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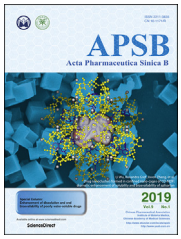

REVIEW

Localized delivery of nanomedicine and antibodies for combating COVID-19

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Abstract The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has been a major health burden in the world. So far, many strategies have been investigated to control the spread of COVID-19, including social distancing, disinfection protocols, vaccines, and antiviral treatments. Despite the significant achievement, due to the constantly emerging new variants, COVID-19 is still a great challenge to the global healthcare system. It is an urgent demand for the development of new therapeutics and technologies for containing the wild spread of SARS-CoV-2. Inhaled administration is useful for the treatment of lung and respiratory diseases, and enables the drugs to reach the site of action directly with benefits of decreased dose, improved safety, and enhanced patient compliance. Nanotechnology has been extensively applied in the prevention and treatment of COVID-19. In this review, the inhaled nanomedicines and antibodies, as well as intranasal nanodrugs, for the prevention and treatment of COVID-19 are summarized.

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1. Introduction

Coronavirus disease 2019 (COVID-19) is a highly contagious respiratory infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[1]. SARS-CoV-2 has caused an unprecedented public health crisis. As of June 29, 2022, COVID-19 has caused 543,352,927 confirmed cases and 6,331,059 deaths globally[2]. Vaccination is the most effective means of COVID-19 prevention. A variety of vaccines have been marketed in various forms including mRNA (*e.g.*, the Pfizer-BioNTech COVID-19 vaccine)[3]; inactivated virus (*e.g.*, Covaxin)[4]; recombinant subunit (*e.g.*, Covovax)[5]; and adenovirus vector vaccines (*e.g.*, Janssen COVID-19 vaccine)[6]. However, the prevalent mutated strains have caused vaccine breakthrough infection and a high transmission rate, thereby aggravating the global pandemic[7]. Normally, COVID-19 vaccines are administrated by intramuscular injection[8]. However, SARS-CoV-2 enters the human body mainly through the nose and mouth to the lung. Intramuscular vaccination cannot efficiently induce mucosal immunity[9]. It was reported that inhaled vaccination, *via* mimicking the SARS-CoV-2 infection route, can induce potent mucosal immunity[10], and a clinical trial showed that atomized COVID-19 vaccine can induce both humoral and mucosal antibody responses[11]. Moreover, a variety of specific drugs for COVID-19 treatment are administered orally and intravenously[12]. Yet, the lung is the main lesion of COVID-19 and inhalation could achieve maximum pharmacological targeting with minimal systemic exposure and avoid first-pass metabolism in the liver. Thus, inhalation could be an appropriate drug delivery route for COVID-19 prevention and treatment. In early September, 2022, China approved the world's first inhaled COVID-19 vaccine (Convidecia Air), which has been granted emergency use as a booster.

Infection cases and deaths of COVID-19 are continuing to rise as the mutant strains emerge and spread. It is of great significance to develop new medications against the infection. Nanotechnology has been extensively studied in the medical field due to its unique properties, *e.g.*, small size, large surface area, multifunctionality, surface adaptability, and enhanced drug solubility[13]. Nanotechnology can improve the detection performance and the therapeutic effect compared with traditional diagnosis and treatment techniques. For example, the nanotechnology-based SARS-CoV-2 detection methods may provide a viable platform for improving the diagnosis efficiency of SARS-CoV-2[14,15]. Moreover, long-term antiviral therapy usually leads to gradually weakened effectiveness and side effects[16,17]. Nanomedicines can improve the treatments by increasing the solubility of hydrophobic drugs, prolonging the blood circulation time, delivering to the target tissues, and releasing drugs in a controlled manner[18]. Therefore, nanotechnology-based antiviral strategies may optimize pharmacokinetics and pharmacodynamics, reduce toxic and side effects, and improve therapeutic efficacy[19].

In this review, we summarized the inhaled delivery strategies including pulmonary and intranasal administration for anti-COVID-19 and discussed the benefits of inhaled nanomedicines for the prevention and treatment of COVID-19. Meanwhile, neutralizing antibodies also play an important role in blocking the infection of SARS-CoV-2. The benefits of inhaled antibodies for the treatment of COVID-19 have also been discussed.

2. The process of the COVID-19 infection

In the past two decades, there have been three coronavirus epidemics: SARS-CoV[20], the Middle East respiratory syndrome coronavirus (MERS-CoV)[21], and SARS-CoV-2. There are four subgroups of the coronavirus family (*i.e.*, alpha, beta, gamma, and delta coronaviruses). SARS-CoV-2, with a small diameter ranging from 65 to 125 nm[22], belongs to the beta subgroup[23]. SARS-CoV-2 shares a certain similar sequence and structural homology with SARS-CoV and MERS-CoV; these three belong to coronaviridae with positive-sense single-stranded RNA genomes that are highly conserved. Phylogenetic analysis clustered SARS-CoV-2 in the same group of SARS-CoV and MERS-CoV with similarity scores of 79% and 50%, respectively[24]. The gene sequences (5′−3′) of SARS-CoV-2 are as follows: open reading frame 1 ab (ORF1ab), spike (S), ORF3a, envelope (E), membrane (M), ORF6, ORF7a, ORF7b, ORF8, nucleocapsid (N), and ORF10[25].

Inhalation of viral particles from respiratory droplets expelled by sneezing, coughing, or talking is the most important transmission route of SARS-CoV-2[26]. Furthermore, SARS-CoV-2 infection can occur *via* hand-to-eye, hand-to-nose, or hand-to-mouth routes after touching virus-laden surfaces, *e.g.*, metal, paper, plastic, and cloth, where viruses could be viable for hours depending on temperature, humidity, and chemical and topological properties of the solid surface[27]. The possibility of fecal-oral transmission of SARS-CoV-2 was also reported[28,29].

SARS-CoV-2 infects the host cells relying on the S protein on the surface of the virus[30]. The S protein is the main immunodominant antigen of SARS-CoV-2 and consists of three heterodimers of S1 and S2 subunits[31]. S1 is responsible for the recognition and binding of the host cell surface receptors and S2 mediates the fusion of the viral envelope with the host cell membrane[32]. The S1 subunit can bind to heparan sulfate (HS) on the cell surface, and open the RBD of the S protein, thus facilitating the binding of the S protein to angiotensin-converting enzyme 2 (ACE2) on the host cell surface (Fig. 1A)[33]. ACE2 is a transmembrane protein and is highly expressed on the epithelial cells of the heart, stomach, nasal mucosa, lungs, bladder, intestine, and kidneys[34], which indicates that these tissues are susceptibly infected by SARS-CoV-2. During infection, the S protein is cleaved at two sites: the cleavage at S1/S2 by furin induces a conformational change and facilitates recognition by ACE2, and the S2' cleavage by transmembrane protease, serine 2 (TMPRSS2) initiates virus/cell membrane fusion and intracellular entry[35]. These cleavage steps, called priming, are critical for the virus to bind to the host cell membrane and enter the cell (Fig. 1B)[36]. Subsequently, SARS-CoV-2 hijacks the host cell transcription and initiates replication inside the cells. Finally, the mature viruses are released from the infected cells and infect others. Besides HS and ACE2, other host cell receptors have also been found to be involved in facilitating SARS-CoV-2 entry,

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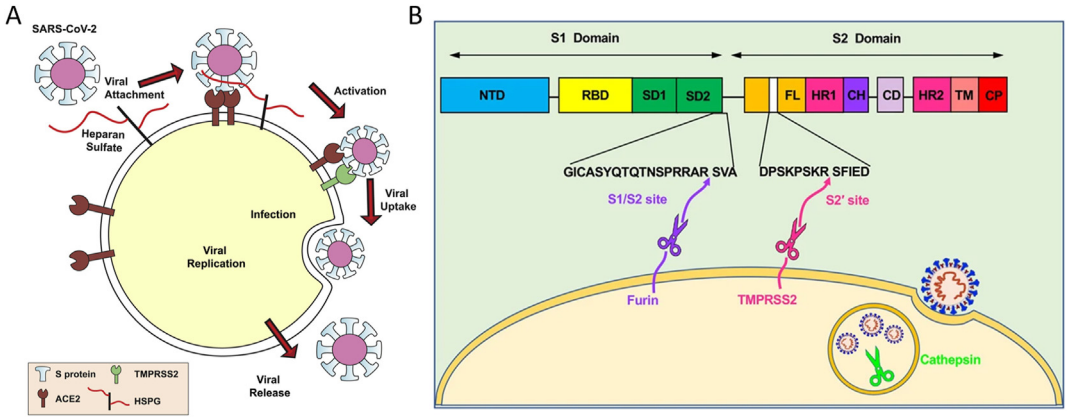

Figure 1 The pathway of the SARS-CoV-2 entry into cells. (A) The process of SARS-CoV-2 infecting the host cells. Reprinted with permission from Ref. 33. Copyright © 2020 Elsevier. (B) The cleavage of the S protein by furin and TMPRSS2. Reprinted from Ref. 36, CC BY 4.0. Copyright © 2021 The Author(s).

e.g., the neuropilin-1 (NRP1) receptor[37] and the tyrosine-protein kinase receptor UFO (AXL)[38].

During the early stages of infection, SARS-CoV-2 enters the respiratory tract and infects the goblet cells and ciliated cells by depositing on the airway epithelium[39]. At this stage, SARS-CoV-2 amplifies in the cells, and patients could develop mild symptoms. With the rapid reproduction of the virus, the goblet and ciliated cells die off and release a large number of viruses that cause strong immune responses and severe symptoms like cough, fever, difficult breathing, and coagulation[40]. Meanwhile, hyper inflammation would occur because the overactivated immune system leads to cytokine storms and progressive damage to the tissues and organs[41]. A detailed description of this process can be referred to a recent review[42]. Infectiousness and disease severity are changed among different strains of SARS-CoV-2 and have been summarized in Table 1[43–67].

