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Review article

Strategies for fighting pandemic virus infections: Integration of virology and drug delivery

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ABSTRACT

Respiratory viruses have sometimes resulted in worldwide pandemics, with the influenza virus and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) being major participants. Long-term efforts have made it possible to control the influenza virus, but seasonal influenza continues to take many lives each year, and a pandemic influenza virus sometimes emerges. Although vaccines for coronavirus disease 2019 (COVID-19) have been developed, we are not yet able to coexist with the SARS-CoV-2. To overcome such viruses, it is necessary to obtain knowledge about international surveillance systems, virology, ecology and to determine that immune responses are effective. The information must then be transferred to drugs. Delivery systems would be expected to contribute to the rational development of drugs. In this review, virologist and drug delivery system (DDS) researchers discuss drug delivery strategies, especially the use of lipid-based nanocarriers, for fighting to respiratory virus infections.

1. Introduction

Respiratory viruses, which are spread by droplets, have often cased pandemics. The first influenza pandemic encountered by humankind was the 1918 Spanish flu [1]. At least 40 million people died in this pandemic, and humanity suffered historic damage. Since then, there have been several pandemics caused by the 1957 Asian flu; the 1968 Hong Kong flu; the 2009 swine flu, which were less serious than the Spanish flu. The world has been fighting the novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that is currently causing a historic pandemic since December 2019. SARS-CoV-2 infections are caused by the coronavirus disease 2019 (COVID-19). As of December 16, 2021, the World Health Organization (WHO) reported 271 million cases worldwide including 5.3 million deaths [2]. This pandemic has had a great impact on the lives of people around the world, and the convergence of the pandemic is the most important issue now facing the world.

These pandemic respiratory viruses predominantly circulate within non-human hosts such as mammals and avian species, namely by zoonotic infections. For example, the influenza virus that caused the 1918 Spanish flu was transmitted from an avian [1]. The SARS-CoV-2 appears to be derived from bats and pangolins [3,4]. Thus, in order to prevent the outbreak of a pandemic, it is important to identify the natural reservoir of pathogens that can be transmitted to humans. For the influenza virus, WHO has an excellent global system, namely the Global Influenza Surveillance and Response System (GISRS). GISRS has the position of global leadership for surveillance, preparedness, response, epidemiology and disease against seasonal, pandemic and zoonotic influenza, and issues alerts for novel influenza viruses and other respiratory pathogens [5]. Due to the large number of member countries, global monitoring and quick responses are a possibility. Furthermore, humanity have been developing antiviral drugs and vaccines for use against the influenza virus and have succeeded in obtaining some effective formulations. Therefore, we can say that humanity now have some control over the influenza virus.

According to the data issued by the WHO, 290,000–650,000 deaths by seasonal influenza are estimated. Although vaccination is the cornerstone for controlling seasonal influenza and to achieve the quick containment of an influenza pandemic, the present vaccine strategies are inadequate. Because the efficiency of a vaccine can vary widely, such

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as from 10% to 60% in the US from 2004 to 2018 [6], and in Japan, the vaccine efficiency during the 2018–2019 season was less than 50% [7]. The currently available vaccines are not sufficient to protect the elderly who are at high risk. The reasons behind the low vaccine efficacy are the use of inactivated vaccines (low immunogenicity and insufficient induction of cellular immunity), the variety of our immune responses, the antigenic drift and the antigenic shift. Many of these issues are also related to vaccines against COVID-19. Thus, a new vaccine strategy is needed for the effective immune induction and the response to viral revolutions.

The big differences between COVID-19 and influenza are as follows: the incubation period of SARS-CoV-2 is longer than that of the influenza virus [8]; In SARS-CoV-2 infections, at least one third of the infected subjects are asymptomatic [9]; As a result, asymptomatic or pre-symptomatic subjects largely contribute to the transmission of SARS-CoV-2 [10]. In addition, similar to influenza virus, the response to new variants of SARS-CoV-2 is essential. These features of SARS-CoV-2 infection are the reason why we are still struggling to control SARS-CoV-2 even with the currently available COVID-19 vaccines. The current mainstay COVID-19 vaccines are mRNA-based vaccines that make full use of recent drug delivery system (DDS) technology [11]. However, to solve the sticky points, it is necessary to improve the COVID-19 vaccines based on our understanding of the characteristics of SARS-CoV-2 infection.

Nano carrier based DDS (nano-DDS) is extremely useful as a technology for controlling the dynamics of a wide range of drugs from small molecules to macromolecules. The nano-DDS can also be used to develop new vaccines against future pandemic respiratory viruses. In this review, virologist and nano-DDS researchers discuss drug delivery strategies, especially lipid-based nano-DDS, for use in fighting pandemic respiratory virus infections. The integration of virology and nano-DDS are rational strategies and the design of vaccines based on our understanding of viral characteristics (virus spreading, ecology, infections, host immune response and evolution, etc), leading to the accelerated development of new-types of nano-vaccine against them.

2. Influenza (Fig. 1)

2.1. Virology

The influenza virus belongs to the *Orthomyxovirus* family, possesses segmented negative-sense, single-stranded RNAs (vRNAs) and are classified into four types A to D, based on the antigenicity of the nucleoprotein (NP) [12,13]. Influenza type A viruses (IAVs) are isolated from a wide range of mammalian and avian species whereas influenza type B (IBV) and type D viruses are limited in humans and seals, and in cattle, goats and pigs, respectively [14–16]. The influenza type C virus can only infect humans and usually causes mild respiratory symptoms [16]. IAV contains eight vRNAs that encode for ten major proteins and several minor optional proteins [17]. There are two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and the proton channel matrix protein 2 (M2) is located on the surface of the IAV envelop. The HA can bind to sialic acid residues on the terminal of the glycan chains of glycoproteins and glycolipids (glycoconjugates). According to the structure of sialylated glycoconjugates, cell susceptibility to IAV through the specific binding potential of HA to the sialic acid receptor needs to be determined [18]. The NA poses the sialidase activity at the head of the virus and cleaves off the sialic acid of the glycoconjugates of the cell, thus detaching progeny virus particles from the cell surface at virus budding step [19]. Based on the reactivities against antiserum of hosts infected with IAV or similarity of amino acid sequence, the HA and NA are classified to 18 (H1–18) and 11 (N1−11) subtypes, respectively [13]. Matrix protein 2 (M2) is also a membrane protein on the envelop whereas matrix 1 protein (M1) lines the inner surface of the viral

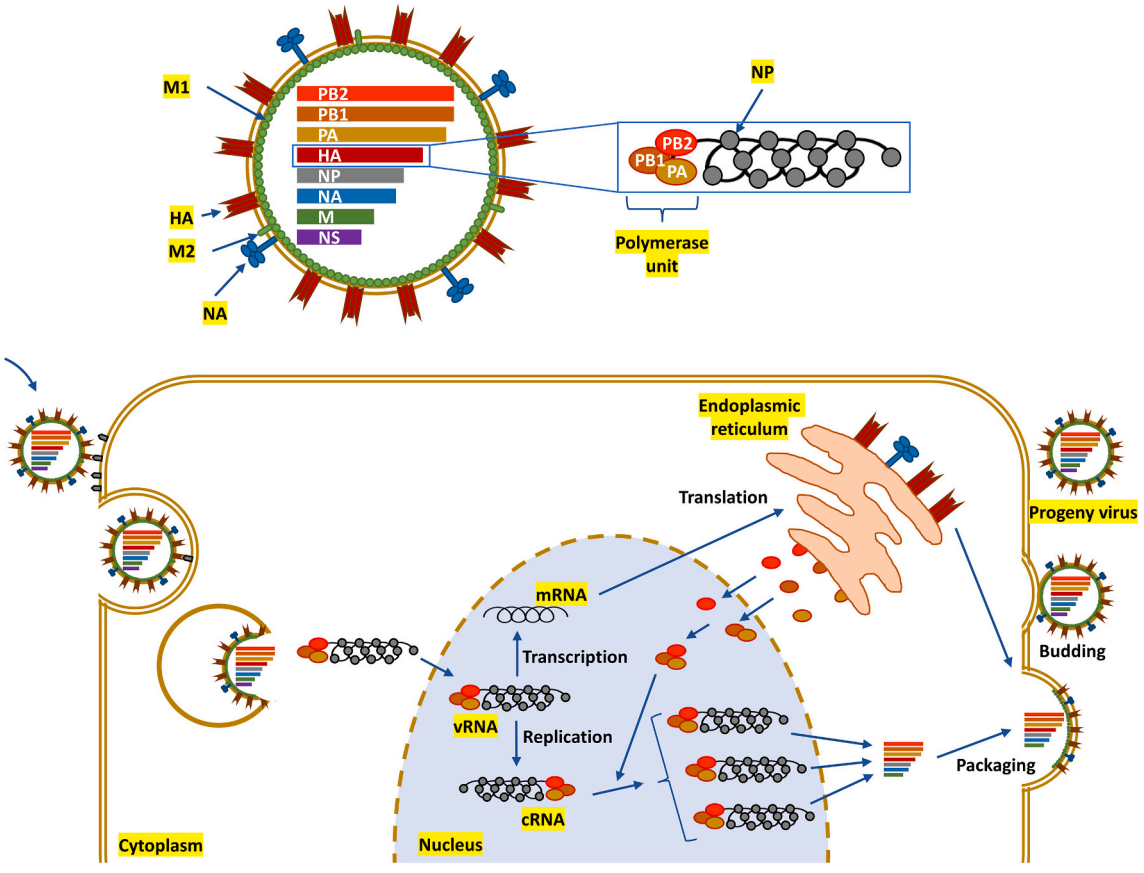

Fig. 1. Structure of virion and viral ribonucleoprotein (vRNP), and lifecycle of Influenza type A virus (IAV).

