Asian Journal of Pharmaceutical Sciences 18 (2023) 100855

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Review Article

Leveraging immunoliposomes as nanocarriers against SARS-CoV-2 and its emerging variants

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ARTICLE INFO

Article history:
Received 3 June 2023
Revised 20 August 2023
Accepted 21 August 2023
Available online 20 October 2023

Keywords:
Coronavirus
COVID-19
SARS-CoV-2
Liposomes
Immunoliposomes

ABSTRACT

The global COVID-19 pandemic arising from SARS-CoV-2 has impacted many lives, gaining interest worldwide ever since it was first identified in December 2019. Till 2023, 752 million cumulative cases and 6.8 million deaths were documented globally. COVID-19 has been rapidly evolving, affecting virus transmissibility and properties and contributing to increased disease severity. The Omicron is the most circulating variant of concern. Although success in its treatment has indicated progress in tackling the virus, limitations in delivering the current antiviral agents in battling emerging variants remain remarkable. With the latest advancements in nanotechnology for controlling infectious diseases, liposomes have the potential to counteract SARS-CoV-2 because of their ability to employ different targeting strategies, incorporating monoclonal antibodies for the active and passive targeting of infected patients. This review will present a concise summary of the possible strategies for utilizing immunoliposomes to improve current treatment against the occurrence of SARS-CoV-2 and its variants.

1. Emergence, epidemiology, and progression of SARS-CoV-2 and its variants

The occurrence of the novel coronavirus disease (COVID-19) pandemic from the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has contributed to 752 million cases and 6.8 million deaths globally. Starting from being announced as a global pandemic by the World Health Organization, although massive efforts on the production of therapeutics and prophylactics such as vaccines and antiviral agents have been taken to eradicate the virus

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Peer review under responsibility of Shenyang Pharmaceutical University.

https://doi.org/10.1016/j.ajps.2023.100855

transmissibility, the number of active cases remain escalating.

Similar to the other viruses, COVID-19 mutates and infects new geographical areas. The effectiveness of current treatment strategies and vaccination programs is hindered by the appearance of mutations in SARS-CoV-2, resulting in multiple huge waves of COVID-19. These mutations aid in better accommodation of the virus within hosts and locations. The evolution of COVID-19 not only increases transmissibility, but also contributes to the severity of the infection. New variants of SARS-CoV-2 became more prevalent, leading to a significant surge in the number of COVID-19 cases in numerous countries. The classification of the SARS-CoV-2 variants has evolved with time. Previously, the World Health Organization has identified SARS-CoV-2 variants of concerns (VOC) as Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529). As of March 2023, the only announced VOC was the most mutated and highly transmissible Omicron variant [1–4].

Unfortunately, these VOCs have shown resistance to existing monoclonal antibody (mAb) therapies that are currently available *in vitro* and may show less efficacy of treatment in clinical settings. The possibility of SARS-CoV-2 to acquire mutations is high, allowing it to evade mAbs, resulting in adverse prognosis and reducing the effectiveness as well as durability of mAbs in controlling the current global pandemic crisis.

With the plethora of advancements in drug delivery technologies in the last decade, the use of a targeted delivery system may be explored to improve current treatment strategies for SARS-CoV-2 and its evolving mutations. Liposomes, which are renowned for their versatility and flexibility in incorporating therapeutic biomolecules either by encapsulation or conjugation to their surface, are the most suitable candidates for this purpose. Liposomes with antibodies conjugated on their surface are called immunoliposomes and have been successful in the targeted, active delivery of drugs and therapeutics in many studies over the years.

In this context, this review integrates a thorough perspective and discussion on the appearance and epidemiology of SARS-CoV-2 and its variants and focuses on the characteristics of immunoliposomes as a possible treatment strategy for COVID-19. In addition, this review provides an elaborate overview of functionalization and conjugation strategies in the development of immunoliposomes and discusses the possible targeting strategies of immunoliposomes in tackling SARS-CoV-2 disease progression.

1.1. *Biological mechanism of infections by coronaviruses (CoVs)*

To design a carrier for targeted vaccines and therapy, the pathogenesis of CoV, particularly COVID-19 must be considered. COVID-19 shared 79.6 % genomic sequence similarity with SARS-CoV. COVID-19 viral genome classification was defined by sequence similarity to two conserved members of the genus *Betacoronavirus*: a human-related Cov and a bat-related Cov. In accordance with this sequence alignment and open reading frame (ORF) projection, untranslated regions and ORF of COVID-19 were assigned.

The replicase ORF1ab gene of COVID-19 consists of 16 non-structural proteins and 13 downstream ORFs. The COVID-19 genes in spike (S), ORF3a, envelope (E), membrane (M) and nucleocapsid (N) are 3822 nt, 828 nt, 228 nt, 669 nt and 1260 nt, respectively [5]. On top of these ORF regions that are similar with members of the subgenus *Sarbecovirus*, COVID-19 shares similarity with SARS-CoV as it bears a projected ORF8 gene (366 nt in length) [5]. The role of each COVID-19 ORF was hypothesized centered by those established Covs.

The gene order (5′ to 3′) in COVID-19 was organized as follows: ORF1ab replicase, S, E, M and N, with nearly 16 non-structural proteins and a maximum of eight accessory proteins. The S protein is the main structure of focus, as it determines the virus access into the infected host cells. It is a large glycoprotein that weighs ~180 kDa, is present as a prominent trimer on the surface of the virus and consists of S1 and S2 subunit [6]. S1 is known by its two distinctive domains, the receptor-binding domain (RBD) and the N-terminal domain. The RBD of S1 subunit regulates binding of receptor and is activated by the N-terminal S1 subunit, whereas virus-cell membrane fusion is driven by the C-terminal (also known as the C-domain) S2 subunit [5,7–9].

SARS-CoV-2 directly interact with the host cell by binding to angiotensin-converting enzyme 2 (ACE2) (*e.g.*, pneumocytes) on host cells. The mechanism of viral entry occurs when CoV enters the host cell during infection by the binding between its cell membrane receptor and S1-RBD, and a spike protein sheds its S1 subunit after it is connected to the ACE2 receptor. The remaining S2 component changes its shape or “conformation”, which enables the fusion of outer membrane with viral envelope initiating the conformational changes in the S2 subunit and enabling the entry into the target cell [10,11].

2. Current medications and drugs for treatment of COVID-19

2.1. *Broad-spectrum antiviral against COVID-19*

A myriad of antiviral medications has been proposed as SARS-CoV-2 infection treatment options; however, many of these interventions have yet to improve the disease progression or are inconveniently expensive and technically infeasible as broad treatment options [12]. The precise course of treatment depends on the stage of the illness and the condition of the patient. The emergency use of Paxlovid, that includes nirmatrelvir, a protease inhibitor of SAR-CoV-2, and ritonavir, a HIV-1 protease inhibitor and CYP3A inhibitor, to treat adult and pediatric patients has been approved and announced by the U.S. Food and Drug Administration (FDA) [13]. To assess the efficacy of Paxlovid in minimizing disease progression of COVID-19 and death, a large retrospective cohort study of high-risk infected patients was conducted in Israel between January and February 2022 [14].