Therefore, through the infection process of SARS-CoV-2, the following therapeutic targets have been investigated: the S protein of SARS-CoV-2, the targets on the host cells (*e.g.*, ACE2 and heparan sulfate proteoglycans, HSPGs), the essential enzymes for virus replication (*e.g.*, RNA dependent RNA polymerase, RdRp), the targets of the virus-associated inflammation (*e.g.*, IL-6 blockage). Moreover, given the transmission nature and the primary target organ (*i.e.*, the lung) of SARS-CoV-2, inhalation would be an ideal drug delivery route for both the prevention and treatment of COVID-19. The International Society for Aerosol Medicine (ISAM) has called for the development of inhaled therapies for COVID-19[68]. Moreover, a variety of drugs for the prevention and treatment of COVID-19 have been developed in inhalations, *e.g.*, Ad5-nCoV inhaled vaccine[69], ivermectin inhalation[70,71], ciclesonide inhalation[72], and inhaled antibody therapy[35].

3. Inhalations for lung or respiratory tract delivery

Inhalation is a traditional method and an important drug delivery strategy for the treatment of lung and respiratory diseases, *e.g.*, asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), pneumonia, and pulmonary hypertension[73,74]. In addition to small molecules, inhalation is also suitable for the delivery of biologics, *e.g.*, peptides, proteins, nucleic acids, and exosomes[75–78]. Recently, vaccination through inhalation has received great attention. Nebulized measles vaccine eliminated morbidity and mortality in young children and reduced the severity of associated pneumonia, and pulmonary delivery of measles vaccines has shown the promise (detailed information can be referred to a review[79]). Furthermore, inhalation is an effective and non-invasive strategy, which is patient-friendly, and self-administered inhalation can remove the need for healthcare workers and facilities.

3.1. *Orally inhaled delivery*

The drugs can be directly delivered through the mouth to the lower respiratory tract by oral inhalation. Moreover, the complex physiological structure in the nose and the mechanical obstruction are also part of the reasons for less pulmonary drug deposition[80]. Therefore, orally-inhaled delivery is preferable for inhalations. An aerosolized adenovirus type-5 vector-based COVID-19 vaccine (Ad5-nCoV) has passed phase I clinical trial and the result shows good safety, tolerability, and immunogenicity[11]. It is inspired that only 1/5 of the intramuscular injection dose by inhalation can reach the same level of immune response[11]. Moreover, the latest clinical data show that orally administered aerosolized heterologous vaccination, serving as the 2nd booster, has good safety, and its immunogenicity to the wild-type and delta strains is significantly higher than that of homologous inactivated vaccine[81].

Normally, orally-inhaled drugs could directly pass through the throat and enter the lower respiratory tract with the help of a device. However, a part of inhaled drugs will be trapped in the larynx due to the limitations of airway anatomy[82]. The drugs deposited in the lung may undergo pulmonary absorption and metabolism, which is dependent on the physiological characteristics of the biological membranes and the properties of the drugs[83].

Stabilizers are commonly used to maintain particle size and shape, and prevent particle aggregation in a given aqueous suspension medium[84]. However, the stabilizers can be a source of cytotoxicity. For example, Solutol HS 15 can contribute to the cytotoxicity of nanoparticles measured in a cell-based assay[85]. In this regard, aerosol is an effective method to prevent particle agglomeration. Metered-dose inhalers (MDI), dry powder inhalers (DPI), and nebulizers are commonly used to generate aerosols for enhanced pulmonary delivery and reduced side effects[86]. The

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Table 1 The basic information of the SARS-CoV-2 (wild-type and variants of concern, as of April 2022).

Variant	Pangolin name	Number of mutations	Infectiousness	Disease severity	Therapeutic effectiveness	Vaccine effectiveness	Ref.
Wild-type	–	–	High transmissibility	Pneumonia, supplemental oxygen requirement, and ICU admission	Be effectively neutralized by monoclonal antibodies (mAbs) for clinical use	High antibody neutralizing titers elicited by mRNA vaccine (mRNA-1273 and BNT162b2)	43,44
Alpha	B.1.1.7	Spike: 10 Overall: 24	43%–90% more transmissible than the previous lineages	Cough, sore throat, fatigue, and myalgia occur more frequently and anosmia is less common; higher risk of hospitalization and increased mortality than in previous variants	Just be resistant to a few mAbs	Only a modest reduction in neutralizing titers elicited by mRNA-1273 or BNT162b2 (less than 3-fold)	43,45–50
Beta	B.1.351	Spike: 10 Overall: 20	1.5 times more transmissible than the previous linages	More frequent symptomatic cases than delta; higher risk of hospitalization and ICU admission than delta and non-VOC cases	Be resistant to most mAbs	4.9-fold reduction in neutralizing titers elicited by 2-dose BNT162b2	43,49-53
Gamma	P.1	Spike: 12 Overall: 24	1.4–2.2 times more transmissible than the previous lineages	Higher risk of hospitalization and ICU admission than delta and non-VOC cases and higher mortality than the previous lineages	Be similar to beta	Significant reduction in neutralizing titers elicited by mRNA-1273 and BNT162b2 (4.5- and 6.7-fold)	43,52,54–56
Delta	B.1.617.2	Spike: 9 Overall: 22	Higher transmissibility than the previous lineages	Higher risk of pneumonia than the wild-type; higher rates of ICU admission and death than other variants.	Be highly resistant to bamlanivimab but retain susceptibility to many mAbs	5.8-fold reduction in neutralizing titers elicited by 2-dose BNT162b2	43,53,57–60
Omicron	B.1.1.529	Spike: more than 30 Overall: more than 60	Higher transmissibility than delta	Low risk of pneumonia and symptoms are mostly upper respiratory tract infection; lower rates of hospitalization, ICU admission, and death than delta	Be resistant to many commercial mAbs in various degree	22-fold reduction in neutralizing titers elicited by 2-dose BNT162b2	43,61–67

–, not applicable.

MDI, also named pressurized metered-dose inhalers (pMDI), can atomize drug solutions and the DPI atomizes drug powders; both require patients to cooperate with inhalation or actuation. The power-driven nebulizers can convert a drug solution or suspension into an aerosol, which is friendly to the elderly, children, and unconscious patients. Therefore, it is important to select an appropriate inhaler according to the nature of the drugs, the age of the patients, and the disease stages.

3.2. *Intranasal delivery*

The intranasal route is another inhalation option. Notably, the intranasal vaccination can simulate a respiratory infection of a virus and elicit potent mucosal immunity, which has been clinically used for influenza vaccination[87]. The dNS1-RBD, based on a live attenuated influenza virus vector, is the first reported intranasal vaccine entering clinical trials to prevent COVID-19[88]. The

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results of phase I/II clinical trials showed that the intranasal dNS1-RBD vaccine was safe and activated a variety of immune responses[88]. These data also indicate that the intranasal dNS1-RBD vaccine could become an important supplement to the current vaccines for COVID-19.

The nose is structurally complex and the nasal cavity can be separated into three parts: the vestibular region, the respiratory region, and the olfactory region[89]. It is expected that the drugs deposited in the respiratory mucosa could provide a local action against viral infection through the nasal epithelium. The main factors affecting the biodistribution of intranasal drugs are their molecular weight, the hydrophilicity/hydrophobicity, and the ionization degree of the drugs[90]. Excipients are often applied to intranasal formulations, such as absorption enhancers, mucoadhesives, and preservatives, which might raise a safety concern[91,92]. For instance, as a preservative, benzalkonium chloride could induce lung irritation, inflammation, and pneumonia[93]. Drugs themselves can also impose toxicity concerns. For example, corticosteroids may cause nose bleeding and nasal septal peroration in the treatment of seasonal rhinitis[94].

4. Pharmacokinetic and biodistribution issues of inhalations

For the pharmacokinetics of inhaled therapies, the drugs in the blood are often not sufficient to evaluate drug exposure in the lung from an equivalence perspective[95]. For example, a clinical investigation revealed that inhaled salbutamol yielded an approximately 100-time higher concentration in the lung than in the plasma, while the pulmonary concentration of salmeterol and fluticasone showed a much higher ratio in the lung versus plasma[96]. To illustrate the regional lung targeting efficiency, typically, a ratio between the drug exposure in a certain lung compartment and systemic exposure is used. Various lung-sampling methods (*e.g.*, bronchoalveolar lavage, bronchial brushing, bronchosorption, mucosal biopsies, and particles in exhaled air) are often applied for determining drug concentrations in different compartments in the lung. The advance in lung-sampling technology renders human lung spatial pharmacokinetic studies feasible, which can promote the clinical development of lung-targeted drugs; for instance, bronchosorption can be used to determine drug concentration in epithelial lining fluid samples, with the benefit of measurement of both pulmonary pharmacokinetics and pharmacodynamic biomarkers[96]. The *in vivo* imaging technology of small animals has also been applied in the lung pharmacokinetic evaluation of inhalations. The distribution of inhalations in the body can be presented intuitively with fluorescence imaging. With this technology, different studies have revealed that inhaled drugs have a longer retention time and higher concentration in the lung compared with an intravenous preparation[97,98]. Furthermore, detecting the plasma drug concentration is also useful for establishing bioequivalence of inhaled formulations to avoid possible systemic side effect[99]. For example, the bioequivalence of two inhaled tiotropium bromide formulations was investigated by measuring systemic drug exposure[100]. In addition, the inhaled formulations present complex pharmacokinetic characteristics due to many product-related and patient-related factors such as inhalation devices, dosage forms (liquid aerosols versus dry powder inhalations), and the experience of patients using devices, making it more complex to establish the bioequivalence. For example, the lung and systemic delivery of salbutamol was compared in a clinical investigation following inhalation from an MDI, an MDI attached to a spacer, and a nebulizer[101]. It was found that using MDI attached to a spacer resulted in more pulmonary drug deposit and less drug in the systemic circulation than the other two devices, indicating the importance of spacers. Therefore, the abovementioned factors should be taken into consideration in the design of inhaled products.