envelop and encapsulates viral ribonucleoproteins (RNPs) into the envelop [20]. The RNP is composed of eight vRNAs that are covered with the nucleoproteins (NPs) and three subunits of polymerase complex which is a heterotrimer of the polymerase basic protein 2 (PB2), the polymerase basic protein 1 (PB1), and the polymerase advanced protein (PA). The virus virions are endocytosed and release the eight sets of the vRNPs into the cell cytoplasm following the conformation change of the HA under the low pH conditions [21,22]. Each of the polymerase complex subunits, which are synthesized by the initial transcription and translation in the cell cytoplasm, possess the nuclear localization signal which is transferred into the nucleus, where the heterotrimer of the polymerase complex is formulated in the nucleus. The PB2 is then transported into the nucleus whereas the PB1 and PA are likely to be transported with the PB2 independently or form dimers to be transported [23–26]. The polymerase complex mediates the "cap-snatching" mechanism; the PB2 captures the host capped transcripts that are cleaved by a cap-dependent endonuclease in the PA, producing short capped primers which are used for viral mRNA transcription by RNA-dependent RNA polymerase (RdRp) activity in the PB1 [27]. Complementary RNAs (cRNA) are replicated as positive copies of vRNAs and are further used as templates for vRNA synthesis. Messenger RNAs are transcribed from original and synthesized vRNAs in the nucleus. The viral eight segments genome encodes for the nonstructural protein 1 which is a multifunctional small protein that functions to suppress host immunity and for regulation of the polymerase activities [28,29], and nonstructural protein 2 which are located on the inner surface of the virion associated with M1 to export vRNPs to the nucleus [30].

There are three transmembrane proteins on the surface of viral envelop, hemagglutinin (HA), neuraminidase (NA) and matric protein 2 (M2). IAV contains eight segmented negative sense, single strand viral RNA (vRNA). Matrix protein 1 (M1) lines inner surface of the envelop and encapsulates the eight sets of the RNPs comprised of the vRNA, the polymerase basic protein 2 (PB2), the polymerase basic protein 1 (PB1) and the polymerase advanced protein (PA) and the nucleoprotein (NP). The HA attaches the sialylated glycoconjugates on the surface of a targeted cell, and then are endocytosed and release the vRNPs into the cell cytoplasm. The vRNPs are further transferred into the nucleus through nuclear localization signals to mediate viral genome replication and transcription. Each of the PB2, PB1, and PA transcribed and translated in the cytoplasm, are transferred into the nucleus to formulate the heterotrimer of the polymerase complex. Complementary RNAs (cRNAs) are replicated as positive copy of vRNAs and are further used as template for vRNA synthesis. Messenger RNA (mRNA) are transcribed from the vRNAs in the nucleus and translated to each protein in endoplasmic reticulum. Progeny viruses are detached from the cell surface by cleaving off the sialic acid of the cell by the NA.

2.2. Ecology of IAV

The maintenance of H1–16 of IAV in wild aquatic birds, especially ducks clearly demonstrate that wild aquatic birds are a natural reservoir of IAV [31,32], and IAV usually replicates in the lower digestive tract including the colon in these species without any clear disease symptoms being generated [33]. Apathogenic IAVs are occasionally transmitted to terrestrial birds such as geese, quails and turkeys, and acquire more virulence, thus allowing them to replicate in the upper respiratory tracts with apparent but low pathogenicity of respiratory disorders during the recurrent circulation among their population. High pathogenicity avian influenza viruses emerge in the chicken following the continuous circulation of low pathogenicity avian influenza viruses (LPAIV) among the chicken population and can cause severe pathogenicity in host birds due to the insertion of more than two pairs of basic amino acids at the cleavage site of the HA [34]. LPAIV infects host cells by cleaving the HA with a trypsin-like enzyme, however, the HA of HPAIV can cleave ubiquitous proteases which are systemically expressed in the host, such as furin [35], thereby, HPAIV causes a systemic infection in the body of the host especially in endothelial cells, which results in a high mortality [36]. The HA subtype of HPAIV is limited in H5 and H7, which is supported by AIV surveillance reports. In commercialized farming, IAV infections in pigs cause serious economic damage sometimes involving co-infections with bacteria. The pig is one of the critical hosts of IAV infections crossing into new host species, especially humans due to the expression of both of the sialylated glycoconjugates on the surface of respiratory mucosal cells; α2–6-linked sialic acid and α2–3-linked sialic acid, that are likely to interact with human-adapted and avian-adapted IAVs, respectively [18,37]. Co-infection of two different type of IAVs in pig respiratory cells potentially cause reassortment which is the partial exchange of viral genes, posing a great concern for generating antigenically shifted IAVs [38].

2.3. Pandemic influenza

An influenza pandemic involves the circulation of antigenically different IAV in an immunologically naïve population, most of which is produced by genetic reassortment rather than antigenic drift due to amino acid point mutations in the IAV [39]. Historically, the first pandemic influenza occurred in 1918 due to the circulation of the Spanish flu virus subtype H1N1, which is estimated to have killed 50–100 million people worldwide. In 1957, the second influenza pandemic, caused by infection by the Asian flu virus subtype H2N2 killed 1.1 million people worldwide in the next 3 years [40]. The Hong Kong flu, the third influenza pandemic, was caused by the circulation of the H3N2 subtype IAV, which was identified as the outcome of a reassortment between the H2N2 Asian flu virus and the H3 IAV derived from avian species [41]. The latest pandemic influenza, reported in 2019, is attributed to the circulation of A(H1N1)pdm2009 derived from pigs and killed approximately 18 thousand people during the pandemic phase [42]. All of the four pandemic influenza viruses were continuously infecting humans as the subsequent seasonal influenza competed with the previous seasonal influenza viruses, although two (Spanish flu and Asian flu viruses) are now rarely circulating in the world. The World Health Organization estimated approximately 1 billion seasonal influenza patients including 3 to 5 million severe cases, and 290,000 to 650,000 deaths annually [38].

In addition to the loss due to seasonal influenza, humans had been exposed to several zoonotic IAV risks since 1997. Transition of husbandry style and marketing practice augmented the risks of pathogen circulation among them, especially the H5N1 and H7N9 avian influenza viruses (AIVs), well as poultry production. As of 15 April 2021, a total of 862 cases of H5N1 AIV virus infections in humans with 455 deaths were reported since 2003, most of which occurred in limited countries, indicating that the risk of H5N1 AIV infections in humans still remains high but appears to occur within very limited countries and situations [43]. H7N9 AIV infections were also limited in several countries mainly China. Sporadic human infections with H7N9 AIV have been reported since March 2013, and accounts for 1568 cases with 616 deaths as of 1 December 2021 [44]. A number of AIV infections in humans, either HPAIV or LPAIV, but limited to certain areas could cause developing zoonotic transmission cycles in the geographically or anthropologically specific conditions.

2.4. Vaccines

Similar to other respiratory diseases, vaccinations are still the primary and effective control measures against IAV infections, both in humans and animals, and serve to weaken virulence and reduce the transmission of a virus in the general population. However, each of the three types of authorized vaccines in many countries cannot address the disadvantages [45]. Inactivated vaccines are widely applied for controlling IAV infections in humans in many countries due to low costs and high safety, but accidental antigenicity drifts in the glycoproteins of IAV through egg adaptation process cannot be avoided [46,47]. The whole

virion vaccine prepared by inactivation of the virus virion with a chemical reagent induces a higher immunogenicity but is less frequently applied compared to split virion vaccines prepared by the purification of distributed virion particles [17]. Live attenuated vaccines can activate the immune system in a host similar to a natural infection but cannot regulate the excessive level of immunogenicity following efficient viral replication in high risk subjects including infants, the elderly and immunocompromised subjects [17]. Though an HA recombinant vaccine does not require live viruses and can confer protective immunity to hosts without the change of antigenicity which may frequently be caused through the egg adaptation process, the introduced immunogenicity is generally lower compared with that for an inactivated vaccine. Another issue in vaccine development is that the adjuvant which is a chemical or natural substance supplemented to enhance immunogenicity by activating mainly the local immune system, can cause a variety of side effects.

2.5. Antiviral drugs

Differing from inducing appropriate immunity in antigen-specificity and immunogenicity by a vaccine, antiviral drugs can protect hosts from developing IAV infections without limiting the antigenicity. This is especially true for the HA subtype of prevalent IAVs and activating host immune system excessively. Furthermore, the antiviral effect of an antiviral drug in a shorter period (generally several days) compared to a vaccine (more than a week) represents a great advantage especially in emergency situations such as zoonotic infections in humans with unfamiliar subtypes. To date, substantial efforts have been expended in the development of antiviral drug against IAV, and four classes of antiviral drugs were or are likely to be released for therapeutic and prophylactic purposes [48,49]. Amantadine and rimantadine were first developed as anti-IAV drugs and were used for a long time to reduce pathogenicity due to IAV infections by blocking the activity of the M2 proton channel which contributes to uncoating the virion with the subsequent release of vRNAs into the host cell [50]. The antiviral efficacy of these drugs for treatment and prevention against seasonal IAV were originally very high; up to 90%. However, the ratio of the resistance of seasonal IAV against adamantanes was drastically elevated after 2000 and, in 2013, reached a global level of approximately 45% [51]. The admission of adamantanes to IAVs both for treatment and prophylaxis purposes is currently restricted due to the high resistant ratio in seasonal IAVs [52]. A variety of neuraminidase inhibitors were currently used as first-line therapies and include oseltamivir via oral administration, zanamivir via inhaled administration, and peramivir via intravenous administration. Interactions of neuraminidase inhibitors and the NA active site competitively suppress the neuraminidase activities of IAV, thereby, preventing the progeny viruses from budding from the infected cells. The administration of oseltamivir at 24 and 36 h after disease onset shortened the duration of the disease by 56% and 70%, respectively [53,54]. Even for prophylaxis purposes, the neuraminidase inhibitors can reduce the likelihood of being infected with AIV to approximately 10–30% [55]. In 2007–2008, resistance IAV against oseltamivir was suddenly confirmed in patients, many of whom had not received it, and were horizontally transmitted in the population [56]. Furthermore, in 2008–2009, in limited areas, more than 90% of the oseltamivir-resistant H1N1 seasonal influenza was reported [57].