In vaccinated and non-vaccinated patients with COVID-19, treatment with Paxlovid for the first five days of infection has shown a significantly reduced risk of disease progression

Table 1 – List of mAbs available with their effectiveness against Omicron variant of SARS-CoV-2.

Generic name	Status (FDA)	Effectiveness against Omicron variants	Ref
Bebtelovimab	Not currently authorized in the U.S.	• Effective against Omicron BA.5 • Not effective against Omicron BQ.1 and BQ.1.1.	[18]
Sotrovimab	Not currently authorized in the U.S.	• Not effective against BA.2.12.1, BA.4, or BA.5	[3]
Bamlanivimab with etesevimab	Authorized for use but limited to susceptible strains	• Not effective against Omicron BA.1 and BA.2.	[19]
Casirivimab with imdevimab	Authorized for use but limited to susceptible strains	• Not effective against Omicron BA.1 and BA.2.	[1]

from developing to a severe state and death. This study, which reported that Omicron was the prevalent variety at the time it was performed in Israel, demonstrated the strong efficiency of Paxlovid against infection with the variant. However, the concurrent use of Paxlovid together with other medications may cause possibly harmful drug interactions owing to the presence of ritonavir [12]. For example, rifampin is contraindicated in Paxlovid. Nirmatrelvir and ritonavir concentrations may decrease because of the delayed offset of enzyme induction, making these medications ineffective against SARS-CoV-2.

Remdesivir is an antiviral, repurposed drug used as one of the therapeutic options for hospitalized COVID-19 patients. It functions as a prodrug of an adenosine nucleotide analog when inhibiting viral replication [15]. Piccicacco et al. [4] reported the efficiency of remdesivir treatment in patients with COVID-19 that require hospitalizations during the Omicron surge. According to this reported study, early treatment with remdesivir or sotrovimab decreased the likelihood of hospitalization in outpatients with severe COVID-19 and numerous risk factors. In addition, this study supports the clinical SARS-CoV-2 variants of mAbs, such as sotrovimab, as a therapy against SARS-CoV-2 infection [4].

However, various monoclonal antibodies are licensed under the EUA for the treatment of COVID-19, adding to sotrovimab (Table 1). Patients with severe COVID-19 may benefit from mAbs, which are crucial for high-risk patients for whom immunization is not an option. However, due to the ability of SARS-CoV-2 to mutate by changing its amino acid in non-epitope sites, epitope alterations and combinations of mutations aid in the escape of the neutralization action of mAbs, promoting mAb evasion [16]. Despite their effectiveness in combating strains before Omicron infection, most therapeutic monoclonal antibodies available for SARS-CoV-2 are ineffective against Omicron variants [2]. Therefore, continuous monitoring of mAb resistance is necessary to improve treatment efficacy [17].

3. Immunoliposomes

3.1. *Immunoliposomes as nanocarriers in eradicating viral infections*

Over the last few decades, nanocarriers have been explored for drug and vaccine development to achieve targeted delivery to infection sites. For instance, the applications of immunoliposomes as nanocarriers have been reported in eradicating viral infections like the human immunodeficiency virus (HIV), as well as the SARS-CoV-2 [20,21]. However, some nanocarriers have certain limitations, such as disruption of payload release before reaching the intended locations, inadequate capacity for loading, inefficient biodistribution and cytotoxicity [22].

Liposomes are one choice of the nanocarriers, because of their ideal ability to entrap both hydrophilic and hydrophobic therapeutics, thus can be loaded with a myriad of therapeutic moieties [23]. Several liposomal formulations have been developed by the entrapment of therapeutics in the core of liposomes as well as between their bilayers, especially for the management of COVID-19 [24]. For example, liposomes loaded with efavirenz and mefloquine, as well as small interfering RNAs (siRNAs), have been developed as therapeutics for HIV and CoV infections [25,26]. Conventional liposomes consist of phospholipid bilayers, which are typically comprised of phospholipids and other additional composition such as cholesterol. Liposomes may be further manipulated to achieve desired properties by selecting the lipid composition, which may consist of many types of lipids. The use of a variety of lipids in liposomes, such as 1, 2-dipalmitoyl-sn–glycero-3-phosphocholine, 1,2-dioleoyl-sn–glycero-3-phosphocholine, and phosphatidylcholine, has been revealed in several studies [27,28]. Interesting examples of liposome preparation methods are thin film hydration, freezing, thawing, and the reverse phase, which leads to the transformation of organic solvents into liposomes [29,30].

When administered intravenously, opsonin, a serum protein that recognizes foreign material, will coat liposomes to be presented to the phagocytic cells and later being removed from the bloodstream. Liposomes are eventually engulfed by phagocytes, which is a part of the reticuloendothelial system, and therefore have short circulation times. To prolong the distribution time of liposomes in the bloodstream, the use of a hydrophilic polymer, polyethylene glycol (PEG), can be utilized by coating it onto the surface of liposomes. This modification in liposomes aids to increase the repulsive forces between the liposomes and serum, leading to the development of stealth liposomes [29,30]. Liposomes' surfaces can be modified with enhanced drug-release properties, site-specific targeting, and stealth properties (passive targeting) to avoid identification by the mononuclear phagocyte system, a class of cells that plays a significant role in the immune system [31]. A vast strategy of conjugating antibodies, peptides/proteins, and carbohydrates could also be tailored for targeted drug delivery to designated cells or organs *in vivo* (Fig. 1).

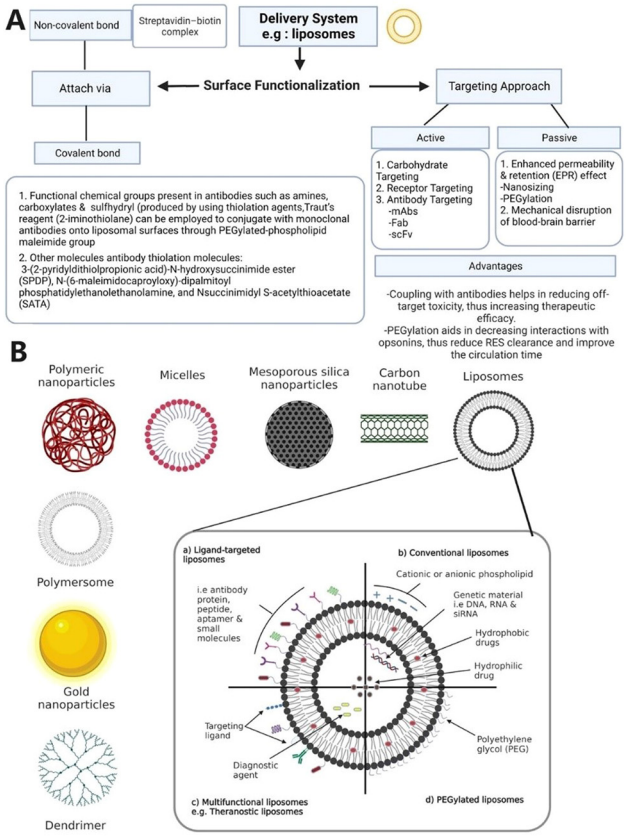

Fig. 1 – (A) Conjugation of monoclonal antibodies or their fragments to liposomes. (B) Nanocarriers with a focus on liposomes as a smart delivery system. a) Liposomes with various targeting strategies b) Standard liposomes where the phospholipids can be changed to obtain different charges. c) Multifunctional liposomes. d) PEGylated liposomes are formed by incorporating PEG to provide stealth properties, subsequently improving the circulation time. (Created with BioRender.com).