It is generally believed that the types and activities of metabolic enzymes in the lung are few and low, and the interaction between pulmonary drugs and metabolic enzymes is often ignored[102]. However, for the inhalations with a long retention time in the lung, it is essential to investigate the interaction between pulmonary inhalations and metabolic enzymes. Detailed information on the phase I and phase II metabolic enzymes in the human lung is summarized in a review[103]. The common cytochrome P450 (CYP) isoforms in the human lung are CYP2B6, CYP1B1, CYP2E1, CYP3A5, CYP2J2, and CYP1A1 (in smokers)[104]. Besides the CYP family, other biotransformation enzymes (*e.g.*, uridine diphosphate glucuronosyltransferase, glutathione-*S*-transferases, flavin monooxygenase, peptidase, esterases, cyclooxygenase, and sulfotransferase) are also expressed in the lung[103]. It should be noted that the metabolic enzymes in the lung differ from those in the liver. For example, CYP3A4 is the most abundant metabolic enzyme in the liver, but its activity in the lung is about 20% compared to that in the liver[105]. In fact, the isoform of CYP3A5 plays an important role in lung metabolism[104,106]. Meanwhile, species differences should be taken into consideration in the study of pulmonary drug metabolism[107]. Furthermore, more metabolism models should be established for the evaluation of the enzyme metabolism process of pulmonary inhalations, which currently are largely dependent on the human lung microsomes or rat lung homogenates[108,109].

Macroscopic pharmacokinetic studies cannot reflect the drug concentration in the target cells, which may lead to a weak correlation between blood concentration and efficacy[110]. Therefore, a cell pharmacokinetic study is helpful for inhaled drug development; for instance, revealing the way and extent of inhalations to enter the target cells in the lung will be beneficial. Some cell lines (*e.g.*, A549) are often used as a model for cellular pharmacokinetic study of inhalations[111,112]. However, if the cells cannot form the tight junctions, it may reduce their utility in drug transport research[113,114]. Meanwhile, the excessive proliferation and the mucus absence of cells may also affect the results[113]. The appropriate cell lines should be selected according to the experimental purposes, and primary cells may be applied if necessary. In addition, the inhaled drugs in the lung could re-distribute to the systemic circulation, and the permeability of drugs should be also evaluated. Often, the Calu-3 cell line is utilized as a model to examine the permeability of drugs in the lung[115,116]. Moreover, inhaled drugs deposited in the lung could be cleared by alveolar macrophages[117,118], thus resulting in off-target delivery.

In addition, *in vitro* simulation methods are often applied to predict the *in vivo* fate of the inhaled drugs. However, the gap between *in vitro* and *in vivo* studies is still difficult to bridge. An in-depth understanding of the whole lung architectures and a clear illustration of the pulmonary distribution patterns and deposition mechanisms of the inhaled drugs at 3D, in situ, and single-particle levels are important. An attractive method was reported for acquiring high-precision cross-scale visualization of the entire lung anatomy by using the advanced Micro-Optical Sectioning Tomography (MOST) system coupled with whole lung Nissl-

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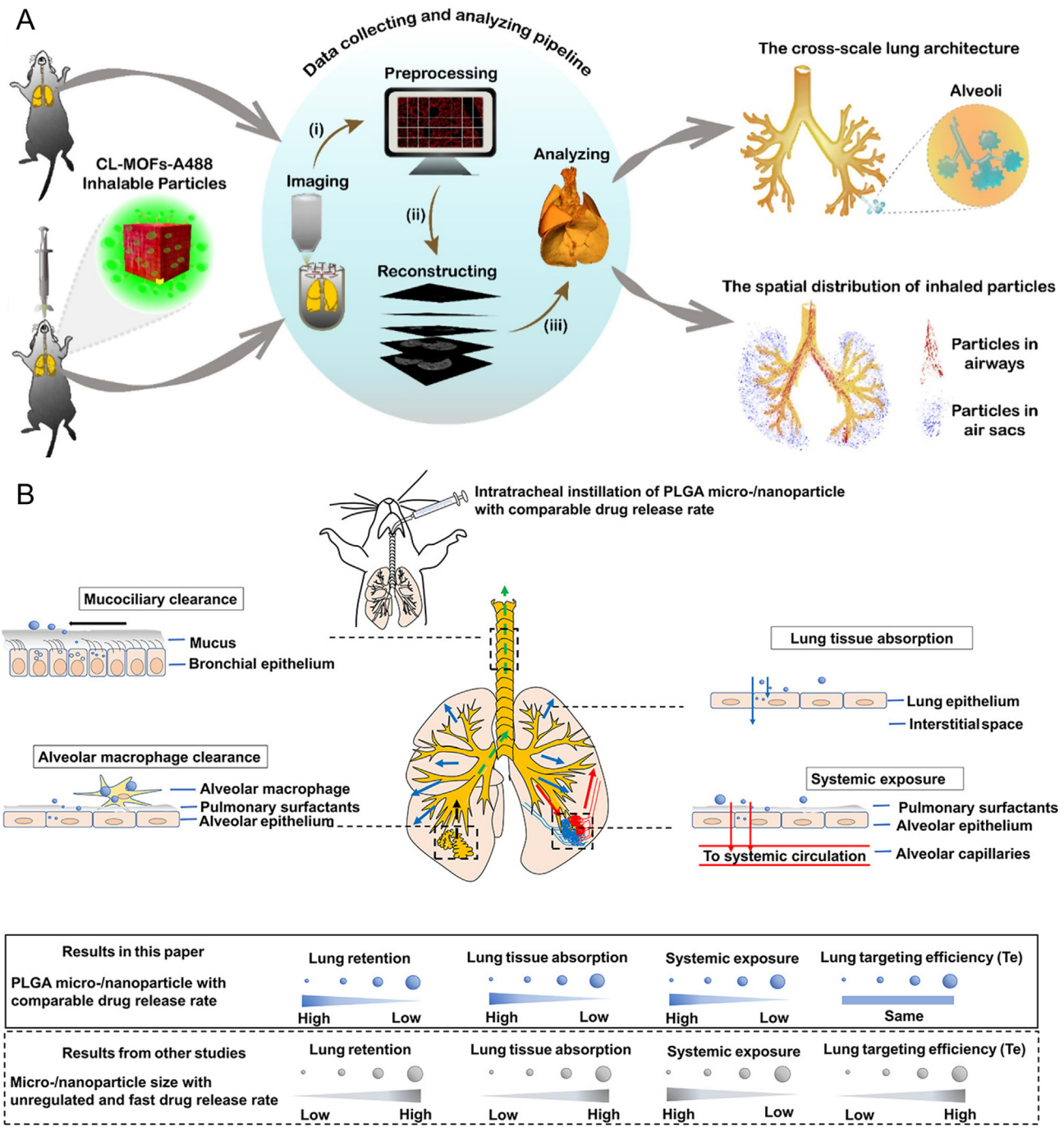

Figure 2 Pharmacokinetic and biodistribution issues of inhalations. (A) Quantitative analysis of the inhaled material deposition in different areas of lung. Reprinted from Ref. 119, CC BY license. Copyright © 2021 The Authors. (B) The influence of particle size itself on the drug fate in the lung after intratracheal administration. Reprinted with permission from Ref. 122. Copyright © 2022 Elsevier.

staining, while the inhaled material was labeled with Alex Fluorescence 488[119]. The cross-scale lung architectures were reconstructed by overlapping about 80,000 coronal images for qualitative analysis of the lung structure (airways, arteries, veins, and alveoli) and quantitative computing of the inhaled material deposition in different areas (Fig. 2A)[119].

Particle size is a critical parameter affecting the pharmacokinetics of inhalations. The wind tunnel studies showed that particles smaller than 5 μm can be inhaled completely and the efficacy of inhalations would decrease with the increasing size of particles[120]. Meanwhile, particles of different sizes would be delivered to different regions in the lung with different mechanisms. For example, particles (5−9 μm, slow inhalation; 3−6 μm fast inhalation) deposited in large airways through impaction, particles (1−5 μm) deposited in smaller airways through gravitational sedimentation, particles (1−3 μm) deposited in respiratory bronchioles through gravitational sedimentation, and particles (≤0.5 μm) deposited in alveoli through Brownian diffusion; detailed information can be found in a review[121]. Moreover, a recent report revealed the influence of particle size on the inhaled

Table 2 The inhaled nanomedicines and antibodies for COVID-19 in the clinical trials (https://clinicaltrials.gov).

Name	Drug types or delivery system	Administration	Clinical stage	Clinical status	NCT number
CSTC-Exo	T cell-derived exosomes	Oral inhalation	Phase 1	Unknown	NCT04389385
MSCs-derived exosomes	Allogenic adipose mesenchymal stem cells	Oral inhalation	Phase 1	Completed	NCT04276987
COVID-19EXO2	Mesenchymal stem cell exosomes	Oral inhalation	Phase 2	Enrolling by invitation	NCT04602442
TLC19	Hydroxychloroquine liposome	Oral inhalation	Phase 1	Completed	NCT04697654
Liposomal lactoferrin	Liposome	Intranasal inhalation	Phase 2/3	Completed	NCT04475120
Remdesivir (GS-5734) and NA-831 (NEUROSIVIR)	Nanoparticle	Oral inhalation	Phase 1	Recruiting	NCT04480333
CT-P63 and CT-P66 combination therapy	Monoclonal antibodies	Oral inhalation	Phase 3	Not yet recruiting	NCT05224856
DZIF-10c	Monoclonal antibody	Oral inhalation	Phase 1/2a	Completed	NCT04631705
STI-2099 (COVI-DROPS™)	Monoclonal antibody	Intranasal inhalation	Phase 2	Completed	NCT04906694
STI-9199	Monoclonal antibody	Intranasal inhalation	Phase 2	Not yet recruiting	NCT05372783
IGM-6268	Immunoglobulin M antibody	Intranasal inhalation	Phase 1	Recruiting	NCT05184218

drug distribution in the lung after intratracheal administration in a mouse model[122]. In that study, the particles with different sizes and similar release rates were used, and the *in vivo* fate of inhaled drugs was vastly different in lung retention, lung tissue absorption, and lung targeting efficiency (Fig. 2B)[122], compared to the previous reports; it suggests that the effect of drug release rate matters.