An amino acid substitution at the 275 position of the N1 NA from a histidine to a tyrosine (H275Y) was revealed as a major mutation that was critical for antiviral activity was confirmed in contemporary seasonal H1N1 IAVs [58,59]. This mutation causes a conformational change in the active neuraminidase site and suppresses the antiviral activity of oseltamivir to approximately 1/400 by inhibiting its binding. On the other hand, the antiviral activity of zanamivir is not influenced by the conformational change due to H275Y [55], but is influenced by an amino acid substitution at the position of 292 of the N2 NA from an arginine to a lysine unit, which also reduces the antiviral effect of oseltamivir. Baloxavir marboxil, referred to as baloxavir, specifically suppress viral polymerase function by inhibiting cap-dependent endonuclease activity in the PA and therefore exhibits antiviral effects in a wide range of seasonal and zoonotic IAV and IBV including resistance to neuraminidase inhibitors [60–62]. Baloxabir is permitted for therapeutic purposes but only in targeted people in limited countries. Favipiravir is another antiviral drug that functions by inhibiting polymerase activity especially inhibiting RdRp activity in the PB1 of IAV and other RNA viruses [63,64]. The use of favipiravir against IAV and IBV infection was approved in Japan with strict conditions due to the potential risk of teratogenicity and embryotoxicity [65]. Pimodivir is also a newly generated antiviral drug that functions by inhibiting cap-binding potential in the PB2, which selectively interacts with IAV [66]. Clinical trials in a double-bind phase 2b study with acute uncomplicated IAV patients demonstrated that pimodivir has a positive effect in virological improvement with or without oseltamivir [67].

3. Corona-virus (Fig. 2)

3.1. Virology

Coronaviruses are a class of important viruses that cause respiratory discharges in several animals including humans, and belong to the *Coronaviridae* family in *Nidovirales*. *Orthocoronavirinae*, which is a *Coronaviridae* subfamily, was further classified into four genera; alphacoronavirus, betacoronavirus, gammacoronavirus, and deltacoronavirus. Several betacoronavirus viruses including severe acute respiratory syndrome coronaviruses (SARS-CoV), Middle East respiratory syndrome coronaviruses (MERS-CoV), and SARS-CoV-2, caused enzootic respiratory pandemics with substantial mortality [68–70]. Coronaviruses contain positive-sense single-strand genomic RNA with a phosphorylated nucleocapsid (N) protein. The first two-thirds at the 5 terminal end of viral genome corresponds to two large open reading frames 1a (ORF1a) and ORF1b translating two overlapping polyproteins; pp1a and pp1ab. These replicase polyproteins were cleaved with the assistance of the 3C-like main protease (3CLpro) or papain-like protease (PLpro) to functionize 16 non-structural proteins (nsps) [71]. All of the nsps, except for nsp1, make up the multi-protein replicase-transcriptase complex (RTC) that contributes to regulating intracellular membrane modulation, host immunity evasion, and replication-transcription function in RNA synthesis, proofreading, and modification [72–74]. All of the structural and accessory proteins occupy one-third of the 3 terminal region of the viral genome and are encoded by the subgenomic (sg) RNA of Coronaviruses [71,75]. Four main structural proteins including N, spike (S), membrane (M), and envelop proteins (E) are common in Coronaviruses whereas accessory proteins are scattered among the structural genes, and the numbers and their functions are restricted by each Coronavirus [71,75,76]. The S, E, and M proteins are embedded into the membrane bilayer of the viral envelop. The SARS-CoV-2 S protein is a homotrimeric fusion glycoprotein composed of subunits S1 and S2, which is pivotal for virus entry by binding to the angiotensin-converting enzyme 2 (ACE2) on the host cell surface [77,78]. The surface S1 and transmembrane S2 subunits contain the receptor-binding domain and the heptad repeat region and fusion region, respectively [79,80]. The receptor-bound SARS-CoV-2 particle invades into the cell following the proteolytic cleavage of the S proteins by cell-derived proteases including transmembrane serine protease 2 (TMPRSS2), which is widely expressed on the surface of human lung cells and is thought to play a critical role for virus entry [77,81,82]. Besides other coronaviruses, SARS-CoV and SARS-CoV-2 possess a poly basic amino acid insertion at the junction of the S1-S2 subunits (RRAR), which enables the cleavage of the S protein by the host furin like ubiquitous protease [83]. Viral and host lysosomal membrane fusion is mediated by the S2 protein through endocytosis of the host cell, and facilitates the release of viral genome into the cytoplasm and subsequent structural change [71,84]. Genomic RNA is translated in the ribosomes

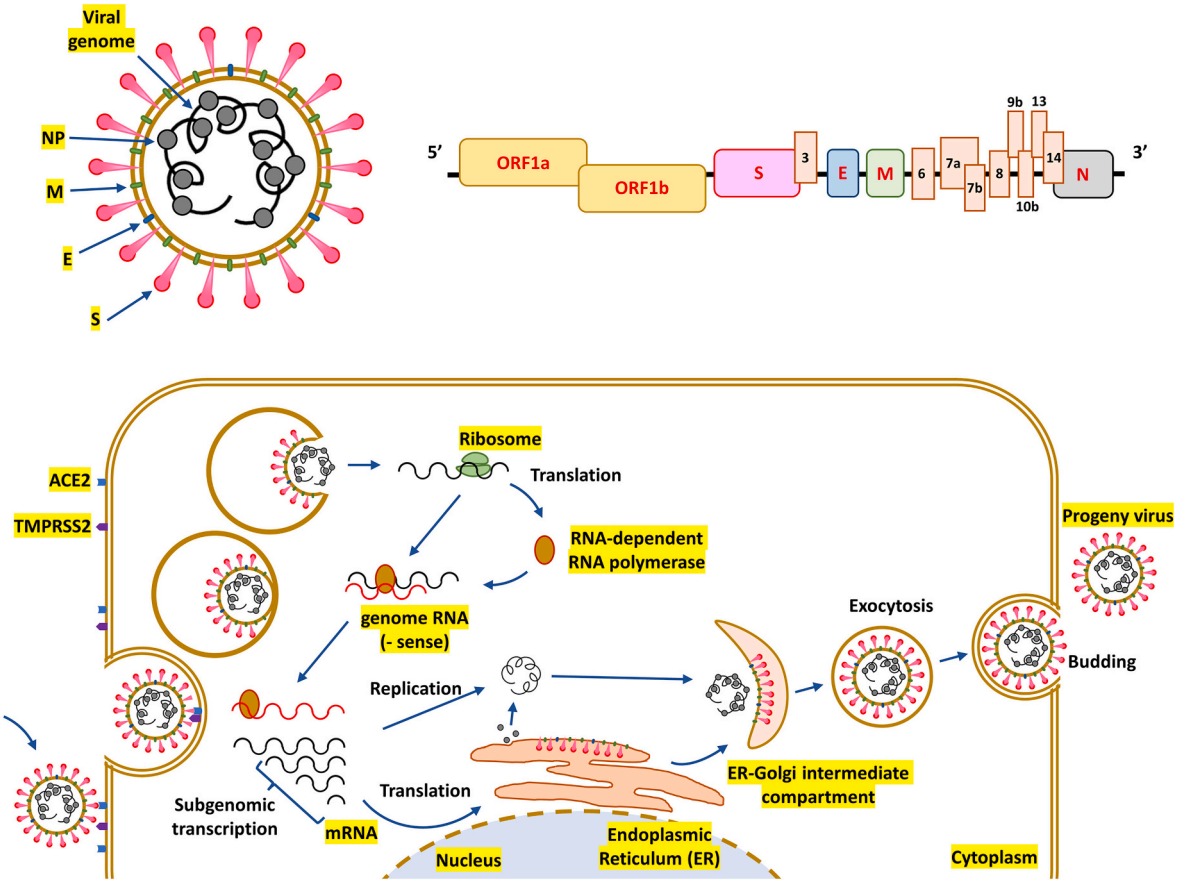

Fig. 2. Structure of virion and viral genome, and lifecycle of severe acute respiratory syndrome coronaviruses 2 (SARS-CoV-2).

of cells and produces structural, non-structural, and accessory proteins. The RdRp which is contained in the RTC, replicates the negative-sense genomic RNA from the viral genome and transcribes it into the positive-sense of subgenomic mRNA fragments [71]. The full-length positive-sense genomic RNA, which is polyadenylated at the 3 terminal and capped at the 5 terminal is combined with N proteins to produce a ribonucleoprotein (RNP) complex, and all of the S, E, and M proteins as well as the RNP are transferred to the endoplasmic reticulum-Golgi intermediate compartment to be released from the host cell by exocytosis [85–88].

