Chitosan-Based Nanoparticles Against Viral Infections

Homa Boroumand[1†], Fereshteh Badie[2†], Samaneh Mazaheri[3], Zeynab Sadat Seyedi[4], Javid Sadri Nahand[5], Majid Nejati[6], Hossein Bannazadeh Baghi[7,8], Mohammad Abbasi-Kolli[9], Bita Badehnoosh[10], Maryam Ghandali[11*], Michael R. Hamblin[12*] and Hamed Mirzaei[13*]

[1] School of Medicine, Kashan University of Medical Sciences, Kashan, Iran, [2] Department of Microbiology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran, [3] Department of Analytical Chemistry, Faculty of Chemistry, University of Kashan, Kashan, Iran, [4] Department of Cell and Molecular Biology, Faculty of Chemistry, University of Kashan, Kashan, Iran, [5] Department of Virology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran, [6] Anatomical Sciences Research Center, Institute for Basic Sciences, Kashan University of Medical Sciences, Kashan, Iran, [7] Department of Microbiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran, [8] Infectious and Tropical Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran, [9] Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran, [10] Department of Gynecology and Obstetrics, Dietary Supplements and Probiotic Research Center, Alborz University of Medical Sciences, Karaj, Iran, [11] School of Medicine, Iran University of Medical Sciences, Tehran, Iran, [12] Laser Research Centre, Faculty of Health Science, University of Johannesburg, Doornfontein, South Africa, [13] Research Center for Biochemistry and Nutrition in Metabolic Diseases, Institute for Basic Sciences, Kashan University of Medical Sciences, Kashan, Iran

Viral infections, in addition to damaging host cells, can compromise the host immune system, leading to frequent relapse or long-term persistence. Viruses have the capacity to destroy the host cell while liberating their own RNA or DNA in order to replicate within additional host cells. The viral life cycle makes it challenging to develop anti-viral drugs. Nanotechnology-based approaches have been suggested to deal effectively with viral diseases, and overcome some limitations of anti-viral drugs. Nanotechnology has enabled scientists to overcome the challenges of solubility and toxicity of anti-viral drugs, and can enhance their selectivity towards viruses and virally infected cells, while preserving healthy host cells. Chitosan is a naturally occurring polymer that has been used to construct nanoparticles (NPs), which are biocompatible, biodegradable, less toxic, easy to prepare, and can function as effective drug delivery systems (DDSs). Furthermore, chitosan is Generally Recognized as Safe (GRAS) by the US Food and Drug Administration (U.S. FDA). Chitosan NPs have been used in drug delivery by the oral, ocular, pulmonary, nasal, mucosal, buccal, or vaginal routes. They have also been studied for gene delivery, vaccine delivery, and advanced cancer therapy. Multiple lines of evidence suggest that chitosan NPs could be used as new therapeutic tools against viral infections. In this review we summarize reports concerning the therapeutic potential of chitosan NPs against various viral infections.

Keywords: viral infection, chitosan, nanoparticles, delivery system, therapy

INTRODUCTION

Infectious diseases caused by bacteria, fungi, viruses, and parasites are responsible for nearly 15 million deaths globally, of which human immunodeficiency virus (HIV), malaria, tuberculosis, as well as acute respiratory infection including the newly emerged COVID-19 have been considered to be the key causes of death (Panácek et al., 2009; Li et al., 2011; Qasim et al., 2014). Viral infections cause substantial health concerns that affect millions of individuals globally, and negatively impact human health and socio-economic development (Mehendale et al., 2013; Nahand et al., 2020a; Nahand et al., 2020b). Effective treatments of viral infections are limited by increased incidence of drug resistance, particularly related to HIV (Little et al., 2002; Shafer et al., 2007; Lockhat et al., 2016; Maseko et al., 2016) and influenza (Hayden, 2009). Drug resistant viral infections are a public health threat, causing widespread morbidity as well as mortality (Cosgrove, 2006), and additional costs caused by expensive drugs (Li et al., 2011; Qasim et al., 2014). Therefore, there is an urgent need to develop novel approaches to prevent and treat these viral infections.

Nanotechnology is concerned with particles with sizes within the nanometer range from 10^{-9} or 1 billionth of a meter (Nalwa, 1999; Parboosing et al., 2012). Nanobiotechnology refers to interactions of nanoscience with biological systems (Scheller et al., 1995; Niemeyer, 2006). Nanomedicine involves the use of nano-structured materials for the diagnosis, prevention, or treatment of diseases (Medepalli, 2008). The first use of nanoparticles (NPs) in medicine was to increase the solubility and stability of drugs with low bioavailability (Schütz et al., 2013). NPs can exert antiviral activity through several mechanisms. Firstly, NPs are useful in viral treatment due to their specific features, such as small particle size (Kumar et al., 2012; Parboosing et al., 2012), high ratio of surface area to volume (McNeil, 2011), and adjustable surface charge (Caron et al., 2010; Petros and DeSimone, 2010). Secondly, it has been shown that NPs can display biomimicry features (Bowman et al., 2008; Sanvicens and Marco, 2008; Gagliardi, 2017), and therefore can exert intrinsic antiviral activity; e.g. silver nanoparticles (AgNPs) (Sun et al., 2005; Lara et al., 2010) or dendrimers (Wang et al., 2006; Mallipeddi and Rohan, 2010). Thirdly, NPs can also increase the stability of the loaded drug, allowing dose optimization and more predictable delivery, which all stem from the drug encapsulation ability (Chiappetta et al., 2011; Kumar et al., 2012). NPs can be tailored to create stable structures (Goldberg et al., 2007), or modified by attachment of polyethylene glycol (PEG) (Gabizon et al., 1994; Alexis et al., 2008; McNeil, 2011; Santos-Martinez et al., 2014). Fourthly, engineered NPs can significantly enhance drug delivery by the use of targeting moieties that recognize receptors or biomarkers to increase the specificity for target cells, target tissues, or subcellular compartments (Muthu and Singh, 2009; Petros and DeSimone, 2010; McNeil, 2011).