Recent advancements in the design of liposomal formulation have been reported to improve localization of the delivery of therapeutics agents, by coupling or conjugating antibodies to the surface of liposomes, creating immunoliposomes. Immunoliposomes have great potential for targeted delivery of drugs or other biomolecules for the treatment of infections, particularly viral infections. The use of immunoliposomes as delivery systems for viral infection was reported in [32], where a single-chain Fv antibody against the H5N1 avian influenza virus was conjugated to the surface of liposomes with cationic property to further enhance the delivery of siRNA to suppress viral infection. In their study, the delivery of siRNA immunoliposomes resulted in a 10-fold reduction in viral titers compared with normal liposomes, proving the efficiency of targeted immunoliposomes as a delivery system for tackling viral infections. Other studies reporting the development of immunoliposomes for viral infections have also been conducted. For example, melittin-loaded immunoliposomes for the treatment of haemorrhagic septicaemia rhabdovirus (VHSV) and si-RNA loaded immunoliposomes for HIV [33–35].

Immunoliposomes can be developed by chemically conjugating antibodies or their fragments onto the liposomal surface through interactions between reactive groups on the surface of the liposomes and different groups present in the ligand, resulting in high specificity for their target antigens [36,37]. Modifications of diverse forms of block-copolymers and lipids can be carried prior to the incorporation of functional groups, *e.g.*, in the commercially available formulation of modified dibenzocyclooctyne (DBCO) liposome (Immunosome®-DBCO), functional DBCO groups are introduced directly through the phospholipid before the liposomes self-assembled.

4. Addressing disease progression of SARS-CoV-2 variants as primary targeting possibilities for immunoliposomes

4.1. *Cytokine storm release (CRS), acute respiratory distress syndrome (ARDS) and acute lung injury (ALI)*

Inflammatory cytokines are normally produced by the innate immune system of macrophages, endothelial cells, and epithelial cells. However, in patients with COVID-19, an abnormal inflammatory response is believed to cause CRS. Inflammatory cytokines are secreted in response to infections [38]. CRS is one of the causative agents of COVID-19 progression, particularly towards a more severe form. Park [39] also reported excessive production of interleukin-6 (IL-6), which is a primary factor of the inflammatory response in COVID-19 [40]. In clinical settings, COVID-19-related-ARDS shows elevated levels of inflammatory cytokines. Through the overproduction of inflammatory cytokines, CRS attracts inflammatory cells such as neutrophils and monocytes towards the lungs, consequently causing edema and reducing gas exchange within the alveoli, which can lead to ARDS.

Till date, it has been clearly manifested that the infection of COVID-19 into the host cells is integrated via the ACE2 receptors in several organs and most importantly, the lungs. Numerous studies have also highlighted that COVID-19 affects the brain via myriad mechanisms, disrupting the blood-brain barrier and facilitating the introduction of virus into the brain and spinal cord [9,41–44].

ARDS stands out as the most critical clinical effect of COVID-19 and is linked with an alarming death rate. ARDS is an outcome of CRS in lung tissue, wherein the respiratory epithelium is damaged. Severe COVID-19 may cause reduced lung function, like that observed in ARDS as well as inflammation and dysfunction of endothelial cells. These effects may ultimately result in respiratory failure, multi-organ dysfunction and death. A meta-analysis of 38 studies on patients with COVID-19 reported an ARDS occurrence rate of 19.5 % and 5.5 % fatality rate. According to Gibson et al. [45], the COVID-19-induced ARDS has poorer prognosis than ARDS influenced by other conditions, such as pneumonia and serious systemic infections. The fatality rates for patients with COVID-19 ARDS in the ICU are estimated from 26 % to 61.5 %, and those receiving ventilation support ranged from 65.7 % to 94 %. Analysis shows that COVID-19 ARDS shares similar

pathophysiological features with ARDS from other diseases, such as decreased respiratory compliance, and low levels of oxygen in the blood.

4.2. *Targeting possibilities for immunoliposomes*

According to the most recent treatment guidelines by the National Institutes of Health, published on March 6, 2023, positive outcomes have been observed in the treatment of COVID-19 with mAbs neutralizing the SARS-CoV-2 S protein. However, laboratory studies have shown that these mAbs have different antiviral capabilities towards variants and subvariants. Therefore, these mAbs are expected to be less effective in treating COVID-19. The panel advises COVID-19 treatment guidelines not to recommend the use of mAbs for treating COVID-19 (AIII). This is due to the prevalent of Omicron subvariants have been predicted to be unaffected by the listed mAbs treatments. Despite being authorized by the FDA for mild to moderate COVID-19 in outpatients, four anti-SARS-CoV-2 mAb products, namely bamlanivimab plus etesevimab, casirivimab plus imdevimab, sotrovimab and bebatelovimab, are currently not opted as treatment options in the United States. This is because the dominant Omicron micron-subvariants are expected to be ineffective by these therapies. At present, Evusheld, a combination of tixagevimab and ciligavimab, is the sole authorized anti-SARS-CoV-2 mAb product utilized for pre-exposure prophylaxis. These mAbs were engineered to bind to different regions of the RBD, but the efficacy of tixagevimab and cilgavimab against Omicron sub-variants, including those prevalent in the United States are anticipated to be less effective.

Immunoliposome may be a possible candidate for addressing the issue of current antiviral mAbs as a therapeutic option for COVID-19 infection, as well as for emerging variants. COVID-19 clinical manifestations, such as CRS, ALI and ARDS, may become possible targets for immunoliposomes. In a recent study by Zhou et al. [20], immunoliposomes were developed by attaching SARS-CoV-2 variable heavy domain of heavy chain to the liposomal surface and revealed good neutralizing ability against SARS-CoV-2. Immunoliposomes, owing to their characteristics; targeting ability, allow for specific delivery of mAbs to infected cells or tissues, which will increase the efficacy of the therapeutics; thus, they may be conjugated with not just one but various mAbs on their surface to overcome issues challenging traditional mAbs treatment. For instance, the design of immunoliposomes conjugated with tocilizumab on their surface or encapsulated in their core may reduce the release of IL-6, subsequently reducing the risk of COVID-19 complications.