Spike (S), membrane (M), and envelop (E) proteins are embedded into the viral envelop. Coronavirus contain positive-sense single strand genomic RNA with phosphorylated nucleocapsid (N) protein. The majority at the 5 terminal end of genome corresponds to open leading frame 1a and 1b (ORF1a and ORF1b, respectively), which is cleaved to 16 non-structural proteins including RNA-dependent RNA polymerase (RdRp). All the structural (in red character) and accessory proteins (in black character) are encoded at the remaining region of the 3 terminal end. The S protein of the SARS-CoV-2 binds to the angiotensin-converting enzyme 2 (ACE2) on the cell surface and are cleaved with cell-derived protease including transmembrane serine protease 2 (TMPRSS2). The virus particle invade into the cell. Genomic RNA is translated in the ribosome to produce viral proteins. The RdRp replicates the negative-sense genomic RNA and transcribe it into positive-sense of subgenomic mRNAs. The full-length positive-sense genomic RNA and N proteins compose ribonucleoprotein complex (vRNP). All the S, E, M and vRNP are transferred endoplasmic reticulum-Golgi intermediate compartment to be released from the host cell by exocytosis.

3.2. *Ecology of coronavirus*

The origin of SARS-CoV had been intensively investigated in efforts to identify the natural host and the transmission dynamics of the virus, and was speculated to originate from horseshoe bats because of the isolation of discovery of SARS-like coronaviruses from these hosts [89,90]. Genetic analysis revealed that MERS-CoV is also originated from bat coronaviruses [91]. These two viruses are derived from bats and transmitted to humans via intermediate host animals such as palm civets and dromedary camels [92–95]. To date, RaTG13 detected from *Rhinolophus affinis* bat in Yunnan, China is regarded as the most relative virus to SARS-CoV-2 with a 96% genome sequence homology throughout the genome and more than a 90% homology in all ORFs [3]. Thereafter, another coronavirus that was related to SARS-CoV-2 was detected from *Rhinolophus malayanus* bat and designated as RmYN02, which has a 93% genetical homology throughout the genome to SARS-CoV-2 [96]. One of the genomic features of RmYN02 is the insertion of three amino acid residues (PAA) at the cleavage site of the S1 and S2 subunits, an insertion that has not been reported in coronaviruses except for SARS-CoV-2, which contains RRAR [96,97]. While the insertion of three amino acid resides at the cleavage site of the S1-S2 does not confer a functional advantage in RmYN02, the insertion of polybasic amino acids in the conjunction of S1-S2 allow effective cleavage by furin and others [97]. Phylogenetic analyses demonstrated that bat RaTG13 and RmYN02 as well and ZC45 and ZXC21, both of which had been detected in *Rhinolophus pusillus* bats in eastern China are categorized into the same lineage as SARS-CoV-2. The detection of bat coronaviruses with genetically close or similar characteristics to SARS-CoV-2 implies that the natural reservoir of SARS-CoV-2 is likely the bat [98]. The pangolin was also considered to be a potential reservoir of SARS-CoV-2 due to repeated identification of its related virus in the pangolin [99].

However, symptomatic infections of SARS-CoV-2 related viruses with histopathological abnormalities in the pangolin could allow us to conclude that the pangolin should not be a natural host but a definitive host of SARS-CoV-2 after being spilled over from other, natural hosts. Determining the host of the CoV-2 virus, however, will take time, because some information is not available because it is considered to be confidential by many government agencies.

3.3. Pandemic coronavirues

3.3.1. SARS and SARS-CoV

The report of an initial patient with SARS in November 2002, in China, triggered the global pandemic, with 8096 cases with 774 fatalities in 28 countries [100]. This was considered to be a zoonotic infection because SARS-CoV or a genetically SARS-CoV related virus was detected in several animals including masked palm civets (*Paguma larvata*), the racoon dog (*Nyctereutes procyonoides*) and rhinolophid bats (Rphinolophus spp.) [89,90,95,101].

The S protein of SARS-CoV mainly binds to ACE2 but alternatives include CD 209 L, which is a C-type lectin [102]. The ACE2 receptor is commonly distributed on the surface of epithelial cells in the respiratory tract (trachea, bronchi, bronchial serous glands, and alveoli), alveolar monocytes and macrophages, and is also found on endothelial cells, cerebral neurons, tubular epithelial cells of the kidneys, mucosal cells of intestines [103,104]. Due to the wide dissemination of ACE2 receptors in the body, SARS-CoV infections in humans can cause a variety of symptoms.

3.3.2. MERS and MERS-CoV

An unrecognized coronavirus (MERS-CoV) infection was initially reported for a dead patient due to severe respiratory illness in Jeddah, Saudi Arabia, in June 2012. MERS-CoV is genetically close to bat coronaviruses including the Tylonycteris bat coronavirus HKU4 and the Pipistrellus bat coronavirus HKU5 [92,105,106], although the MERS-CoV had not isolated from bats [107]. In contrast to SARS-CoV, MERS-CoV binds to the Dipeptidyl peptidase 4 (DDP4) and invades the host cells [108]. DDP4 is also widely expressed in many tissues including kidneys, intestine, and liver as well as the respiratory tract [108,109]. In the respiratory tract, DDP4 is frequently confirmed in alveolar regions but rarely in nasal cavities, implying that MERS causes the lower respiratory tract discharge, one of the main primary symptoms and rarely causes human-to-human transmission [110].

Compared to ACE2, DDP4 is expressed only in limited animals including nonhuman primates, bats and camels [109]. The identification of an anti-MERS-CoV antibody in the dromedary camel would confirm that it is a potential host of MERS-CoV, but it has not been isolated from dromedary camels yet [111,112]. These results suggest that the dromedary camel is also a definitive host of MERS-CoV, the same as humans, and that MERS-CoV should have already invaded into the dromedary camel population before the announcements of the MERS outbreaks in humans. Human MERS cases were considered to due to the direct or indirect exposure to dromedary camels, especially via ingesting their milk or by direct and close exposure to diseased patients as secondary infections, although human-to-human infections of MERS-CoV occurred in limited settings including within-household transmission and transmission in health care settings in hospitals [107,113–115].

3.4. Dysregulation of host immunity

Innate immunity is initial host defense system for removing pathogens in hosts through the induction of cytokines and chemokines especially, type I and III interferons (IFNs), which specifically have an antiviral role by facilitating the primary adaptive immune response against viruses in the cell [116,117]. However, in ideal conditions, the efficient replication of SARS-CoV-2 in a host involves evading the host defense against virus infections. Innate and adaptive immunity of the host is modulated by viral proteins and genome materials. Structural proteins including S and M proteins, nsps (nsp1, nsp3) and ORF6 interfere with the expression of retinoic acid-inducible gene-I (RIG-1) or melanoma differentiation-associated gene 5 (MDA-5), thereby leading to a decreased type I IFN induction [118–121]. ORF6 also prevents IFN induction by interfering with the nuclear translocation of IFN regulatory factors 3 (IRF3) and STAT-1 [118]. Impairment of STAT-1 and STAT-2 phosphorylation are mediated by several nsps and structural proteins [122].

It should also be noted that severe COVID-19 cases often show a dysregulated immune response resulting in the development of hyperinflammation [123]. Excessive secretion of cytokines through the activation of toll-like receptors (TLRs) triggered by virus invasion plays a pivotal in the severe pathogenesis of SARS-CoV-2, especially at the early phase of its infection [124]. The induction of interleukin 1 (IL-1), IL-6, IL-12, IFN-gamma, and tumor necrosis factor α (TNF-α) is increased in patients who are severely infected with SARS-CoV-2, and the level of induction of IL-2 and IFN-gamma is associated with the severity of lung damage in such patients [125].

3.5. Vaccines

Many of efforts have been made to combat the contagious pathogens of global pandemics including SARS and COVID-19. These efforts include alleviating disease symptoms after infection or preventing infection by decreasing the proportion of antibody population in an entire population (herd immunity). According to the potential for inducing effective immunity and of decreasing adverse events following vaccination, a variety of vaccine are currently under development.

3.5.1. Live attenuated vaccines

Live attenuated vaccines are one of the traditional and most effective vaccines for inducing sufficient immunity for a life against a targeted pathogen [126,127]. A vaccine strain is needed to reduce its pathogenicity but intentional infections with a small dose of the vaccine strain can easily induce sufficient humoral and cellular immunity to protect against infections [128]. Intranasal administration of live attenuated vaccines can also introduce local immunity in mucosal tissues of the upper respiratory tract, which can contribute to the prevention or suppression of the initial virus replication in a coronavirus infection. The current COVID-19 live vaccine was attenuated via the codon pair deoptimization method to synthesize a recoded viral genome by revising the positions of the synonymous codons, leading to the replication of a suboptimal viral genome [129,130]. Intentional infection caused by the administration of a live vaccine may cause the spill over of the infectious viruses in the environment, especially to an unvaccinated community; this is a potential disadvantage.

3.5.2. Protein-based vaccines

Protein subunit vaccines are composed of a fragment of harmless but immunogenic proteins and are to be considered safe vaccines due to the fact that they do not cause and infection in the host [131]. However, the development and application of a protein subunit vaccine is generally costly in terms of developing an adequate targeted protein and its purification, and specific adjuvants are needed to stimulate the immune system more efficiently [132]. Because the S protein plays a vital role in virus infections in the host cell and is definitely a target of a neutralizing antibody induced by host immunity. Many of the recombinant protein subunit vaccines were developed by expressing the antigen in cell-line base [133].

3.5.3. Viral like particles (VLPs)

The virus-like particles (VLPs) vaccine is a different type of protein-based vaccine, is composed of viral capsid proteins but not the entire viral genome, and can induce the same immune response as the original virus. A deficiency in the infectivity in a VLPs vaccine does not cause

serious pathogenicity and even induces sufficient immunity. VLP vaccines had already been developed to use in the treatment of many virus infectious diseases like as SARS and MERS as well as influenza, Hepatitis B and Human papillomavirus infection [134–138]. Utilizing the procedure used for preparing past VLP vaccines, several VLP vaccines against SARS-CoV-2 are currently under development.