5. Inhaled administration of nano-formulations for prevention and treatment of COVID-19

Various nanotechnology strategies for the prevention, diagnosis, and treatment of COVID-19 have been widely explored. The inhaled nanomedicines for the prevention and treatment of COVID-19 in the clinical trials were summarized in Table 2. The major roles of nanotechnology-based inhaled delivery in the prevention and treatment of COVID-19 in the preclinical phase are reviewed in this article from five aspects: extracellular vesicles, cell membrane vesicles, liposomal formulations, polymeric nanoparticles, and inorganic nanoparticles.

5.1. Extracellular vesicles for prevention and treatment of COVID-19

Extracellular vesicles (EVs) are heterogeneous membrane structures, which are secreted from almost all human cells. They are categorized into various subgroups including microvesicles, exosomes, and apoptotic bodies. As an important pathway for intercellular communication, EVs can transfer various cargos, such as proteins, lipids, and nucleic acids, to the adjacent or distant cells and exert pathological or physiological effects[123]. EVs can carry both endogenous and exogenous compounds for therapeutic purposes. For example, EVs can be engineered to deliver exogenous proteins or nucleic acids, with the benefits of stability, safety, and biomimetic nature. Exosomes carrying the S protein of SARS-CoV-2 have been investigated as a vaccine candidate[124].

To prevent viral entry into the host cell, the blockage of the ACE2 receptor could be an effective strategy. A study shows that the COVID-19 patient-derived EVs expressing ACE2 can neutralize SARS-CoV-2 by competing with the ACE2-bearing cells[125]. Notably, the neutralization strategy using the EVs displaying ACE2 has been studied extensively[126,127]. The *in vivo* studies were carried out in some reports using intravenous and intraperitoneal administration[126,127]. Of note, as a non-invasive path, the intranasal administration could provide a fast onset of action, which allows a lower dose to achieve a local effect. Our team constructed the engineered HEK293T cells with stable expression of hACE2, from which the EVs bearing hACE2 (termed EVs-ACE2) were obtained[128]. The intranasally inhaled EVs-ACE2 can competitively bind with the *S*-pseudovirus to prevent viral entry into the host cells (Fig. 3C)[128]. The EVs-ACE2 effectively inhibited the cell entry of pseudovirus in various cell lines with ACE2 expression, and the intracellular entry efficiency was correlated with the expression level of ACE2 (Fig. 3A)[128]. The pseudovirus could be successfully captured by the EVs-ACE2 that was intranasally pre-administered to the mice, reflected by the major overlap of the fluorescence of the pseudovirus and EVs-ACE2 (Fig. 3B)[128]. The pseudovirus-infected cells in the nasal epithelium tissues were measured and the positive percentage was significantly reduced in the mice that were treated with the EVs-ACE2 (Fig. 3D)[128], indicating that the intranasal pre-treatment with EVs-ACE2 could block the viruses to enter the nasal epithelium. The intranasal EVs-based nanodecoy with the benefit of self-administration may be an efficient platform for preventing infection for those who expose to or work in a highly contagious environment.

Infection of SARS-CoV-2 could result in hyperinflammation. EVs were also investigated to relieve lung inflammation. It has been reported that the mesenchymal stem cells (MSCs)-derived EVs can mitigate acute lung injury by transferring proteins, lipids, and RNA from MSCs to the injured cells[129]. Meanwhile, MSC-EVs administered intravascularly could relieve pro-inflammatory cytokine secretion and respiratory dysfunction *in vivo*[130], which

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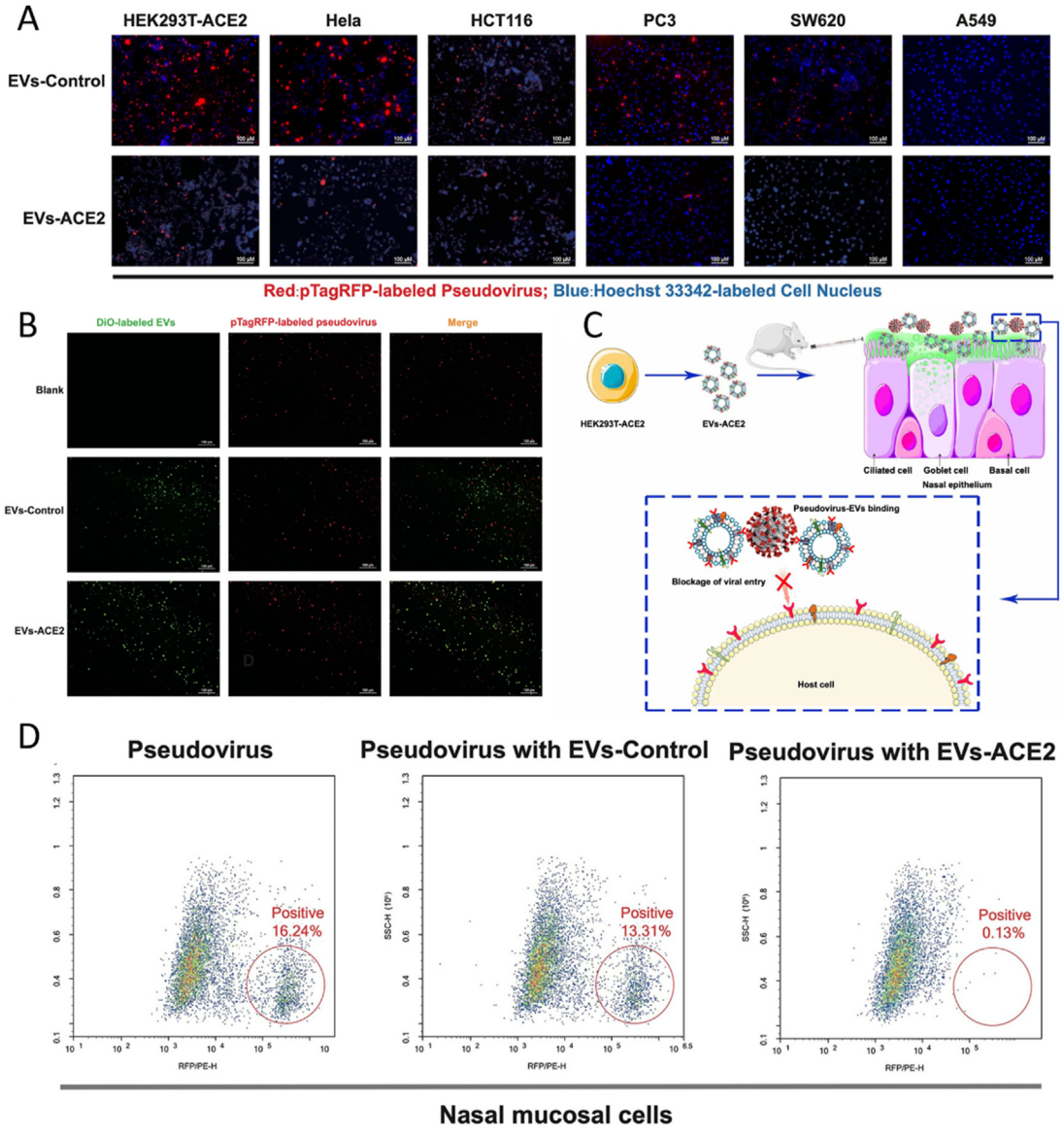

Figure 3 Intranasally administered ACE2-engineered extracellular vesicles neutralize the SARS-CoV-2 pseudovirus. (A) EVs-ACE2 inhibited the cell entry of pseudovirus. (HEK293T-ACE2, HeLa, and PC3 cells with ACE2 expression.) (Scale bar, 100 μm). (B) Fluorescence images of the nasal mucosa cryosection slices from the mice challenged by the S-pseudovirus with the DiO-labeled EVs-ACE2/EVs-Control pretreatment. Scale bar, 100 μm. (C) Schematic illustration of EVs-ACE2 inhibiting SARS-CoV-2 infection. The EVs-ACE2 were derived from the engineered HEK293T cells with stable ACE2 expression. EVs-ACE2 can competitively bind with the viruses, thus blocking the virus to enter the host cells. (D) Flow cytometry assay of the pseudovirus-infected cells in the nasal epithelium tissues. Reprinted from Ref. 128, CC BY-NC-ND 4.0. Copyright © 2022 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences.

indicated the implication of MSC-EVs for relieving the lung inflammation of severe patients infected by SARS-CoV-2. Clinical trials of MSC-EVs for the treatment of COVID-19 have been described by Yan et al[131]. Furthermore, the effectiveness of MSCs exosomes by inhalation was examined in a small sample size of COVID-19 patients, and the results showed nebulization of MSCs exosomes reduced the hospitalization time for COVID-19 patients, without inducing toxic and side effects[132].