5. Functionalization strategies for immunoliposomes

5.1. *Succinimidyl-ester conjugation*

Immunoliposomes can be synthesized using various strategies. Monoclonal antibodies or its fragments combined with liposomes to create immunoliposomes are considered

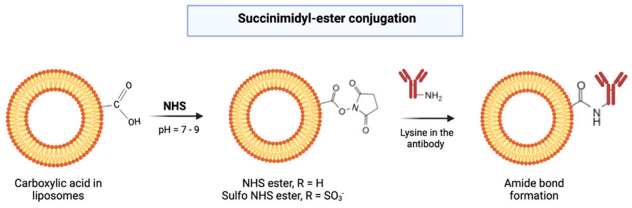

Fig. 2 – Activation of a carboxylic acid in a liposome followed by coupling reaction with lysine residue in a protein. (Created with BioRender.com).

the leading techniques for targeted delivery. Synthesis using N–hydroxy succinimide (NHS) is a commonly used approach. Primary amine groups are commonly used in coupling reactions with carboxylic moieties because of the reactivity of nucleophiles and stability of the formed conjugation products. Due to its exposed surface, the amino group of a lysine residue within a protein can easily interact and form binds with the carboxylic moiety in lipids and antibodies. For an amide coupling reaction between the amine and carboxylic groups, the carboxylic acid of the lipid is first activated using a coupling reagent, *i.e.*, NHS or *N*–hydroxy-sulfo-succinimide (sulfo-NHS) with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC). Subsequently, the activated NHS/sulfo-NHS ester reacts with the primary amine of the protein, creating a stable amide bond, resulting in antibody-drug conjugates, drug delivery agents, and antimicrobial vaccines, a tool to fight against antimicrobial resistance (Fig. 2).

Some commercially available NHS esters containing an activated acyl group are highly reactive, and are known to promptly form amide bonds at physiological pH [46]. Xiao et al. [47] incubated NHS-labeled lipid nanoparticles with wheat germ agglutinin protein, and the obtained nanoconjugates were reported to improve the delivery of drugs across the blood-brain barrier and increase their targeting effect on tumor cells [47]. Lactoferrin (Lf) is an innate protein present in milk and other body fluids that exhibits multiple physiological actions and works as a potential target for the transferrin receptor. Zhang et al. [48] produced PEG-functionalized liposomes as nanocarriers to encapsulate holo-Lf and the anticancer drug doxorubicin for tumor-targeting and imaging-guided combined radio chemotherapy. In this study, the lipid bilayer was first decorated with 1,2-distearoyl-sn–glycero-3-phosphoethanolamine (DSPE)-PEG-COOH, and then Holo-Lf was conjugated with the PEGylated liposomal bilayers through an amide bond using the EDC/NHS conjugation method (Fig. 3). These nanocomposites exhibited high cellular accumulation at the tumor site post-intravenous injection, indicated by imaging results from *in vivo* fluorescence, which also catalyzed the conversion of hydrogen peroxide (H_2O_2) to oxygen [48]. In another study, a lactoferrin-modified liposome (LF-lipo) was successfully synthesized *via* Lf/DSPE-PEG-NHS conjugation to liposomes and used to deliver patchoulol (PA), a tricyclic sesquiterpenoid isolated from a Chinese herb, to treat inflammatory bowel disease. In this case, the modification of Lf conferred a biomimetic delivery ability to the liposomes [49].

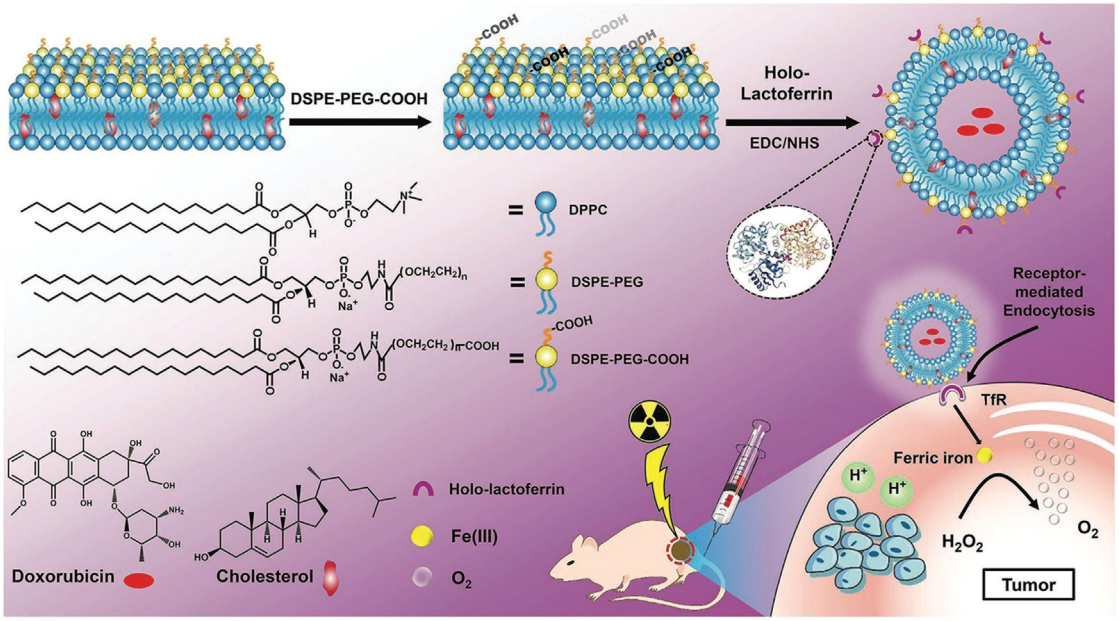

Fig. 3 – Schematic representation of EDC/NHS activated liposomes. Adapted from [48].

Although antibody-modified liposomes, or immunoliposomes, are known to specifically deliver encapsulated drugs to cells through the interaction of cell receptors and ligands with antibodies, the standard techniques used for chemically modifying the antibodies and phospholipids are time-consuming, decrease the binding affinity of antibodies, and alter the physicochemical properties of liposomes. In the study by Hama et al. [50] protein A-R28 (PAR28), a protein derived from bacteria which has high affinity to immunoglobulin G (IgG), were incorporated as one of the liposomes' compositions to avoid disruption in protein conformation by organic solvents. In this study, protein-conjugated phospholipids were first synthesized by succinimidyl-ester conjugation by treating the protein (PAR28) solution with DSPE–PEG-NHS ester. The isolated PAR28-conjugated were reported to be capable of selective drug delivery to the cell, depending on the antibodies [50]. Singh et al. [51] designed and developed surface-functionalized bosutinib liposomes that exhibit estrogen-positive cancer activity via estrogen response elements, that contributes to the malignancy of cancer cells. DSPC-based liposomes were synthesized by activating the biotin carboxylic end using NHS and DCC. The activated biotin-NHS was then allowed to react with the amino-PEG-modified lipid surface (DSPC-PEG-NH_2) to form liposome derivatives that have been reported to be effective for targeted delivery [51]. Lian et al. [52] synthesized galactose-modified liposomes (Gal-LPs) for the dual-delivery of doxorubicin and combretastatin A4 phosphate (CA4P) for anti-hepatoma therapy. In their study, succinimidyl-ester coupling between DSPE-PEG-NHS and aminated glycyrrhetinic acid (GA) was performed, as shown in Fig. 4.