3.5.4. Viral-vector vaccines

Viral vector vaccines are originated from modified viruses including adenovirus or poxviruses, which loads a genetic fragment corresponding to a targeted viral antigen. The advantage of a viral vector vaccine is its efficient major histocompatibility complex (MHC) class I and II antigen presentation within the host cell [139]. Non-replicating viral-vector vaccines cannot produce new infectious viruses in the cell but produce target proteins as antigens. Alternatively, in self-replicating viral-vector based vaccines, new viral particles are produced in the original cell and are then transmitted to other cells, leading to an enhanced antigen production in the host [140]. Neither of these vaccines can produce the original vector virus or loaded viruses [141]. Two viral vector vaccines including an adenovirus serotype 26 vector vaccine and a chimpanzee adenovirus vector have provided prominent results for practical application for reducing the risk associated with SARS-CoV-2 infection [142].

3.5.5. Nucleic-acid vaccines

In nucleic acid vaccines, only a fragment of a nucleic acid (DNA and RNA) is inserted into human cells and the resulting translated proteins induce an immune response [140]. Although safe but prolonged immunity is conferred to the host with a lower cost in a short duration, the efficacy of immunogenicity of this vaccine in humans is insufficient [143]. In addition, an mRNA vaccine has the ability to elicit high immune responses, since it is rapidly, safely and efficiently delivered to the host cell. A major concern of the mRNA vaccine against SARS-CoV-2 lies in uncertainties associated with potential adverse effects due to eliciting excessive mucosal immunity following intramuscular administration, and of the capability to viruses to produce mutated forms of the S protein [144,145]. Delivery of mRNA contained within lipid nanoparticles (LNPs) protects the target nucleic fragment from degradation by host enzymes and promotes endocytosis, thus resulting in a sufficient induction of host immunity [146].

3.5.6. Challenges in vaccines and commercialization against COVID-19

The emergence of a pandemic results in a physically and mentally scared public. The prompt development and application of effective vaccines is typically expected by the public. In the case of SARS-CoV-2 vaccines, clinical trials were performed, and the results were evaluated with a short duration. The public might doubt the safety and effectiveness of newly developed SARS-CoV-2 vaccines. Establishing and manufacturing of vaccines against emerging pathogens represents a challenge in terms of satisfying public demand and suppling a safe and effective vaccine as quickly as possible.

3.6. Antiviral drugs

The antiviral therapies against SARS-CoV-2 involved developing specific agents to inhibit or suppress its replication in humans. Blocking the entry of the virus to host cells, inhibiting viral genome replication, or modulating the host immunity are typical targets of anti-viral agents. Many of the drugs that were originally developed or investigated for use against other virus infections with similar properties were further utilized for SARS-CoV-2.

3.6.1. Remdesivir

Remdesivir is a monophosphate nucleoside analog prodrug that is metabolized to an active triphosphate nucleoside from by the action of an intracellular kinase and shows antiviral effects against a broad range of RNA viruses by inhibiting RdRp [147]. The origin of Remdesivir is from earlier research on hepatitis B, for the treatment of AIDS and for the prevention of HIV [148]. The active form of triphosphate nucleoside competes with natural adenosine triphosphate by mimicking an ATP analog, resulting in the specific interference of viral RNA extension by RdRp the infected cells [149]. Clinical trials of remdesivir on adult and child SARS-CoV-2 patients demonstrated that an intensive treatment with remdesivir for 5 days compared to no specific treatment and a normal treatment in 10 days significantly alleviated the clinical conditions of the patients [150,151]. Since the therapeutic efficacy of remdesivir was not ensured, application of remdesivir was only conditionally recommended by the WHO [152].

3.6.2. Favipiravir

Favipiravir is a pyrazine analog that displays effective antiviral activity against RNA viruses, including influenza viruses, bunyaviruses, filoviruses and arenaviruses by being metabolized by cellular enzymes into ribofuranosyltriposphate, the active therapeutic form [64,65]. Favipiravir also suppress viral RdRp activity through preventing viral RNA elongation in the cell. However, although some countries had already approved it for the treatment of SARS-CoV-2, positive results of Favipiravir against SARS-CoV-2 were not confirmed in virus shedding, oxygen requirement support, and side effect profiles [153]. The repeated oral administrations of favipiravir showed apparent toxicity in an animal model including a reduction in red blood cell production and an increase in indicators of liver function. These results suggest that Favipiravir should not be administrated to pregnant women [154].

3.6.3. Ivermectin

Ivermectin is an anti-parasitic agent that is used to treat river blindness, lymphatic filariasis, scabies, and lice but has also been used as a therapeutic agent against flaviviruses infection in humans [155–157]. Ivermectin shows anti-helminthic activity through blocking several vital ion channels in parasites. The importin-α/β1 heterodimer (Imp-α/β1) is a main target of Ivermectin against virus infections in the cell to suppress the transport of viral proteins to the nucleus [158]. In the case of SARS-CoV-2 infection, Ivermectin is thought to interact with RdRp to inhibit its activity [159]. In addition, Ivermectin enhances the immune responses in the host by enhancing the induction of IL-6 and the translation of the C-reactive protein and by the activation of neutrophils [160].

3.6.4. Lopinavir and ritonavir

A mixture of lopinavir, interferon, ritonavir, and ribavirin showed positive effects against early stages of SARS-CoV-2 infections [161]. Lopinavir is viral protease inhibitor that was originally developed for the treatment of HIV infections by mimicking the normal peptide linkage which is a target of the HIV protease [162]. Ritonavir was developed as a second protease inhibitor by preventing the action of cytochrome P450 3A4 (CYP3A) and is used with lopinavir for the treatment of HIV, where it functions to prevent lopinavir from being metabolized by CYP3A and was used together with lopinavir [163].

3.6.5. Hydroxychloroquine and chloroquine

Hydroxychloroquine and chloroquine are very familiar as specific agents for the treatment of malaria and chronic inflammation [152]. The main mechanism of the anti-helminthic activity of these drugs involves raising the intracellular pH by a protonation agent, which may also affect endosomal pH, thus inhibiting the initiation of the viral fusion steps [164]. Moreover, these agents are known to prevent the glycosylation of the ACE2 receptor in SARS-CoV infections [165], which may also contribute to reducing the receptor-binding affinity between host cells and SARS-CoV-2, resulting in the inhibition of progeny virus production in the infected cells. In addition, these agents prevent antigen processing, T-cell activation and toll-like receptor activation, leading to the down-regulation of the expression of pro-inflammatory genes [166–168].

4. Current challenges for controlling the pandemic respiratory viruses

4.1. Current challenges in influenza virus infection

In seasonal influenza, the severity of the disease such as pneumonia largely depends on the potential for the virus to replicate and the location of growth (lower respiratory tract) [169]. Children (<2 years old) and the elderly (>65 years old) are at particularly high risk [8]. The facts clearly show the importance of vaccination for suppressing virus replication. However, the vaccine efficacy is not high enough to suppress the infection in all cases [6,170]. The efficacy of vaccines was greatly biased in certain years [170] and there are several reasons for this. First, inactivated vaccines are broadly used for seasonal influenza. The inactivated vaccines mainly induce humoral immunity, but rarely cellular immunity. In particular, in children and the elderly, the efficacy of such preparations is extremely limited. In contrast, live-attenuated vaccines are intranasally administered in some countries. Although live-attenuated vaccines can induce strong mucosal immune responses and cellular immunity, their use is limited to subjects between 2 and 49 years of age and has a risk of genetic reversion into a high pathogenic strain [171]. The second is the antigenic change of the influenza virus [172]. Since the influenza virus is an RNA virus and has eight segmented RNA genomes, amino acid substitution following nucleotide mutation (antigenic drift) and genetic reassortment (antigenic shift) can occur during an infection. As a result, the selection of effective vaccine strains is, in practice, difficult, and the emergence of new subtypes of influenza viruses due to genetic reassortment can cause pandemics. Third, the production of current seasonal vaccines is a lengthy process. An egg-based production method is typically employed and represents the highest share in the global market, because of their high production capacity and low cost [173]. However, the entire process from strain selection by WHO to vaccine availability takes around 8 months. Thus, it is necessary to select the strains that will be prevalent in the next season at an early stage, and the prediction may be in error. In contrast, we are in the process of selecting vaccine strains to accommodate any subtype of influenza virus by establishing an influenza virus library that contains 4580 strains [174]. This strategy, though not yet global, can make a significant contribution to vaccine preparation and the diagnosis of future pandemic influenza viruses.

As mentioned above, various antiviral drugs, such as amantadine, oseltamivir, zanamivir, peramivir, baloxavir and favipiravir have been licensed for use. These drugs can strongly suppress virus replication and exert non-specific antiviral effects within several days. Unfortunately, resistant virus strains have emerged for these drugs, and the development of new antiviral drugs is clearly needed. In addition, it will be necessary to develop guidelines for the proper use these drugs to prevent the emergence of resistant virus strains.