5.2. *Cell membrane-modified vesicles for prevention and treatment of COVID-19*

The cell membrane-based systems have great promise as biomimetic carriers[133]. Derived from various cells, the membrane can be extruded into small vesicles or encapsulated on the surface of the synthetic nanoparticles. Various kinds of cell membranes have been used for therapeutic or drug delivery purposes, including red blood cells, platelets, cancer cells, immune cells, and bacterial membranes[134]. In the case of COVID-19, the specific ligands can be displayed on the cell membranes for neutralizing the virus. A cell membrane-based nanosystem for COVID-19 treatment was developed by using the cell membrane of the human lung epithelial type II cells or human macrophages that express ACE2 to coat the poly (lactic-*co*-glycolic acid) (PLGA) nanoparticles[135]. Such a system served as the nanosponges (NS) for neutralizing SARS-CoV-2 and displayed a concentration-dependent manner *in vitro*. In another report, the hACE2-containing nanocatchers (NCs) with a mucoadhesive excipient hyaluronic acid were

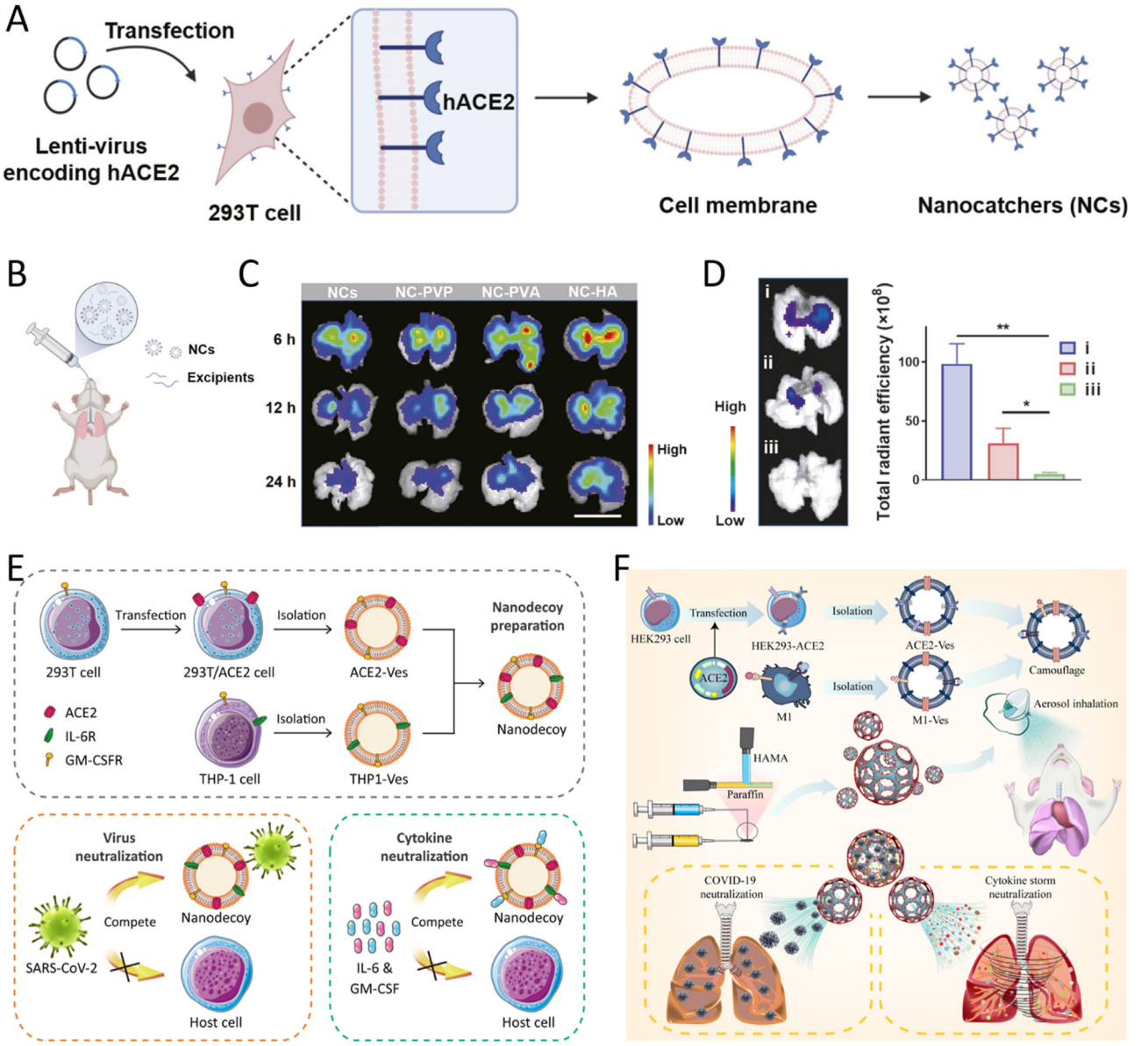

Figure 4 Inhaled cell-membrane vesicles for prevention and treatment of COVID-19. (A−D) Inhalable nanocatchers (NCs) for SARS-CoV-2 inhibition. Reprinted with permission from Ref. 136. Copyright © 2022 National Academy of Science. (A) Schematic showing the preparation process of NCs. (B) Scheme of inhalation of NCs with excipients. (C) Fluorescence IVIS images of lungs reflect the retention degree of different formulations. The mixture of NCs and HA showed the best retention effect (Scale bar, 1 cm). PVP, poly (vinyl pyrrolidone); PVA, poly (vinyl alcohol); HA, hyaluronic acid. (D) Bioluminescence of LUCI from pseudovirus in lungs collected at 48 h post-PBS (blue), NC−sucrose (red), and NC−HA−sucrose (green) inhalation. (E) Preparation of nanodecoys by fusing cell membrane vesicles derived from engineered 293T/ACE2 and THP-1 cells. The nanodecoys could neutralize SARS-CoV-2 and inflammatory cytokines, such as IL-6 and GM-CSF. Reprinted with permission from Ref. 137. Copyright © 2022 National Academy of Science. (F) Schematic illustration of the inhaled ACE2-engineered microfluidic microsphere for neutralization of COVID-19 and alleviation of the cytokine storm. Reprinted with permission from Ref. 138. Copyright © 2022 Elsevier.

developed into an inhalable dry formulation *via* lyophilization for a prolonged retention effect in the lung *via* intratracheal inhalation (Fig. 4A−D)[136]. The nanocatchers were prepared by extruding the cell membranes derived from the engineered 293 T cells with stable hACE2 expression, and the neutralization potency against the pseudoviruses of wild-type SARS-CoV-2 and D614G variants was demonstrated. The *in vivo* investigations showed that the intratracheally administered nanocatchers efficiently inhibited the infection of pseudoviruses.

In addition to blocking the virus infection, the cell membrane-based nanomedicine can also be used to treat COVID-19 complications. Cytokine storm is a potentially severe consequence of COVID-19[137]. To address this problem, the two-step neutralizable cell-membrane-based nanovesicles were designed by Rao et al[138]. Two types of cells were used in this study, *i.e.*, ACE2-engineered 293 T cells and precursor human myeloid mononuclear THP-1 cells. The two types of cell membranes were extracted and fused into a final nanodecoy (Fig. 4E)[138]. The results showed that the

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nanodecoy not only neutralized the pseudovirus and authentic SARS-CoV-2 but also neutralized the inflammatory cytokines interleukin-6 (IL-6) and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the lungs, because of the inherent expression of IL-6 receptor on the THP-1 cell membrane and GM-CSF receptor on both types of cell membranes[138]. With intratracheal administration of the nanodecoy to the mice, the immune disorder and lung injury were effectively improved in an acute lung inflammation mouse model[138]. To further improve the treatment, Wang, et al.[139] developed a microfluidic microsphere-based inhaled aerosol (termed iAE-PMS) in which two kinds of cell membrane nanovesicles (one from the ACE2-expressed HEK293 cells, another from the pro-inflammatory M1 macrophages that expressed inflammatory cytokine receptors) were fused. The hybrid nanovesicles were loaded into the pores of the negatively charged methacrylate hyaluronic acid hydrogel microspheres *via* electrostatic interaction to neutralize the complex immunoregulatory molecules (Fig. 4F)[139]. The mice inhaled the iAE-PMS through a mask of the atomizer and the iAE-PMS was distributed throughout the whole respiratory system, including the nasopharynx, trachea, and alveolus, where the iAE-PMS competitively bound with SARS-CoV-2 *via* ACE2/S protein interaction, thus protecting the body against infection. Meanwhile, the inflammatory cytokines were neutralized *via* binding with the iAE-PMS and the hyperinflammatory state was alleviated.

Apart from treatment, the cell-membrane vesicles can also be applied for vaccination against COVID-19. The outer membrane vesicles (OMVs), naturally released by gram-negative bacteria, are mainly comprised of lipids, lipopolysaccharide (LPS), integral membrane proteins, and lipoproteins[140]. OMVs have been explored as a vaccination platform[141]. For instance, *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC) were genetically engineered to produce the detoxified OMVs displaying a receptor-binding domain (RBD) of the S protein[142]. Intranasal immunization with the RBD-OMVs induced a robust S protein-specific immune response, with a high antibody titer against the S protein[142].

The abovementioned results demonstrate that the cell membrane-based formulations *via* intranasal or orally inhaled administration may be the potential approaches for the prevention and treatment of COVID-19.