Immunoliposomes synthesized by the succinimidyl-ester conjugation approach are not only effective drug delivery vehicles against cancerous cells in the body, but

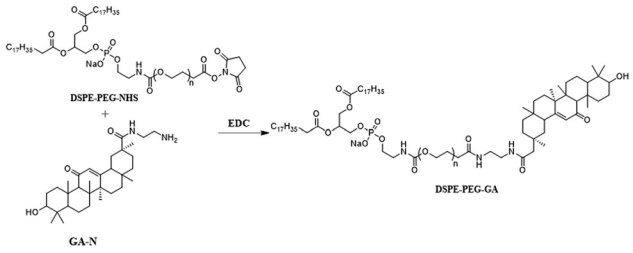

Fig. 4 – Synthesis of GA-N and DSPE-PEG-GA.

are also efficient in delivering drugs to the sites of viral cells. For example, Hertogs and colleagues [33] developed immunoliposomes for site-specific delivery of a HIV-1 protease inhibitor, PI1, by encapsulating the antiretroviral drug-pegylated liposomes conjugated to F105 Fab' fragment as a targeting agent. The carboxylic groups in the dextran matrix were activated by EDC and NHS and the reactive succinimide esters products were reacted with the neutral amino groups of the targeting ligands synthesized from the Fab fragment of the HIV-gp120-directed monoclonal antibody F105 at pH-7.4. Drug-encapsulated liposomes conjugated with targeting moiety have been reported to result in better and more prolonged antiviral activity than the non-targeted liposomes [33]. The discovery of native-like HIV type 1 envelope (HIV-1 Env) trimer-based liposomes has significantly aided advancements in production of HIV-1 prophylactic vaccines. Env-liposome conjugates were synthesized by Damm et al. [53] using Env trimers containing a specific functional group, which in turn was accomplished using EDC/sulfo-NHS-based chemistry comprising two steps. In addition, Ringe et al. [54] developed HIV-1 Env SOSIP trimer-based iron oxide nanoparticles to produce a particulate immunogen for the induction of neutralizing antibodies. In

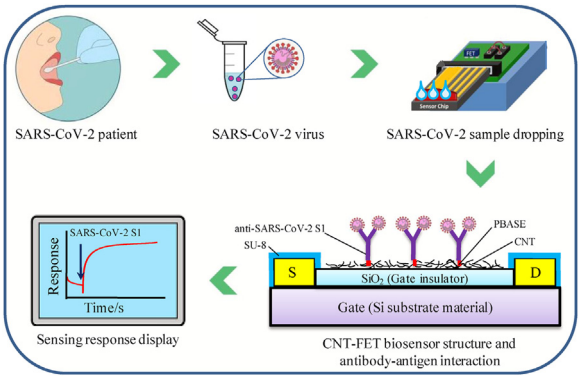

Fig. 5 – Schematic diagram of steps for testing of CNT-FET biosensor and SARS-CoV-2 S1. Adapted from [56]. PBASE: 1-pyrene butanoic acid succinimidyl-ester.

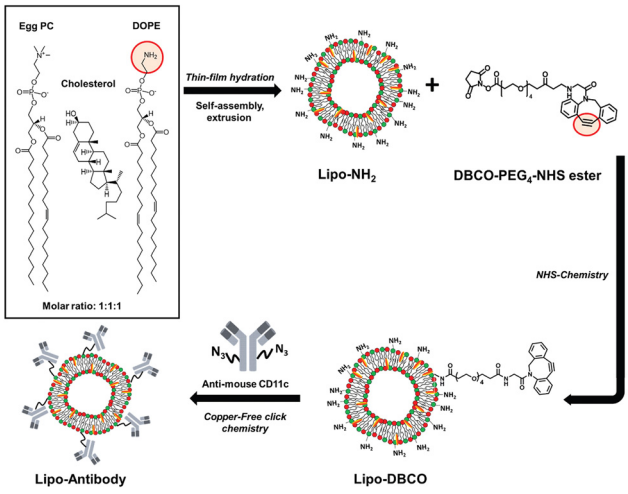

Fig. 6 – Schematic diagram of surface engineered liposomes with site-specific antibodies. The lipid bilayer, consisting of cholesterol and two different phospholipids-2-dioleoyl-sn–glycero-3-phosphoethanolamine (DOPE) and L-α-phosphatidylcholine (egg PC) was formed with a molar ratio of 1:1:1. Effective coupling of CD11c to the DBCO-functionalized liposomes was efficiently performed through the SPAAC approach, and the conjugation was further confirmed by a binding assay based on flow cytometry using fluorescent labeled secondary antibodies. Adapted from [57].

this trimer-based nanoparticle, the carboxylic acid of the oleic acid-coated iron oxide nanoparticles was activated using EDC and sulfo-NHS coupling reagents, and the activated sulfo-NHS ester reacted with the amine group of the Env trimer.

Shao et al. [55] created a nanobiosensor for the detection of SARS-COV-2 S antigen by a carbon nanotube field-effect transistor (CNT-FET) modified with an antibody against SARS-COV-2 spike. The nanobiosensor demonstrated a broad detection range, enabling detection of even lower concentrations of the S antigen. In this study, the carboxylic acid groups of semiconducting single-walled carbon nanotubes were conjugated with the amine group of the SARS-COV-2S antibody *via* sulfo-NHS coupling [55]. Zamzami et al. [56] developed a CNT-FET-based electrochemical biosensor to detect the SARS-CoV-2 S1 virus. In this study, CNT-FET chips were fabricated using a succinimidyl-ester linker. This 1-pyrene butanoic acid succinimidyl-ester-modified CNT was conjugated to the amine group of an anti-SARS-COV-2S antibody. This CNT-FET biosensor was reported to be highly sensitive which enabled it to identify the SARS-COV-2 antigen among other antigens (Fig. 5).

A recent study by Gai et al. [57] described the development of site-specific conjugation of antibody on the surface of liposomes by combining surface primary amine groups (–NH_2) with dibenzocyclooctyne–PEG4–NHS esters to introduce strained alkynes (DBCO) into the liposome region under various conditions. Anti-mouse CD11c antibodies were site-specifically modified using a transferase and UDP-N-azidoacetylgalactosamine (UDP-GalNaz) with azide groups. Subsequently, using the SPAAC approach, the antibody bearing azide reactive group was successfully conjugated to liposomes with activated DBCO group [57] (Fig. 6).

5.2. *Sortase A-mediated click-chemistry*

Modifying proteins at specific locations is important for creating unique protein derivatives for different uses, including therapeutics and scientific research such as for protein labeling and/or adding nongenetically encoded functional groups [58]. There are various methods for this, but sortase-mediated ligation is often used [59]. Sortase is a transpeptidase, an enzyme found in many gram-positive bacteria that is necessary for bacterial peptidoglycan crosslinking [60]. Sortase A is a transpeptidase derived from a gram-positive bacteria *Staphylococcus aureus* and is an enzyme responsible for cell wall sorting and secretion of proteins on the cell surface [61]. The ligation reaction of sortase A occurs when it identifies the specific C-terminal sequence "LPXTG" in a protein, and breaking the bond between threonine and glycine, linking the carboxyl group of threonine to an amino group of pentaglycine on the cell wall. Hence, a chain of glycine amino acids is attached to the C-terminal end of that protein through a peptide bond [62]. Using sortase-mediated ligation, a wide range of modifications can be made to target proteins. This method has numerous applications such as detecting interactions between cells, creating biomaterials such as cell-laden hydrogels with proteins that are site-specifically modified. These proteins are modified by addition of reactive groups onto proteins required for biomaterial decoration and synthesizing ubiquitylated/SUMOylated proteins for producing Ub conjugates of complex, proteins that are unfoldable and to observe the ubiquitylation and SUMOylation events in living cells. This is due to the thioester-linked Ubiquitin-Sortase A (Ub-SrtA) intermediate that can be attacked by its nucleophile by the -amino group of the terminal glycine residue of GGK. This terminal residue is located specifically into a protein of interest (POI), by changing the unstructured Ub C terminus from its native sequence [63–65].