4.2. Current challenges in SARS-CoV-2 infection

The success of developing effective COVID-19 vaccines in about a year after the emergence of SARS-CoV-2 is attributed to great efforts of human research and development, and we should all be very pleased with the occurrence. Although these vaccines have been shown to be effective on COVID-19, this may not dramatically change the infections or deaths reported by WHO (December 2, 2021). This is due to properties that are peculiar to COVID-19, current vaccine challenges and the lack of effective antiviral drugs. The most important challenge appears to be virus transmission even by presymptomatic and asymptomatic infection [175,176]. Unless a PCR test is performed, it can be inferred that pre-symptomatic or asymptomatic subjects will have no behavioral restrictions and are likely to contribute to virus spread as well. Second, potency in preventing infections by the current vaccines is unclear. The BNT162b2 COVID-19 mRNA vaccine showed a 92% protective effect against SARS-CoV-2 infections and subjects who were vaccinated with the mRNA-1273 COVID-19 mRNA vaccine had SARS-CoV-2 specific IgA in their saliva [177,178]. Although the possibility has been suggested, no conclusion has been reached. Third, due to rapid appearance of this emergency, the effective duration of the vaccine was not fully investigated in clinical trials. Studies using predictive models suggests that the effects of infection prevention and onset prevention are significantly reduced at 250 days after the vaccination, but the effects of prevention from severe disease may last for more than 2 years [179]. Fourth, possible adverse events following vaccination by new modality vaccines such as mRNA vaccines have also not been fully investigated. Compared with conventional vaccines against other infectious diseases, the incidence of side effects in the mRNA vaccinations appears to be high. Fifth, similar to influenza vaccine, the potency of vaccines against SARS-CoV-2 variants, due to antigenic drift is uncertain [180]. In addition, there are no effective antiviral drugs. Currently, various candidate drugs are in clinical trials, and we look forward to the emergence of effective antiviral drugs.

Taken together, vaccine and effective antiviral drugs are important for controlling this pandemic. The ideal vaccine would be a universal vaccine that can prevent infection (induction of mucosal immunity), has high antibody induction, long-term persistence, and a high level of safety.

5. Role of nano-DDS in the pandemic respiratory virus infections

As mentioned earlier, the successes of mRNA vaccines and adenoviral vector vaccines have given a significant advantage to the battle between humans and SARS-CoV-2. Both of these vaccines use DDS technology. In particular, the latest LNP technology is now being used for mRNA vaccines. Ionizable lipids are essential LNP components for the efficient delivery of mRNA to the cytoplasm. ALC-0315 is used for BNT162b and SM-102 is used for mRNA-1273 [11]. The principle of these ionizable lipids is based on the use of an LNP for siRNA delivery, and an LNP containing DLin-MC3-DMA has been put into practical use as the world's first siRNA drug [181,182]. Since mRNA-loaded LNPs are a platform that need to be clinically used within a short time, there is no doubt that it will become the mainstream vaccine against pandemic respiratory virus infections in the future. More information on mRNA-LNP vaccines can be found in other excellent reviews [11,183].

Nano-DDS technologies have long been used to control immunological processes. For example, in 1991, the first report of the loading of an antigen on a membrane-fused liposome that promoted escape from endosomes and promoted MHC class I antigen presentation of foreign antigens appeared [184]. The use of nano-DDS is extremely beneficial, because many immunofunctional molecules that affect immunological processes, such as antigens and adjuvants, are easily degraded, have low cell uptake, and are sparingly soluble [185]. In particular, a lipid-based nano DDS, namely liposomes and LNPs, have already been put to practical use and appear to have an advantage over other nano-DDS platforms. Liposomes have been often employed delivering antigens (proteins and peptides) to antigen presenting cells (APCs), especially dendritic cells (DCs) [186–188]. Modifying the liposomal surface with a cell-penetrating peptide (CPP) drastically enhanced the cellular uptake of antigens by DCs, and the lipid composition largely influenced the intracellular fate of the liposomes, leading to the ability to control MHC class I/II antigen presentation [186]. Moreover, liposomes and LNPs can be used to efficiently deliver various adjuvants such as lipid-based adjuvants [189], nucleic acid-based adjuvants [187,190–193], bacterial components [194]. In addition to mRNA vaccines, LNPs show excellent performance in the delivery of mRNA and small interfering RNA (siRNA) [181,182,195–197]. Although introducing nucleic acids into immune cells is challenging for nano-DDS, the LNP systems developed in our laboratories that contain ionizable lipids can efficiently deliver siRNA into various immune cells [198–203]. Ionizable lipids are not the only key to the success of LNPs. It is also the establishment of a mass manufacturing method using flow systems and microfluidic devices.

Microfluidic devices have micrometer scaled structures and allow continuous mixing by flow, resulting in the scalability of LNPs to be improved greatly [204,205]. Furthermore, the use of microfluidic devices permits sub-50 nm sized LNPs to be prepared and these products have been applied to efficient lymphatic delivery [206,207]. Taken together, the lipid-based nano-DDS would be a useful technology for appropriately enhancing immune responses against pandemic respiratory virus infections.

Appropriate control of the immune process based on the characteristics of the viruses is important for ending the current COVID-19 pandemic, the control of seasonal influenza, and the countermeasure against the new pandemic virus. In the following sections, we discuss the lipid-based nano-DDS strategies that are designed to achieve the above three goals in two immune processes, namely innate immunity and adaptive immunity.

6. Innate immunity (Fig. 3)

6.1. Importance of innate immunity in the early stages of infection

Innate immunity is a non-specific and comprehensive defense system, and immediately responds to invading pathogens. Thus, innate immunity is the first immunological process for the treatment of this pandemic respiratory virus. In innate immunity, phagocytes (DCs, macrophages, neutrophils) and innate lymphoid cells (natural killer (NK) cells) mainly function to recognize and then clear pathogens. Cells have various types of pattern-recognition receptors (PRRs) that recognize the specific molecular patterns of pathogens, and recognition by PRRs leads to the activation of immune cells and cytokine production. The innate immune responses are largely associated with viral proliferation and the severity of the disease [208]. The main cause of the severe influenza and COVID-19 infections is an excessive innate immune response to the propagated virus. Age and some underlying illnesses are known as risk factors for influenza and COVID-19 [180]. Infants with an unestablished immune system, elderly people with weakened immune systems, and patients with immunosuppression or autoimmune diseases have difficulty in eliciting a sufficient natural immune response to suppress early viral growth. Two doses of the mRNA vaccines prevent the onset and aggravation of COVID-19 at all ages [209]. However, antibody titers are low in the elderly and diabetics, and the risk of aggravation must still be considered [210,211]. In addition, the only weapon in the emergence of new pandemic viruses is the innate immune response. Therefore, it is essential to induce sufficient innate immune responses and reduce viral load in the early stages of infection.

Adjuvant-loaded lipid-based nano-DDS enhances innate immunity based on the production of type I IFNs. Boosting innate immunity at an early phase would change the severity of a viral infection.

6.2. Innate cytokine booster in the early stages of infection

A key factor in the innate immunity booster should be type I and III IFNs [212,213]. For example, in experiments using SARS-CoV or MARS-CoV infected mice, a delayed type I IFN response led rapid virus replication, and the early administration of type I IFN prevented this severe disease [214,215]. To boost innate immunity, the use of ligands for the PRRs in endosomes and the cytosol is promising. They respond to viruses that invade into cells, leading to the production of type I IFN via the activation of IRFs [216]. The well-known combinations of PPRs and ligands (PRRs/ligands) are the follows: TLR3/double stranded RNA (dsRNA) [217], TLR7/single stranded RNA (ssRNA) [218], TLR9/CpG-ODN [219], RIG-I like receptors (RLRs)/dsRNA and ssRNA [220], stimulator of IFN genes (STING)/ cyclic dinucleotides (CDNs) [221]. Since these ligands are recognized by the intracellular PPRs and are difficult to reach inside the cell on their own, the use of lipid-based nano-DDS would be suitable.

In an early study with phosphatidylcholine/cholesterol/stearylamine liposomes, a pretreatment of polyinosinic-polycytidylic acid (poly I:C) loaded liposomes into the nasal cavity of mice completely protected that from an IAV (H1N1) challenge [222]. Poly I:C is recognized by TLR3 in the endosomes and RLRs in the cytosol [220]. This suggests that selective recognition via RLRs by the cytosolic delivery of poly I:C induces type I IFN production with low inflammatory cytokines and enhances antitumor immunity [187,223]. That is, by controlling the intracellular dynamics of the ligand based on the localization of PRRs, it is possible to control the output of the immune response. In SARS-CoV-2 infections, an inhalable liposomal vaccine containing poly I:C and the spike protein of SARS-CoV-2 efficiently elevated type I IFN production and induced a high titer of secretory immunoglobulin A (sIgA) in

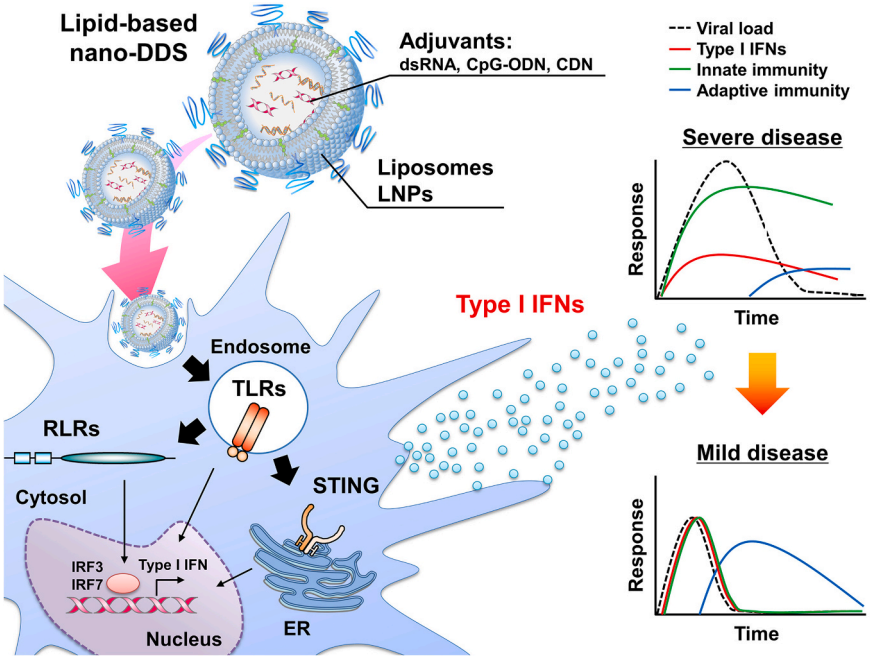

Fig. 3. Boosting innate immunity by lipid-based nano-DDS.

respiratory secretions, resulting in the neutralization of virus and protection from infection [224].

