threats.

Nanodiscs can be engineered to incorporate specific receptors that are recognized by viral particles. These receptors can act as decoys, attracting and binding to the viruses, thereby preventing them from infecting host cells. For instance, the hemagglutinin (HA) protein on the surface of influenza viruses binds to sialic acid (SA) receptors on host cells. By incorporating SA receptors into nanodiscs, these artificial lipoparticles can effectively bind and neutralize influenza viruses. The flexibility of nanodisc engineering allows for the creation of different types of receptors that can be tailored to specific viruses. For example, nanodiscs can be designed with glycan receptors for enveloped viruses, where the viral envelope glycoproteins interact with host cell glycans. By using glycan-bearing nanodiscs, these viral particles can be intercepted and prevented from entering host cells.

Nanodiscs with receptors offer enhanced targeting and specificity. By designing receptors that precisely match the viral attachment sites, the probability of viral binding to the nanodiscs is increased. This high specificity reduces the risk of off-target interactions and side effects, making it a safer approach compared to broad-spectrum antivirals. An approach to maintain specificity while preserving a broad range, particularly in the context of rapid viral mutations, involves the incorporation of both the inserted SA glycan and the Fc-III region connected to MSP. In scenarios where the virus manages to evade the specific binding of HA to SA, the nanodisc can still bind to the viral particle through antibodies. However, it is crucial to conduct tests to ensure that these two mechanisms do not interfere with each other when both are connected to the nanodisc. Conducting binding assays, employing size exclusion chromatography (SEC), and utilizing single-particle analysis cryo-electron microscopy (cryo-EM) can help assess whether the presence of an extra antibody influences the binding affinity between the nanodisc-inserted SA receptor and the viral HA.

The effects of HA-ND are comparable to those of split-virus and whole-virus-based vaccines[68]. Nevertheless, opting for recombinant vaccines offers several advantages. The cloning, expression, and manufacturing processes of rHAs are less time-consuming compared to whole-virus vaccines, enabling faster and more scalable vaccine production in response to emerging viruses. Additionally, as no live influenza virus is involved in the manufacturing process, the necessity for biocontainment during vaccine production is eliminated [76,77]. Another options is using nanodisc-based antiviral therapy in combination with existing antiviral drugs. In the study by Kong et al. (2019), the combination of membrane targeting with ostelmivir, a NA inhibitor, demonstrated nearly complete eradication of the

virus. This suggests potential cooperation between the membrane-targeting nanodisc and a protein-targeting antiviral. Additionally, Bhattacharya et al. (2010) found that Fluzone, a commercially available inactivated "split-virus" vaccine, elicited a comparable immune response to HA-ND. However, a notable difference was observed in the IgG subclass response. HA-ND primarily induced the IgG1 subclass, whereas the Fluzone-immunized group exhibited dominance of the IgG2a subclass. Combining Fluzone with HA-ND could potentially broaden the immune response and enhance overall effectiveness. So, combining nanodiscs with receptors with traditional antiviral drugs may enhance treatment efficacy by targeting multiple stages of the viral life cycle.

Various glycans, each with distinct properties, can be incorporated into the lipid bilayer, contributing to the versatility of the nanodiscs. Covalent attachment of antibodies to the membrane scaffold proteins (MSP) further expands the repertoire of antiviral agents. Additionally, the inclusion of lectins within the membrane adds another dimension to the therapeutic potential of nanodiscs. Furthermore, small molecules and approved drugs can be strategically placed within the nanodisc, based on their chemical properties. Hydrophobic components may be embedded within the lipid bilayer, while others can be attached to the MSP, allowing for a tailored and multifaceted approach in drug delivery. This integration of nanodisc-based therapy with conventional antiviral drugs holds promise for optimizing treatment strategies, offering a comprehensive and adaptable approach to combat viral infections.

The promising findings from this research pave the way for future exploration. It's important to note that, currently, the experiments have been conducted solely by a specific research group. Expanding the scope of these studies through collaboration or independent validation could further strengthen the credibility and applicability of the results. Diversifying the sources of experimentation would contribute to a more comprehensive understanding of the potential implications and robustness of the presented findings. Despite the antiviral-nanodisc promise, there are challenges to overcome, such as optimizing nanodisc receptor design, delivery methods, and ensuring safety. Future research should focus on refining these technologies and conducting rigorous preclinical and clinical trials to establish their efficacy and safety in humans.

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