As it effectively catalyzes ligation to an aminoglycine-ended moiety, a previous study used this enzymatic method

to attach azide/alkyne orthogonal labels to proteins to conjugate them to complementary divalent or tetravalent multiple antigenic peptide scaffolds via copper-catalyzed azide-alkyne cycloaddition click chemistry [66]. As most protein dendrimers are restricted to a valency of two to four and are produced on branching lysine scaffolds, cyclodextrin (CD), a naturally occurring scaffold made up of 6–8 glucose units, provides the potential for extending the valency. Hence, this previous study reported the use of sortase-A mediated ligation followed by click chemistry to synthesis a heptavalent protein using the covalent display of peptides and proteins which were found on β-cyclodextrin (β-CD) template. PspA (pneumococcal surface protein A) and RrgB (a pilus protein) were the two proteins that have been selected for future vaccine development. Instead of the conventional two-step immunohistochemistry, single-step immunohistochemistry has been made possible by labeling the nanobody with Alexa Fluor 488 using the first method that uses sortase A to add an alkyne-containing peptide to nanobodies, and the second method uses para-azido phenylalanine at the C-terminus of the nanobodies covalently and site-specifically attached to the fluorophore [67].

The use of sortase A in a specific site conjugation approach has been well established for adding small molecules to proteins, connecting proteins to DNA, and for lipid-protein conjugates, artificially altering the lipids on a protein and is used in the development of drug delivery systems using antibodies anchored on a lipid carrier [59]. The use of sortase-mediated transpeptidation may be an attractive option for attaching proteins to liposomes at a specific location, with minimal limitations of the protein being attached and requiring only the addition of a small coupling sequence to the substrate protein [68]. Additionally, the modification of protein lipids is also important in the generation of synthetic lipid bilayers. This technique can be utilized to produce liposomes coupled with ligand proteins, where the incorporated lipids act as colipids [69].

It has been shown that sortase A is versatile and utilized to promote the conjugation of proteins to various amine nucleophiles, another study demonstrated the use of sortase A to ligate proteins to liposome sites specifically in the presence of glycine residues [70]. According to a previous study, although the LPXTG motif must be present close to the C-terminus of proteins to be ligated into liposomes, making this approach plausible. Hence, enhanced green fluorescent protein was successfully attached to liposomes coated with phospholipids with a diglycine motif by biologically modifying the protein to carry the LPATG motif at C-terminus; the motif would then be recognizable by sortase A. This was exhibited through enzymatic reactions, and it was also shown that as the concentration and proximity of the diglycine motif on and from the liposome surface increased, the effectiveness of protein modification of liposomes also increased significantly. This proved that the use of sortase A could be beneficial for liposome modification with other proteins. Another successful sortase ligation method is exhibited in the development of pentaglycine liposomes that were prepared with varying degrees of PEGylation, influencing the steric accessibility of the pentaglycine motif using a pentaglycine modification and a scalable solvent injection technique [71] which helps avoid the issue of protein heterogeneity, allows for more precise control over the modification process, and produces consistent and efficient sortaggable liposomes [71,72].

Furthermore, sortase A was used to generate a bispecific antibody-binding fragment (BiFab) [73]. Two clickable groups (such as N3 and DBCO) were inserted into the C-terminal LPETG tag of Fabs via sortase A-mediated ligation. BiFab was then produced by the conjugation of two click handle-modified Fabs via sortase A-mediated click handle functionalization. The effective sortase A-mediated "bio-click" chemistry in producing a variety of potent BiFabs was shown by the BiFabs against distinct targets, which has shown excellent role in inducing T lymphocytes to target and kill tumor cells. Hence, this study suggests that utilizing this method shows the efficient creation of a Fab-derived library for personalized tumor immunotherapy.

Moreover, another study involving sortase A-mediated ligation, followed by click chemistry, involved the conjugation of a heavy chain antibody (VHH). In a previous study, VHH was modified with an azide group using a sortase A-mediated transpeptidation method [74]. Alteration of specific ligands with an azide group is pivotal for click chemistry reactions, which can be achieved by the sortase A-mediated transpeptidation of glycine amino acids from VHH-LPETG, to form VHH-azide. The VHH-azide was then clicked with DBCO-PEG, which is frequently employed to extend the therapeutic half-life of proteins. The binding capacity was compared between clicked products and products conjugated by maleimide-thiol conjugation, and the results showed that clicked products had better binding capacity, suggesting that clicked products are advantageous for producing homogeneous VHH conjugates that maintain their antigen-binding capacity.

One drawback of using sortase-mediated reactions and related conjugation strategies, such as intein chemistry, is that they only allow the production of N-to-C- and C-to-N-fused proteins. However, some proteins, like antibodies, require at least one end to retain the functional properties. Standard genetic fusion of these proteins decreases or eliminates their activity. Nevertheless, an alternative is to fuse the same ends of the proteins (N-to-N or C-to-C). Hence, a previous study described a method that combined transacylation, catalyzed by sortase A with a strain-promoted click reaction to produce such synthetic linked proteins [75]. To summarize, although there are limited studies on the combination of sortase A method ligation with click chemistry on liposomes, previous studies have shown that this could be a promising method for developing immunoliposomes decorated with antibodies. This site-specific and well-established approach can be applied as a functionalization strategy for immunoliposomes, which can then be used for therapeutic and pharmaceutical applications.

5.3. *Maleimide-thiol conjugation*

Maleimide-thiol chemistry is the standard method used to synthesize immunoliposomes. In maleimide-thiol chemistry, the disulfide bonds in monoclonal antibodies are reduced, hence thiol groups will be available as an active

site for conjugation with other biomolecules, polymers or nanocarriers with maleimide group [76]. This coupling method was developed since maleimide and thiol groups react quickly and effectively. This reaction is often utilized in bioconjugate chemistry (a linking process of biomolecules to desired moieties) to enable the conjugation of a thiolated antibody to liposomes grafted with maleimide [77]. One of the examples of the application for this reaction is the development of paclitaxel, an anticancer drug for breast cancer, in PE-consisting liposomes conjugated with Herceptin, a recombinant anti-human epidermal growth factor receptor-2 (HER2) antibody that was specifically designed to target human breast cancer cells, which increases the effectiveness of paclitaxel's intracellular distribution through receptor-mediated endocytosis [78]. In this study, thiolated Herceptin was successfully conjugated to maleimide group of PEG via maleimide-thiol chemistry, then incorporated into the liposomal bilayers. The results showed that in the cancer cells (BT-474 and SK-BR-3) overexpressing HER2, the PEGylated immunoliposomes demonstrated significantly better cellular uptake than the stealth liposomes, whereas indistinguishable difference was observed in cells expressing low HER2 (MDA-MB-231) or at low temperature (4 °C). Therefore, tumor-specific cancer therapies for breast tumors expressing the HER2 may benefit from this formulation, in which selective delivery of anticancer drugs can be accomplished by incorporation of therapeutic antibody, Herceptin to the liposomal formulation, or called as immunoliposomes [78].