CpG-ODN is clinically used in hepatitis B virus vaccines (HEPLISAV-B®). There are three major classes of CpG-ODNs based on their structure (Class A, B and C), and the balance of production of type I IFNs and inflammatory cytokines varies from class to class. [225]. Because the class A CpG-ODNs have an excellent ability to produce type I IFN, its application as a cancer adjuvant is currently being considered [226–228]. Nano-DDSs are also used in this case. At this time, it seems that the effectiveness of using lipid-based nano-DDS containing CpG-ODNs against the pandemic virus has not been verified. However, a recent study clarified that the addition of aluminum hydroxide and CpG-ODN to the subunit vaccine using SARS-CoV-2 spike protein induced the same level of antibody titer as the mRNA vaccine [229].

Other potential ligands for inducing type I IFNs include ligands for the STING pathway, which functions to sense cytosolic DNAs and CDNs [221]. Cyclic GMP–AMP synthase (cGAS) binds to cytosolic DNA and produces CDNs as a second messenger. The recognition of CDNs by STING triggers the signaling pathway. Since STING is essential for the initiation of antitumor immunity [230], it is anticipated that these ligands might be useful as a cancer adjuvant. In fact, in addition to CDNs, various STING ligands have been developed and tested in pre-clinical and clinical trials [231]. The efficient delivery of STING ligands also requires nano-DDS, because they reach the cytosol with difficulty on their own [232]. We also encapsulated CDNs into a LNP composed of YSK12-C4, an ionizable lipid, and the treatment with the LNP caused elevated levels of serum type I IFN and induced an antitumor effect against lung metastasis [193]. Although there are few verifications of drugs for the treatment of pandemic respiratory virus infections, the use on these drugs in conjunction with a lipid-based nano-DDS as a vaccine adjuvant against seasonal influenza and COVID-19 has been reported [233,234].

Taken together, the combination of an adjuvant and a nano-DDS would be expected to boost innate immunity centered on type I IFN production, resulting in a high suppression of viral replication and a high vaccine effect. However, the biggest concern regarding the use of adjuvants is side effects. Some side effects are tolerated when these are used for cancer treatment. But when used for the purpose of preventing infectious diseases, a high level of safety is required because the administration target is a healthy subject. The use of nano-DDS is expected to not only enhance efficacy but also reduce side effects, whereas there are very few clinical examples of adjuvants including those in the oncology area. From this, it is highly possible that the control of side effects will be the rate-limiting step for clinical use. In contrast, it is considered to be highly valuable for practical use in preventing the aggravation of pandemic virus-infected persons. If the innate immune response centered on IFN production can be enhanced in the early stage for infected persons at high risk, it would be possible to suppress viral growth. In addition, it is expected to be more effective and the only weapon against unknown pandemic viruses. Thus, it is important to identify the type of immune response required against virus infections, the appropriate location for inducing antiviral immunity, and the factors associated with side effects, and then to design a nano-DDS that can precisely control these variables.

6.3. NK cell activation in the early stages of infection

NK cells are innate lymphoid cells that are generally active during innate immunity, and their ability to kill virus-infected cells and the IFN-gamma production are well-established. In addition, memory NK cells with properties similar to adaptive immune cells are currently attracting attention [235,236]. Such memory NK cells are expected to enhance innate immunity against broad pathogen infections. Several mouse studies using respiratory viruses such as IAV have demonstrated that NK cells are associated with viral control and better survival [237]. In patients with severe COVID-19 infections, a decrease of blood NK cells was observed [238,239]. Furthermore, at an early phase of SARS-CoV-2 infections, high levels of blood NK cells are correlated with a rapid decrease in viral load [240]. However, the functions of NK cells in patients with severe infections were largely suppressed by transforming growth factor-β (TGF-β) [240]. Thus, NK cell activation during innate immunity at an early phase of infection represents a potential strategy.

We found that the intravenous treatment of a STING agonist loaded LNPs (STING-LNPs) efficiently induced systemic type I IFN production and NK cell activation [192,193]. The production of type I IFN occurred in liver macrophages and the type I IFN in blood circulation can activate systemic NK cells. Although the selection of proper STING agonists and a thorough verification of side effects are required, STING-LNPs are promising as an early-stage treatment to prevent the aggravation of pandemic respiratory viral infections.

Adoptive NK cell transfer can help to control the virus. Since the functions of NK cells are impaired by TGF-β in a body that is infected with SARS-CoV-2 [240], normal NK cells should be infused. SMAD3 associates with the regulation of TGF-β downstream and the gene silencing of SMAD3 by a viral vector in a human NK cell line (NK-92) showed resistance against TGF-β stimulation [241]. The SMAD3-silenced NK-92 killed tumor cells that were producing TGF-β. We developed a siRNA-loaded LNP that included the CL1H6 lipid, an ionizable lipid, that can efficiently silence the gene expression in NK-92 [203]. By using our LNP, it will be possible to safely and easily produce NK-92 that is resistant to TGF-β stimulation. NK-92 are currently being evaluated in clinical trials as an off-the-shelf NK cell therapy for cancer and may also be used in the treatment of COVID-19.

7. Adaptive immunity (Fig. 4)

Adaptive immunity is the greatest weapon against viral infections. It is a specific and strong response against invading pathogens after innate immunity. The three protagonists in adaptive immunity are B cells, $CD4^+$ T cells and $CD8^+$ T cells. B cells produce neutralizing antibodies against viruses, namely humoral immunity. $CD4^+$ T cells aid in the work of B cells and $CD8^+$ T cells by producing various cytokines. $CD8^+$ T cells kill the virus-infected cells, namely by cellular immunity. We obtain adaptive immunity against viruses, when we are naturally infected with viruses or are vaccinated. The ideal adaptive immunity exhibits protection against long-term infections that are independent of viral revolution. This is a goal of vaccines against pandemic respiratory viruses. In general, natural infection induces strong and long-lasting adaptive immunity. Thus, a live attenuated vaccine is most effective and is traditionally used as mentioned above. In fact, a live attenuated vaccine for IAV is used in clinics, and the development of a live attenuated vaccine for SARS-CoV-2 is now in the pre-clinical stage [242]. However, it poses a high risk as a vaccine platform for pandemic viruses. In contrast, while inactivated vaccines and subunit vaccines are safe, their ability to induce adaptive immunity is insufficient. What vaccine is needed to safely produce ideal adaptive immunity? One answer is the development of live artificial vaccines using nano-DDS. The following sections outline the design strategies for preparing live artificial vaccines using lipid-based nano-DDS. The point of the design is to trace the mode of infection of the virus and the host's immune response to eliminate the virus.

Live artificial vaccines have mucosal stability and dispersibility, and are selective for DCs and M cells. In addition, the on-loaded adjuvant is delivered to the cytosol, leading to the activation of innate immunity through the intracellular recognition of PPR. The vaccines also express universal antigens, promote antigen presentation to MHC class molecules, and activate broad and long-term adaptive immunity.

7.1. Mucosal delivery for mucosal vaccines

Since respiratory viruses infect the upper respiratory tract mucosa, immune defense on the surface of the respiratory tract mucosa is essential for protecting against infections. It is difficult to induce

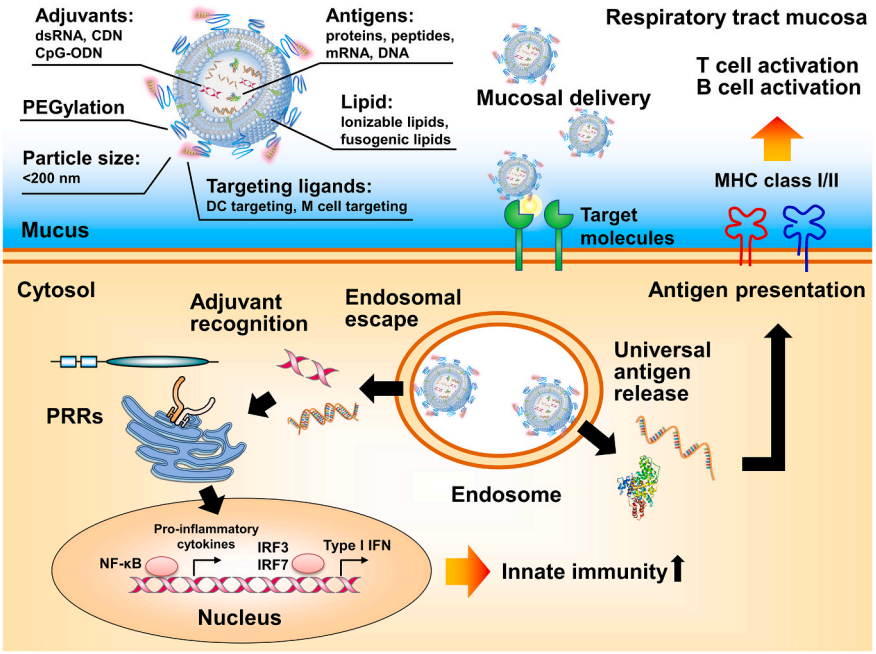

Fig. 4. Live artificial vaccines using lipid-based nano-DDS.

mucosal immunity by intramuscular administration or subcutaneous administration, which are conventional methods for administering a vaccine. Therefore, the development of a mucosal vaccine that induces immune responses at the respiratory tract mucosa by nasal administration or pulmonary administration is currently underway. In fact, there are nine current licensed mucosal vaccines and they are all live-attenuated or inactivated whole-cell types, indicating that infectivity and high immunogenicity are essential for the success of mucosal vaccination [243]. Thus, mucosal delivery systems, mucosal adjuvants and innovative antigens are urgently needed.