5.3. Liposomal formulations for prevention and treatment of COVID-19

In the development of COVID-19 mRNA vaccines, lipid nanoparticles (LNPs) play an essential role in the delivery ability of mRNA. Two LNP vaccines of mRNA have been approved by FDA to prevent the infection of SARS-CoV-2, *e.g.*, Pfizer BioNTech and Moderna[143]. A liposome (LPX)-based mRNA vaccine was developed by Huang et al.[144], which had good immunogenicity with multiple routes of administration including intravenous (i.v.), intramuscular (i.m.), hypodermic (i.h.), intradermal (i.d.), or intraperitoneal (i.p.) injection, which thus provides other options. However, there is no report on inhaled LNP/mRNA COVID-19 vaccine. The low transfection efficacy in the lung seriously hindered the development of inhaled LNP/mRNA vaccine[145]. A recent article illustrates some critical issues of inhaled LNP/mRNA design. Lokugamage et al.[146] reported a cluster-based iterative screening approach for identifying and optimizing different chemical components in lipid nanoparticles for the lung delivery of LNP/mRNA vaccines. In that work, the authors found that the polyethylene glycol (PEG) molarity and the structure and charge of the helper lipid influence the mRNA delivery efficacy in the lung, and showed that LNPs with high PEG density and cationic lipids were more efficient than those with low PEG density because of the PEG steric effect and electronic expelling, which could resist the aggregation of LNPs during nebulization. It was also pointed out that the lung biology issues (*e.g.*, mucus and physiological barriers in the lung) predominates and contributes simultaneously to lung delivery efficiency along with the factors of nebulization[146]. Similarly, Suberi, et al.[147] found that different components and PEG density in the nanoparticles influence the transfection efficacy of the inhaled mRNA vaccine *in vivo* and pointed out the importance of formulation optimization for the inhaled mRNA vaccine delivery.

For blocking the binding between SARS-CoV-2 and ACE2, a liposomal nanotrap platform was designed and functionalized with either recombinant ACE2 protein or neutralizing antibodies of SARS-CoV-2 and phagocytosis-specific phosphatidylserines on the surfaces (Fig. 5A)[148]. The inhaled nanotrap could capture SARS-CoV-2 *via* binding with ACE2 or antibodies and then be cleared by macrophages mediated by phosphatidylserines. Notably, this nanotrap exhibited good safety and inhibited pseudotyped SARS-CoV-2 infection in the dissected human lungs by intratracheal administration.

5.4. Polymeric nanoparticles for prevention and treatment of COVID-19

Compared with the cell membrane vesicles like exosomes, the non-cell-derived nanomaterials can be easier to prepare on a large scale in a controllable and simple manner. The inhaled polymeric nanoparticles developed against COVID-19 can be divided into various applications, including vaccination, blockage of the binding between SARS-CoV-2 and the S protein, and inhibition of SARS-CoV-2 replication.

Inhaled vaccines have the advantages in the prevention of SARS-CoV-2 infections as discussed above. However, biopharmaceutical obstacles and nasal clearing will largely limit the effectiveness of inhaled vaccines, but these limitations could be overcome by adjuvants and delivery systems[149]. Nanoparticle-based vaccines have become an innovative strategy for inhalation delivery of antigens[150]. For instance, an intranasally inhaled nanoparticle vaccine (RBD-TMC NPs) was developed, in which the antigenic spike RBD of SARS-CoV-2 was loaded into the *N*,*N*,*N*-trimethyl chitosan nanoparticles[151]. The RBD-TMC NPs induced a more robust local mucosal immunity, systemic antibody responses, and systemic immune responses compared with the free form of spike RBD antigen[151]. Another work is about the biodegradable poly (amine-*co*-ester) (PACE) polyplexes for an inhalable spike (S) protein mRNA vaccine for SARS-CoV-2[147]. The inhaled PACE nanoparticles of mRNA vaccine could systemically and locally induce immune responses *in vivo*; for example, the draining lymph node germinal center was activated, and S-specific memory B cells, antibody-secreting cells, circulating S-specific $CD8^+$ T cells, and the lung-resident S specific tissue memory $CD8^+$ T cells were induced. The PACE-mRNA vaccination protected K18-hACE2 mice from lethal viral challenges. Notably, PEG density in the PACE nanoparticles influences the inhaled mRNA vaccine efficacy, and the optimal PACE-PEG concentration (10%) for the inhaled

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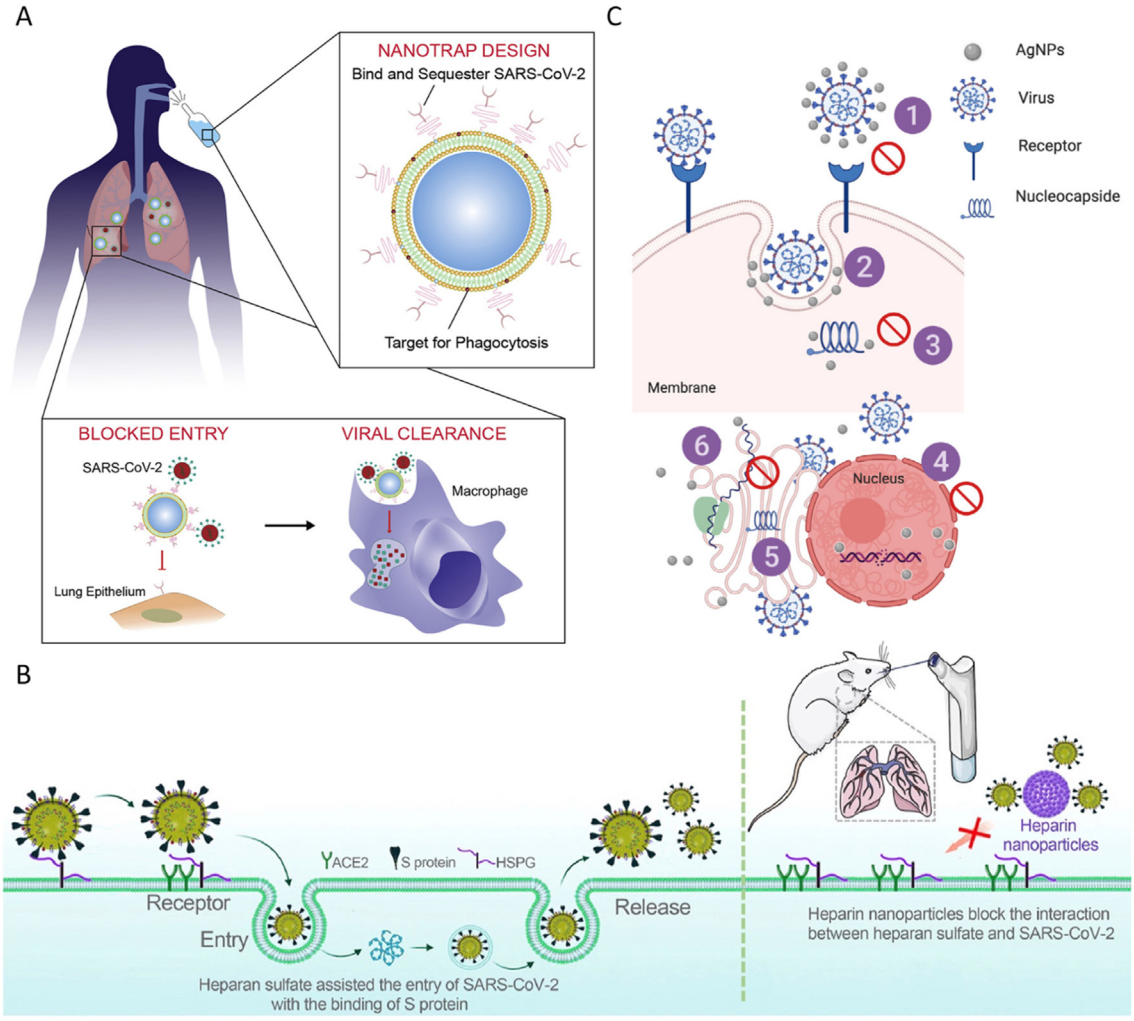

Figure 5 Inhaled nanoparticles for the prevention and treatment of COVID-19. (A) The inhaled nanotrap blocked the infection of SARS-CoV-2. Reprinted with permission from Ref. 148. Copyright © 2022 Elsevier. (B) The inhaled heparin polysaccharide nanodecoy inhibited the infections of SARS-CoV-2 and variants. Reprinted from Ref. 154, CC BY-NC-ND 4.0. Copyright © 2022 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. (C) Mechanism of blocking the infection of SARS-CoV-2 by silver nanoparticles: (1) binding to virus surface, (2) interfering virus attachment, (3) inhibiting virus penetration, (4) binding to viral genome, (5) inhibiting virus genome replication, (6) inhibiting virus protein synthesis. Reprinted with permission from Ref. 161. Copyright © 2021 John Wiley & Sons.

mRNA vaccine delivery achieved high transfection targeted to the lung[147].

In addition, blocking the binding between the S protein of SARS-CoV-2 and heparan sulfate on the cell surface is another promising strategy for interfering interaction between the S and ACE2 protein[33]. Heparin is an analog of heparan sulfate and the most commonly used anticoagulant drug, which may mimic heparan sulfate to interact with the S protein and inhibit the cell entry of SARS-CoV-2. A recent research exhibited that heparin could block SARS-CoV-2 infection by allosterically hindering S protein to bind with the host cell receptor, directly competing with heparan sulfate proteoglycan co-receptors to bind with the S protein, and preventing the S protein from cleavage by furin[152]. The *in vitro* data also revealed that heparin could inhibit the cell entry of SARS-CoV-2 and infection[33]. Meanwhile, it has been reported that heparin could be modified on the cell membrane and suppress the cell entry of SARS-CoV-2[153]. While these studies indicate that heparin has great potential against SARS-CoV-2 infection, there are only heparin injections clinically available. Pulmonary heparin delivery would be superior for the early treatment of COVID-19, because of its self-administrability and reduced systemic exposure and bleeding risk. Our group developed an inhaled heparin nanodecoy to competitively bind the S protein, thus inhibiting the interaction between the virus and the cell surface heparan sulfate (Fig. 5B)[154]. This nanodecoy significantly inhibited the infection of pseudovirus of SARS-CoV-2 and variants by intratracheal administration, and the neutralized pseudovirus as a complex with heparin nanodecoy was quickly phagocytosed and cleared by the macrophages in the lung[154].