Recently, the interest in anti-HER2 immunoliposomes has increased. Rodallec et al. [79] generated trastuzumab (Herceptin)-docetaxel immunoliposomes against HER2 using maleimide-thiol chemistry. *In vitro* studies of this formulation were compared with free docetaxel and free Herceptin using two-dimensional (2D) monolayer models. As this study compared the efficacy and biodistribution of the standard docetaxel liposomes with those of the immunoliposomes, the results showed no difference in tumor cell uptake. The authors suggested that active targeting did not contribute as much to tumor distribution. The reported variations in efficacy were likely attributed by the improved internalization of HER2+ cells by immunoliposomes compared to standard liposomes, rather than their greater specificity for tumor tissue. In another study conducted by Rodallec et al. [80], the results contradicted those of previous studies. They tested the efficacy of trastuzumab-docetaxel immunoliposomes against breast cancer using three-dimensional spheroid models. Immunoliposomes were generated using maleimide chemistry, where trastuzumab was initially thiolated, and then mixed with PEGylated liposomes bearing maleimide group. Compared with current anti-HER2 breast cancer treatments, *in vitro* and *in vivo* studies have demonstrated effective drug delivery with immunoliposomes as well as greater effectiveness and longer life.

Maleimide thiol chemistry has also been used for normal cellular uptake studies [81]. In creation of asymmetric immunoliposomes that maintain their affinity and avidity during the development process, antibodies from both human and rabbit were thiolated and attached to maleimide-functionalized liposomes. These findings demonstrate that immunoliposomes can be internalized by murine

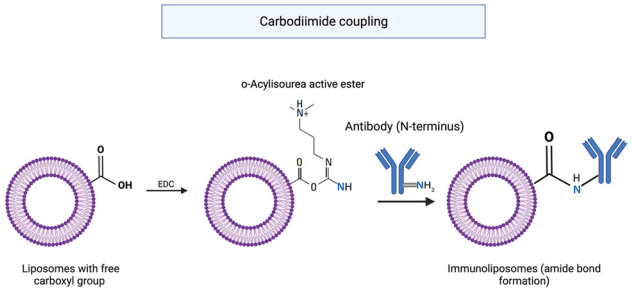

Fig. 7 – Schematic illustration of the production of immunoliposome by using carbodiimide coupling reaction. (Created with BioRender.com).

monocyte macrophage (RAW264.7) to prove broad-spectrum internalization, hepatocarcinoma (HEPG2) as the intended cell for certain pivotal receptors, and monkey kidney cells (CV-1) to show specificity. This suggests that the generated immunoliposomes can be used in the future to improve drug delivery for the targeted delivery of hepatocellular carcinoma. In addition to their application in cancer studies, immunoliposomes generated using this method have been implicated in gene therapy. Kim et al. [82] characterized and optimized plasmid DNA-carrying immunoliposomes synthesized using maleimide chemistry, by studying the effect of the ratio of maleimide-containing PEG to rat IgG on the number of conjugated IgG molecules per liposomes. This was performed to improve the gene delivery system for improved biosafety and increased gene expression. The researchers obtained the highest number of antibodies conjugated to liposomes at a ratio of 10:1; however, the authors suggested that this ratio could be further optimized. In conclusion, various applications of immunoliposomes generated by maleimide chemistry, such as cancer and gene therapy, can be observed.

5.4. *Carbodiimide-mediated conjugation*

Carbodiimide chemistry is one of the most widely used concepts in the field of chemical conjugation. The establishment of amide bonds between the primary amine and active ester group forms the cornerstone of carbodiimide chemistry [83]. It has been demonstrated that this method can affect the properties of the macromolecular structures created as well as the immunogenicity of the conjugate [84]. The reagent most applied in carbodiimide chemistry is EDC, which water soluble and has been found to be stable in aqueous solutions for a minimum of 5 h [83].

By conjugating carboxylates in carboxylic acids directly to the primary amine, this zero-length crosslinker can cross-link biomolecules without being part of the final amide bond. Consequently, the resulting linkage solely consists of an amide [85] (Fig. 7). However, factors such as the solvent, pH, stoichiometry and side reactions have considerable effects on the process and make the formation of amide bonds more complex [85]. In addition, owing to the diverse displacement of primary amine groups on proteins such as antibodies, the final product of the conjugation can be

heterogeneous because of the random polymerization of the polypeptide [83]. However, this reaction is extensively used for modification and conjugation applications [83]. As excess EDC has been established as one of the potential causes of protein degradation, the concentration of EDC has become one of the factors contributing to successful conjugation. Adeagbo et al. [86] reported that degradation of proteins can be observed for EDC concentrations higher than 200 μg/ml the reaction mixture.

In the preparation of immunoliposomes, carbodiimide chemistry can be applied to achieve the desired products by altering phosphatidylethanolamine (PE), one of the components of liposomes; its lipid moiety contains a carboxylic acid spacer needed for the reaction. Manjappa et al. [87] also described the methods for altering the PE and compared the advantages and limitations of these methods. In short, altered PE containing a carboxyl group is added to the liposomal formulation and activated using carbodiimide at a low pH setting. The inclusion of target molecules, such as antibodies, occurs after excess carbodiimide is removed. There is currently a commercial liposome known as the Immunosome®-Carboxylic Acid that features a free carboxyl group on its surface for conjugation. It only requires users to activate the carboxyl group by the addition of carbodiimide, without altering the lipid moiety of the PE.

Several studies have used this method to prepare immunoliposomes. For instance, Raju et al. [88] used carbodiimide chemistry to conjugate trastuzumab with liposomes for breast cancer treatment. The results showed that trastuzumab-conjugated liposomes considerably extended the half-life of the drug compared to PEG-coated liposomes and commercially available forms of docetaxel according to an *in vivo* pharmacokinetics (PK) investigation. Transtuzumab-conjugated liposomes demonstrated greater potential and could meet the demand for prolonged and targeted drug delivery in human breast cancer. In addition to its use in improving therapeutics, this method has also been used to prepare immunoliposomes for diagnostics. Park et al. [89] applied carbodiimide chemistry, where NHS/EDC was used as a crosslinker in the production of polydiacetylene, a PDA-based fluorescence chip. The chip was then used to detect *Cryptosporidium parvum* oocysts. In another study, Lin et al. [90] successfully developed a novel immune sensing protocol for the detection of small-molecule aflatoxin B1, a type of mycotoxin that is harmful to humans and animals. This was made possible by carbodiimide coupling of anti-aflatoxin to the liposomes used in the immunoassay. Only a few studies have discussed the creation of immunoliposomes using EDC chemistry. Though successful conjugation of antibodies via carbodiimide coupling has been reported previously, several drawbacks have also been raised. These limitations include, first, the requirement of a high amount of antibodies and second, a low conjugation yield, typically maximum of 20 % conjugation success rate. Compared to click-chemistry conjugation, the reaction is highly efficient and convenient, as it is does not require any particular reaction conditions [91]. Numerous studies have used comparable techniques to create immunoliposomes that target various antigens and deliver therapeutic drugs.