Antigen capture in the mucus is performed by DCs and microfold (M) cells. M cells are specialized epithelial cells for antigen capture and mucus-specific transport in lymphoid tissues such as Peyer's patch and nasopharynx-associated lymphoid tissue (NALT). Delivering antigens to these types of tissues is the biggest barrier to mucosal vaccine development. This is because mucosal epithelial cells are covered with a thick layer of mucus that functions as a strong physical barrier to foreign substances. Mucus contains mucin, antibacterial peptides, IgA antibodies, etc., and prevents the invasion of viruses and bacteria. If the foreign substances cannot break through the mucus layer, they will be expelled by mucus flow. The main barrier for a nano-DDS appears to be the porous network formed by mucin [244]. The size and surface characteristics of nano-DDS are also important, because they have the effects of size sieving and adsorption. Particles with a size of 200 nm or less are highly dispersible in mucus, because the pore size of the mucin network is thought to be on the order of 200 to 500 nm [245]. It is also believed that modifying the surface of a nano-DDS such as liposomes with PEG reduces interaction with mucosal components and enhances diffusivity in mucus [246,247]. Employing constituents in pulmonary mucus in the composition of liposomes resulted in a pulmonary mucosal vaccine against influenza [233]. Negatively charged lipids and PEGylation were key factors in the balance between excellent mucosal immune responses and toxicity. On the other hand, a cationic polymer such as chitosan electrostatically binds to a negatively charged component in mucus and enhances the binding to the mucosal surface [248]. Liposomes containing cationic lipids induced high levels of IgA production by nasal administration [249,250]. However, the use of cationic lipids has the disadvantages of toxicity and the deterioration of intracellular kinetics due to particle aggregation. Therefore, it is considered that a nano-DDS that has dispersibility properties with particle size being controlled and PEGylation, and furthermore modified with a ligand that targets DCs and M cells may be suitable. The strategy of DC targeting is well-known and the ligands or antibodies against C-type lectins and surface markers are on the DC surface [251]. There are also various reports on targeting ligands for M cells: an antibody against glycoprotein-2 [252]; the M cell specific peptide (CKS9) [253]; the M cell specific antibody (NKM 16–2-4) [254].

7.2. *Proper use of adjuvants*

Mucosal vaccine development uses adjuvants that differ from vaccines that involve other routes of administration. The incorporation of the cholera toxin (CT) and the *Escherichia coli* enterotoxin (LT) into mucosal vaccines induces strong mucosal immunity. The cause for this appears to be the high specific binding to M cells and strong DC activation [255]. Because CT and LT show unacceptable toxicity, studies using mutants with reduced toxicity and B subunits with the toxicity eliminated are underway. CTB-conjugated liposomes enhanced the effect of mucosal vaccination by oral administration [256]. The B subunit may have potential as a targeting ligand.

PRRs ligands that are in use for general vaccines have shown a high potential in mucosal vaccination. In the case of PPR ligands, it will be necessary to incorporate them into nano-DDS, because they are themselves unable to pass through the mucus layer and they lack selectivity for DCs and M cells. A comparison between the free form of CpG-ODN and liposomal CpG-ODN clearly showed that the liposomal CpG-ODN induced a more effective IgA production, cytokine production and protection against IAV (H1N1) challenge at 30 times [257]. A mannose-conjugated liposome encapsulating poly I:C also enhanced mucosal IgA production and inhibited virus infections in vitro compared with the free form of poly I:C [258]. STING ligands have been verified for use in mucosal vaccinations. The above negative PEGylated liposomes based on the constituents in pulmonary mucus internalized the STING ligand [233]. Nasal co-administration of the liposome and whole inactivated H1N1 vaccine induced the production of mucosal IgA and memory $CD8^+$ cells, and showed strong cross-protection against H3N2 and H5N1. In addition to the influenza vaccine, a liposomal STING ligand was employed for a COVID-19 vaccine [234]. The composition of the

liposome was quite similar [233] and the liposome had a negative charge and a PEG lipid. A single-dose nasal vaccination of the liposomal STING ligand elicited strong systemic and mucosal immune responses against SARS-CoV-2 [234]. Taken together, even with PRR ligands, strong mucosal immunity can be induced when appropriate lipid-based nano-DDS is used, and it can be inferred that ligands recognized by intracellular PRRs may be suitable as an adjuvant for mucosal vaccines against the pandemic respiratory virus.

7.3. Selection of antigens: forward to universal vaccines

An antigen is indispensable for the induction of virus-specific adaptive immunity. Although the existing inactivated vaccines and adjuvant-loaded nano-DDS may be co-administered, the general antigen modalities used in nano-DDS based vaccines are proteins, peptides, antigen-corded mRNA and DNA. The choice of antigen modality affects both the productivity and efficacy of the vaccine. In fact, the choice of modality had a major impact on a COVID-19 vaccine. The mRNA vaccine is excellent in terms of speed of production and activation of an immune response, and promises to be the main antigen modality in the future.

On the other hand, the selection of the appropriate antigen has a great influence on the induction of neutralizing antibodies against the virus. In influenza and COVID-19 vaccines, viral surface proteins such as HA proteins and spike proteins are used as target antigens to produce neutralizing antibodies that inhibit adhesion to host cells. However, the surface proteins are prone to mutation, and this has become a major problem in pandemic viruses [172,180]. Under such circumstances, the development of universal vaccines is currently underway to counter virus evolution [259,260]. In current influenza vaccines, HA head domains are used as target antigens, because they are immunodominant [259]. In contrast, they are antigenically variable, resulting in the loss of neutralization. To overcome this, the highly conserved HA stalk domain is used for a universal vaccine target [261,262]. In the COVID-19 pandemic, we have been hit by the worldwide spread of SARS-CoV-2 with mutations in the S protein, and there are concerns that the effectiveness of the current mRNA vaccines will decline [263,264]. Unlike IAV, an antigen shift in SARS-CoV-2 is unlikely to occur, thus, the response to SARS-CoV-2 variants may not be as serious as IAV. In addition, we can generate T cells that recognize a very broad SARS-CoV-2 epitope and diverse antibodies that neutralize the spike protein of SARS-CoV-2 [265]. However, the search for antigens that are amenable for use in universal vaccines is underway. A computational analysis detected immunogenic T cell epitopes and B cell epitopes from all 10 proteins in SARS-CoV-2 [266]. Searching a conserved domain in the spike protein and employing other proteins as an antigen target represent an appropriate response to future SARS-CoV-2 variants and for creating stronger vaccines.

7.4. Control of antigen fate

Controlling the intracellular fate of antigens by a nano-DDS can help to maximize their function. For the antigen-specific activation (namely adaptive immunity) of T cells and B cells, antigen presentations on MHC class I and II and antigen recognition via B cell receptors (BCRs) are essential processes. Antigen presentation on MHC class I requires cytosolic antigens or cross-presentation by DCs [267]. Antigen modalities such as mRNA and DNA can be used to generate the cytosolic antigens. As mentioned above, the mRNA vaccines employ an ionizable LNP technology and the LNPs efficiently deliver mRNA to the cytosol. The control of the intracellular fate of the nRNA is an indispensable step. After intramuscular injection, the mRNA-LNP is taken up by DCs and muscle cells, and the mRNA in the cytosol is translated to antigens [268]. Some of the expressed antigens can be presented to MHC class I and class II on DC, resulting in the activation of $CD4^+$ T cells and $CD8^+$ T cells. In contrast, antigens released from DC and muscle cells can be recognized by BCRs on B cells, and the B cells can be also stimulated by helper T cells to differentiate into plasma cells that produce virus-specific antibodies. Although the detailed mechanism of mRNA-LNP vaccines is still unclear, excellent adaptive immunity would be induced, if the intracellular fate of mRNA can be sufficiently controlled.

When we use proteins and peptides as antigens, controlling the intracellular fate of antigens by nano-DDS is useful. In general, the administered proteins and peptides are taken up by DCs and are presented on MHC class II as exogenous antigens [267]. This explains why subunit vaccines cannot induce cellular immunity. Fusogenic liposomes and CPP-modified liposomes are often used to enhance the MHC class I presentation of exogenous antigens, [184,186]. We modified an octaarginine (R8) peptide, a type of CPP, on the surface of fusogenic liposomes containing an encapsulated antigen, and the R8-modified liposomes efficiently delivered the antigen to the cytosol in DCs and induced MHC class I specific antigen presentation [186]. In another way, TLR signaling enhances the cross-presentation in DCs [269,270]. Incorporating the PRR ligand into a nano-DDS would help to promote the MHC class I presentation of antigens in addition to DC maturation.

8. Perspectives

The SARS-CoV-2 pandemic is the largest pandemic in recorded human history. For this unprecedented pandemic, we should be proud that nano-DDS technology was able to provide a game changer by providing us with the mRNA-LNP vaccine. However, there are still no signs of the end of the SARS-CoV-2 pandemic. It is important to face the challenge of solving the problem while turning the two wheels of accumulating virological knowledge and the technological innovation of delivery systems. In addition, the development of an artificial live vaccine using a nano-DDS is one of the countermeasures against respiratory pandemic viruses including the influenza virus, and it is expected to develop a powerful and safe vaccine that does not yield to viral evolution by combining them with universal antigens. Collaboration between virologists and DDS researchers will be key to this.

Declaration of competing interest

The authors declare no competing interests.

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