Furthermore, small-molecule drugs with anti-SARS-CoV-2 effect can be loaded into the inhaled nanoparticles for improving targeting delivery and reducing side effects. Nanostructure aggregates and liposomes have been applied for preparing the inhaled nanomedicines of Remdesivir (an inhibitor of RNA polymerase)[155,156].

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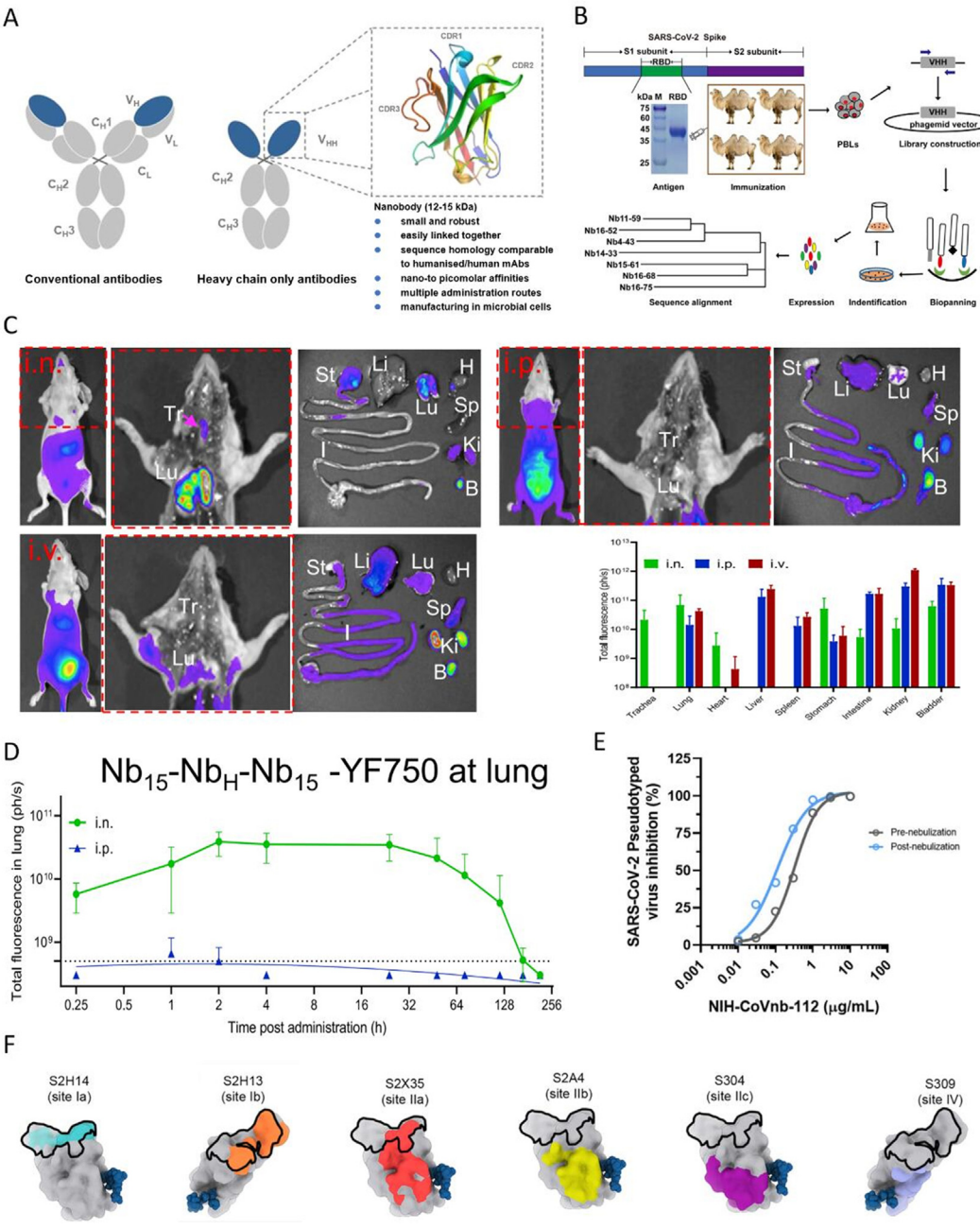

Figure 6 Inhaled nanobodies for prevention and treatment of COVID-19. (A) The model of conventional antibody and nanobody. Reprinted with permission from Ref. 173. Copyright © 2022 Elsevier. (B) The preparation of the Nb11-59 nanobody. Reprinted with permission from Ref. 179. Copyright © 2021 John Wiley & Sons. (C) Spatial distribution of Nb15−NbH−Nb15YF750 after 1 h of intraperitoneal (i.p.), intravascular (i.v.), or intranasal (i.n.) administration, and the fluorescence intensity summary of important organs, $n = 3$. Reprinted with permission from Ref. 97. Copyright © 2022 Elsevier. (D) The fluorescence intensity at the lung location 4 h later. Reprinted with permission from Ref. 97. Copyright © 2022 Elsevier. (E) Nanobodies after nebulization exhibited an enhanced inhibition effect of SARS-CoV-2 *in vitro*. Reprinted with permission from Ref. 181. Copyright © 2022 Springer Nature. (F) The black box represents the binding site of RBM with ACE2, the blue sphere represents the glycan at position N343, and the other six colors represent the binding site of RBD with antibodies, respectively. Reprinted with permission from Ref. 183. Copyright © 2020 Elsevier.

5.5. *Inorganic nanoparticles for prevention and treatment of COVID-19*

Inorganic nanoparticles (*e.g.*, silver and gold nanoparticles) have been explored for the prevention and treatment of COVID-19. Silver nanoparticles have been widely applied in air, water, and surface disinfection because of their broad spectrum of anti-bacterial, anti-fungal, and anti-viral activity[157]. Interestingly, the dynamics of silver nanoparticles by inhalation showed that only a small percentage of silver nanoparticles reached the lungs and most of them are more likely to remain in the upper respiratory tract[158]. Inhaled silver nanoparticles have been investigated *in vivo* to inhibit the infection of viruses, *e.g.*, H3N2 influenza virus[159] and respiratory syncytial virus[160]. The application of silver

nanoparticles against SARS-CoV-2 infection is potential through various anti-virus mechanisms (Fig. 5C)[161]. Inhaled silver nanoparticles for the treatment of COVID-19 were revealed to be feasible by a computation method[162], but the practicability of inhalation of silver nanoparticles for inhibiting SARS-CoV-2 infection needs to be further explored *in vivo*. Another study reported an ultrathin two-dimensional $CuInP_2S_6$ (CIPS) nanosheet could selectively bind with the S protein of wild-type SARS-CoV-2 and its variants (*e.g.*, Delta and Omicron) against infection in the human ACE2-transgenic mice *via* intranasal instillation[163].

As for gold nanoparticles, an inhaled COVID-19 DNA vaccine carried by the chitosan-modified gold nano-star, triggered a strong immune response and induced the formation of memory T cells with good biosafety in the lung[164]. In addition, gold nanoparticles have been studied to enable rapid point-of-care diagnosis and infection monitoring of SARS-CoV-2[165,166]. However, due to the non-degradability, the safety concern of inorganic nanoparticles still is a formidable barrier against clinical translation, and further strict safety evaluation using a standard protocol as drug development must be carried out.

6. Inhaled antibodies and nanobodies for prevention and treatment of COVID-19

Antibody therapy (*e.g.*, bamlanivimab/etesevimab and BRII-196/198) has been approved for clinical use to treat COVID-19, and monoclonal antibody-based treatments have shown therapeutic effects in patients with mild symptoms of COVID-19[167]. However, monoclonal antibodies require very large doses (usually a few grams) for intravenous injection[167]. Moreover, the concentrations of the i.v.-injected antibodies in the lung are hundreds of times lower than in the serum[168]. Inhaled antibodies can partially overcome this problem. Furthermore, multivalent antibodies have shown enhanced potent efficacy in the treatment of COVID-19. Data from Wang et al.[169] exhibited that the anti-SARS-CoV-2 efficacy of IgA monomers was two-fold less than that of IgG, but the anti-SARS-CoV-2 efficacy of IgA dimers was 15 times that of monomers IgA. IgM, another kind of mucosal antibody, is pentamers and has huge potential for the treatment of COVID-19. A study has shown that inhaled IgM antibodies have long-term retention in the nasal cavity and lung, and thus could protect and treat the infection of SARS-CoV-2[170]. Meanwhile, the IgM antibodies also showed the inhibition of SARS-CoV-2 variants *in vitro*[170].

The production of monoclonal antibodies is both time-consuming and pricy. To reduce the cost of the antibody preparation, egg yolk immunoglobulin (IgY) which is the major serum antibody in avians and a counterpart to mammalian IgG, was developed for the neutralization of SARS-CoV-2 and variants[171]. The IgY is thermostable and can be stored at 25 °C for three months. Meanwhile, nasal delivery of the IgY exhibited significant inhibitions of the infection of SARS-CoV-2 in the trachea and lung. Furthermore, the animals infected by SARS-CoV-2 treated with IgY could reduce the risk of cross infections between animals and humans at a low cost. The inhaled antibodies for the prevention and treatment of COVID-19 in the clinical trials are summarized in Table 2.

Nanobodies are a practical alternative to overcome the difficulties of antibody application in the treatment of COVID-19. Conventional antibodies produced by mammals have a heterotetrameric structure and are normally composed of two heavy chains and two light chains. Camels (*e.g.*, alpacas, llamas, and dromedaries) and sharks can produce a heavy chain-only antibody[172], from which nanobody is derived as a single variable domain (VHH) (Fig. 6A)[173]. Although the lack of the variable domain of the light chain might be unfavorable in terms of antigen binding, nanobodies have various advantages such as small size, good stability, cost-effective production, high specificity, low immunogenicity, and identification of variable epitopes[174]. Notably, due to the much smaller size than the full-length antibodies, nanobodies have the advantage of better tissue penetration and extravasation[175].