6. In silico binding of immunoliposomes against SARS-CoV-2

Antibodies against SARS-CoV-2 (nAbs) have revealed several potential candidates for prophylactic and therapeutic use. Two major groups of antibodies characterized by their responses to viral infection include neutralizing antibodies that defend host cells by inhibiting viral invasion, and non-neutralizing antibodies that cannot protect host cells from viral invasion [92]. The incorporation of neutralizing antibodies onto the liposomal surface is one strategy for liposomal surface modification to produce immunoliposomes. The derived immunoliposomes are useful in the drug delivery systems as mentioned earlier in this review.

Here, we focus on the reported nAbs that bind epitopes specific to the SAR-CoV-2 spike (S) protein. The S protein is a trimeric fusion membrane protein. The RBD of the S protein is one of the target regions of nAbs. The inhibitory mechanism of reported nAbs mainly involves disruption of the interaction between the S-RBD domain and ACE2. The S-RBD domain immediately changes its conformation in the up- or down-trimers. The resolved cryo–electron microscopy structure of the infectious state of SARS-CoV-2 has shown some movement of trimers conformation upon binding. When one of the three trimers is rotated up to a receptor expose position, this was defined as up-conformation or receptor accessible conformation of S-RBD domain [93]. The identification of S-RBD trimers' conformations leads to a classification of novel nAbs on a basis of the location and conformation of their binding S-RBD epitopes [94]. Superimposition among crystal structures of nAb-RBD complex and human ACE2-RBD complex could show a competitive binding manner of nAb against ACE2 with or without RBD conformation preferences.

The *in silico* binding study of nAb and S protein of SARS-CoV-2 binding using complex crystallography has revealed interacting residues that are involved in the inhibitory strength against viral attachment. Characterization of broad-spectrum nAbs against SARS-related CoVs, MW06, and the RBD complex revealed that the three-up RBD trimer could bind to MW06 without any clash regions, compared to binding with any down RBD in the S-RBD trimer (PDB ID: 7A98). The clash region in the complex was identified by superimposition of the nAb with one of the down RBD in a trimer. The crystal structure of MW06-RBD complex showed the recognition area of MW06 side chains forming hydrogen bonds with Phe374, Val503, and Ala372 of SARS-CoV-2 RBD as well as hydrophobic interaction with contacted S-RBD residues. In addition to its broader spectrum against both SARS-CoV-2 and SARS-CoV, inhibition by MW06 (K_d 5.48 and 12.3 nM, respectively) did not induce antibody-dependent enhancement, which is a concern for some antibody-based implementations [95].

Regardless of the fluctuations of the S-RBD trimer, the crystal structure of the monoclonal antibody nCoV617 and the S-RBD complex (PDB ID: 7E3O) shows that at least 10 residues of S-RBD are interacting with nCoV617 for potent binding to both up- and down-conformation RBD (K_d 8.02 nM) [6]. The primary interaction of nCoV617 recognized S-RBD region through various hydrophilic and hydrophobic interactions. For example, the complex showed hydrophobic interaction

between nCoV617 and Y449, L452, and F490 in S-RBD. The ϵ-amide group of R97 on heavy chain complementary-determining region 3 (HCDR3) of nCoV617 also forms strong interaction with D-carboxy group of E484 of S-RBD. The S-RBD-interacting residues exclude the Y501 residue that is found in SARS-CoV-2 variant B.1.1.7 (Alpha).

The development of nanobodies as nAbs is ongoing and benefits small molecules by providing easier access to the binding epitopes. A study on inhibition activity of an alpaca SARS-CoV-2 neutralizing nanobody, TY1, reported specifically binding to S-RBD. Ty1 binding epitopes are accessible in both the up and down conformation of the S-RBD trimer [96–98]. Besides functionalization with TY1 nAb on liposomal surface for using of immunoliposomes as nanocarriers, several surface modifications with various functional groups are commercially available for future discovery of targeted delivery of compounds against viral infections. In addition to the RBD of spike proteins that are major targets of the S protein, broad nAbs acting on other domains, including the N-terminal domain of the S1 subunit as well as the system helix and fusion peptide in the S2 subunit, have also been identified [94].

7. Conclusion

With the plethora of advancements in drug delivery technologies in the last decade, the use of a targeted delivery system may be exploited to improve current treatment strategies for SARS-CoV-2 and its evolving variants. Liposomes, which are renowned for their versatility and flexibility in incorporating therapeutic biomolecules either by encapsulation or conjugation to their surface, are the most suitable candidates for this purpose. Recent advancements in the design of liposomal formulation as delivery systems have been reported in literature to enhance the delivery of therapeutic agents, by coupling or conjugating antibodies to the surface of liposomes, creating immunoliposomes. Owing to the flexibility on the design of immunoliposomes, site-targeted and selective delivery of therapeutics can be achieved successfully. It holds great potential for the targeted delivery of antiviral drugs or other biomolecules for the treatment of infections, particularly COVID-19 infections that are still actively circulating.

8. Future perspective

Immunoliposomes may be possible candidates for addressing the issue of current antiviral mAbs as a therapeutic option for COVID-19 infection, as well as for emerging variants. COVID-19 clinical manifestations such as CRS, ALI and ARDS, may become possible targets for immunoliposomes. Their targeting ability allows for the specific delivery of mAbs to infected cells or tissues, which will increase the efficacy of the therapeutics. In this manner, it may be conjugated simultaneously with various mAbs on its surface to overcome issues challenging the traditional mAbs treatment. For instance, the design of immunoliposomes conjugated with tocilizumab on their surface or encapsulated in their core may reduce the release of IL-6, subsequently reducing the risk of COVID-19 complications.

Alternatively, immunoliposomes may encapsulate any antiviral drugs or therapeutics, such as remdesivir, molnupiravir and favipiravir, while having mAbs conjugated to its surface, and present itself as a dual-delivery system for halting viral replication. MAbs neutralize the virus by binding to the S protein of SARS-CoV-2, and antiviral drugs, such as remdesivir, may stop viral replication inside cells by inhibiting the viral replication machinery. This dual delivery system can be designed to target different locations in the host body by utilizing the versatility of liposomes as nanocarriers.

However, various challenges faced during the development of these immunoliposomes such as high temperature and pressure, and mechanical shear forces should also be taken into consideration, as the harsh process involving these antibodies may affect their binding affinity and avidity towards the endpoint when further tested *in vitro* and *in vivo*. To summarize, the use of immunoliposomes in the field of viral infection has been extensively studied; however, the design and effectiveness of immunoliposomes as nanocarriers against SARS-CoV-2 need to be further evaluated to eradicate these emerging cases of COVID-19.

Conflicts of interest

The authors declare that they have no conflicts of interest or financial competing interests.

Acknowledgements

The authors acknowledge the financial support obtained from Universiti Kebangsaan Malaysia (DIP-2021-001) and ASEAN-India Science & Technology Development Fund (AISTDF) (SERB/F/3955/2022-2023). The funder played no part in the paper's design, publication decision, or manuscript writing.

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