Nanobodies have shown great potential for COVID-19 treatment. The S protein, especially the RBD, is the main target for developing therapeutic nanobodies against SARS-CoV-2. Three strategies have been established by distinguishing the interactions between nanobodies and viral S proteins: receptor binding site, non-receptor binding site, and the overlapping cahoots. For receptor binding sites, nanobodies competitively bind to ACE2 receptor-associated epitopes on the RBD, which results in the abrogation of viral entry[176]. Non-receptor binding site means nanobodies interact with a non-RBD part of the S protein, which can also hinder the interaction between ACE2 and the S protein by distorting the conformational freedom of the S protein[177]. For the overlapping cahoots, nanobodies can interact with both the RBD and other parts of the S protein[178].

The biophysical properties of nanobodies, *e.g.*, good stability and small size, could benefit inhalation administration[173]. Inhaled nanobodies have been explored for COVID-19 treatment. For instance, an inhaled nanobody, Nb11-59, was developed (Fig. 6B)[179], with a strong neutralization activity against SARS-CoV-2 by binding the RBD of the S protein (IC_{50} 0.55 μg/mL)[179]. Meanwhile, Nb11-59 showed good stability after nebulization and could be produced on a large scale in Pichia pastoris. In another report, the *in vivo* efficacy of nanobodies (PiN-21 nanobodies) for the inhibition of the SARS-CoV-2 infection was evaluated[180]. The intranasally inhaled PiN-21 nanobodies effectively achieved lung targeting delivery and exhibited high therapeutic efficacy against SARS-CoV-2 infection in *Syrian hamsters*. Importantly, intranasal inhalation of nanobodies can reach the lungs directly and retain a longer time compared to intraperitoneal (i.p.) or intravenous (i.v.) routes (Fig. 6C and D)[97]. The nanobodies after nebulization can retain a high inhibition efficiency against SARS-CoV-2 (Fig. 6E)[181].

Frequent mutations of SARS-CoV-2 have substantially reduced the prevention effect of the currently used vaccines, and elicited antibodies with the high neutralizing activity to the wild-type SARS-CoV-2 showed a sharply decreased efficacy against the mutated strain of Omicron, thus often resulting in vaccine breakthrough infection[182]. It is of great significance to develop antibodies with broadly neutralizing ability. It was reported that there are six different binding sites (Ia, Ib, IIa, IIb, IIc, and IV) on the RBD of the S protein that can interact with antibodies, identified by using cryo-electron microscopy (Fig. 6F)[183]. Both Ia and Ib sites overlap with the receptor-binding motif (RBM) with which ACE2 interacts. Neutralizing antibodies that bind to the Ia and Ib sites can prevent the S protein from entering the host cells by competing with ACE2. IIa, IIb, and IIc, are cryptic sites of the RBDs and could bind with the neutralizing antibodies with open conformations of two or three RBDs. Neutralizing antibodies that bind to the IIa, IIb, and IIc sites do not compete with ACE2, but can create steric hindrance against the virus access to ACE2 and thus disabling the RBD binding with ACE2. Site IV, far from the

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core RBM of RBD, is structurally conserved and could be a target for antibody-drug development, and its affinity to a specific antibody is not affected by the RBD conformation. Antibodies bound to site IV do not compete with ACE2 too, but notably, could induce antibody-dependent cell-mediated cytotoxicity (ADCC). Therefore, the neutralizing antibody to site IV can yield a complementarity with the neutralizing antibody that binds to sites Ia and Ib as a combination therapy for neutralizing SARS-CoV-2 and mutant strains. So far, the ambivirumab (P2C−1F11, BRII-196), binding site Ia[184], and romisevirumab (P2B-1G5, BRII-198), binding site IV[185], have been included in the official guidance of COVID-19 treatment in China.

By searching the conserved sequences of the S proteins between the wild-type and mutant strains and through sequence comparison, it was found that the RBD mutation of Omicron mainly occurred in the RBM region, but the cryptic epitopes hidden or partially hidden inside the trimeric interface and the lateral surface epitopes outside the trimeric interface are relatively conservative, compared with the wild-type S protein[186]. Based on that, two humanized nanobodies (n3113v and n3130v) have been developed with the two conserved regions above, respectively, and then a small bispecific humanized nanobody (bn03) conjugate (MW 27 kD) was obtained by connecting n3113v and n3130v with a flexible polypeptide linker composed of glycine and serine[186]. The developed bn03 can bind and neutralize SARS-CoV-2 and the major mutants *in vitro* and showed a significant neutralizing ability against SARS-CoV-2 *in vivo* by pulmonary administration. Moreover, inhaled bn03 had a higher pulmonary concentration and longer retention than that by intravenous administration.

7. Perspectives

The world has still been struggling with the ongoing pandemic of COVID-19. This global crisis indicates that the development of vaccines and drugs for a quick response to an emerging virus outbreaks is essential for society. The inhaled nanotechnology is a promising measure to combat SARS-CoV-2 infection. On one hand, intranasal or orally inhaled delivery provides the benefit of specific drug distribution to the targeted site (*e.g.*, nasal epithelium and lung) while minimizing systemic exposure; moreover, the easy-handling and self-administrable features offer great convenience for personal care. On the other hand, nanotechnology can improve drug solubility, stability, and drug bioavailability.

Innovative inhaled medicines for lung diseases have been developed and there are a range of opportunities for new inhaled drugs[187]. Inhalation can be a useful intervention to prevent the spread of COVID-19. It should be noted that inhaled medicine relies on the technical support of inhalation devices, *e.g.*, pressurized metered-dose inhalers (pMDIs), dry powder inhalers (DPIs), and nebulizers for inhalation. In addition, the potential safety concern of inhaled nanoparticles should be addressed, too. The inhaled nanoparticles enter the alveolar cells and lung-resident immune cells and possibly induce lung toxicity including the generation of oxidative stress, DNA damage, and inflammation[188].

The intranasal application provides another promising method for the prevention of viral infection. The applied drugs retained in the airway can neutralize the entering virus. The increased viral titer of Omicron in the upper respiratory makes it easy for Omicron to spread from infected patients. The intranasal application could be a promising route for preventative management. The studies have shown that the binding capacity between the S protein and the ACE2 is increased from the wide-type SARS-CoV-2 to Delta and Omicron variants[189,190]. Therefore, the ACE2 protein can potentially inhibit the spread of SARS-CoV-2, especially variants.

It should be noted that variants of SARS-CoV-2 can cause a change in COVID-19 symptoms. Rates of infections and replications of Omicron are much faster than that of original SARS-CoV-2 and Delta variants in human bronchus tissues, while Omicron infects and replicates in lung tissues much slower than Delta and the original strain do[191]. As a result, Omicron typically affects the upper respiratory tract but rarely develops into severe symptoms. Compared to the wide-type SARS-CoV-2 and Delta variants, Omicron is milder and less pathogenic. Patients with Omicron infections resulted in a lower rate of hospitalization (1.9% for Omicron *v.s.* 2.6% for Delta), but an increased rate of upper respiratory tract symptoms (*e.g.*, sore throat and hoarseness)[192]. Therefore, due to the change in Omicron-related pathology, the therapeutic strategy and drug delivery should also be adjusted, *i.e.*, for targeting the upper respiratory tract by using a proper formulation and administration route. Particles with different diameters can deposit in different regions of the respiratory system through oral inhalation[193]. Therefore, fine-tuning the physical properties of inhaled nanodrugs could be a potential method for better improving the prevention and treatment of Omicron-related infection.

The approved vaccines have shown reduced effectiveness against the variants of SARS-CoV-2, especially Omicron. In addition to developing a new vaccine targeting the Omicron variant, improvement of the current vaccination efficacy could also be an alternative method. Considering the infection route of SRAS-CoV-2, mucosal immunity plays an essential role in vaccination protection. Yet, conventional intramuscular immunization is not an ideal way to elicit mucosal immunity (*e.g.*, IgA secretion). In this sense, developing an inhaled vaccine could be a promising vaccination method for enhancing immunological protection against SRAS-CoV-2, of which there would be several advantages. First, self-administrable vaccination can relieve the burden on the healthcare system during the pandemic. Second, robust mucosal immunity can provide specific protection in the respiratory tract. Third, an inhaled vaccine can serve as a part of "hybrid immunization" along with other conventional i.m. injected vaccines. It has been reported that a hybrid immunization (*e.g.*, i.m. + inhaled administration) can induce higher neutralizing antibody responses than a single route of immunization[11].

Additionally, the development of nanomaterials for reducing virus exhalation from patients will play an important role in reducing virus transmission. For example, masks based on nanomaterials can almost completely capture the aerosols containing SARS-CoV-2[194]. Such a design can also reduce the risk of virus infection for susceptible populations.

8. Conclusions

In this review, we focus on the role of orally inhaled and intranasal nanoformulations in the prevention and treatment of COVID-19. There is still a lack of understanding of the *in vivo* fate of orally inhaled or intranasal nanomedicines. To address the complex challenges of applying orally inhaled and intranasal nanoformulations to combat COVID-19, it requires collaboration among researchers from different disciplines including

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pharmaceutical scientists, physicians, and mechanical engineering. The development of formulations in this field will not only combat the current pandemic but also promote better preparation for the future.

Acknowledgments

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Author contributions

Bin Tu and Yanrong Gao wrote the draft. Xinran An and Huiyuan Wang edited the manuscript. Yongzhuo Huang finalized the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts to declare.

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