Chitosan (CH) is a modified biopolymer obtained via partial de-acetylation of the naturally occurring polysaccharide called chitin, which contains randomly distributed (1→ 4)-linked N-acetyl glucosamine and glucosamine units (Rashki et al., 2021). CH can be obtained as a white powder, composed of rigid, inflexible, and nitrogen-containing polysaccharide chains of varying length and molecular weight (Badawy and Rabea, 2011). In addition, CH has multi-faceted applications because of its non-toxicity, bio-degradability, and intrinsic antimicrobial properties. CH has been used in biomedical preparations, genetic engineering, agricultural sector, environmental pollution control, food manufacturing, paper manufacturing, photography, and water treatment (Cheba, 2011). CHNPs show interesting interface and surface effects due to their very small size (Ingle et al., 2008). Several studies have suggested that CH NPs could be used as new therapeutic options against viral infections.

CHITOSAN (CH) AND CH NPS

CH is a straight chain, cationic polysaccharide that is prepared by partial deacetylation of chitin, which is commonly carried out by alkaline hydrolysis (**Figure 1**). CH commonly refers to cationic co-polymers that contain 2 amino 2-deoxy-β-D-glucose residues (60% to 100%) as well as 2 acetamino-2-deoxy-β-D-glucoside residues (0% to 50%), attached together by ß (1→4) bonds (Nasrollahzadeh et al., 2020; Tao et al., 2020). The mean degree of deacetylation is usually >60% (Ng et al., 2002; Varma et al., 2004). The average degree of deacetylation and the molecular weight specifies the total number of amide residues and the number of primary amine residues, and this ratio directly influences the CH solubility and the chemical, biological and physical properties (Kmiec et al., 2017; Jaworska et al., 2020; Rashki et al., 2020; Yang et al., 2020a).

The CH amino groups are more reactive compared to the acetamido-groups of chitin. In fact, the nitrogen free-electron pair in the amino groups can bind to metallic cations, and the average degree of deacetylation determines their number. In addition, protonation of the amino groups in an acidic solution will electrostatically attract surrounding anions (Kmiec et al., 2017).

CH, unlike chitin, can be dissolved in a dilute acidic solution to form a soluble cationic polymer, which has different properties to other polysaccharides (Kmiec et al., 2017). Following a lengthy period of agitation, CH can be dissolved in dilute acids like acetic, formic, lactic, or inorganic acids. Nonetheless, the solubility depends on the degree of deacetylation, molecular weight, bio-polymer concentration, pH, and ionic strength (Varma et al., 2004). In addition to the primary amino groups, CH contains numerous primary and secondary hydroxyl-groups at the C-2, C-3 and C-6 positions, which show robust interactions with water (Peter, 1995).

CH has advantages such as biodegradability, hydrophilicity, biocompatibility, bioactivity, and is obtained from renewable and natural sources. It can be processed into distinct forms, such as solutions, blends, sponges, membrane, gels, paste, tablets, microspheres, or microgranules for different applications (Kumar et al., 2004; Pillai et al., 2009).

Henri Braconnot first discovered chitin in 1811 while researching different mushrooms. Next, Prof. C. Rouget (1859) found that alkaline treatment of chitin produced a material, which could be dissolved in acids, unlike chitin. Hoppe Seiler was the first to call this deacetylated chitin "chitosan" (Badawy and Rabea, 2011). Next to cellulose, chitin is the most widespread
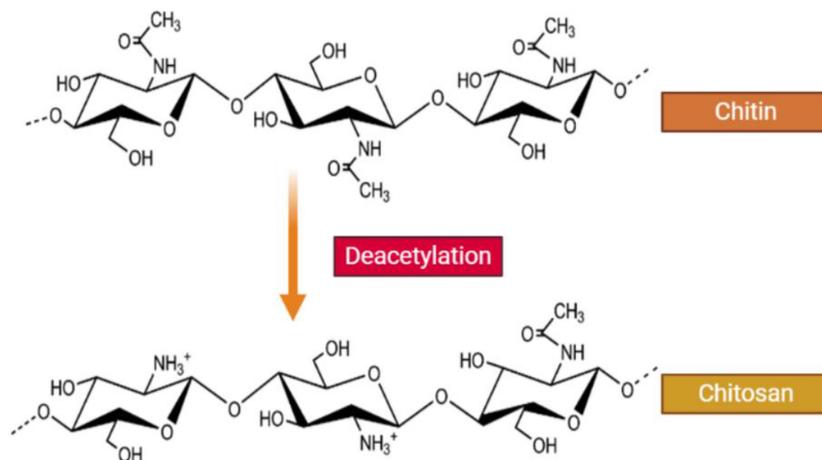
FIGURE 1 | Structure of chitin and chitosan. CH is a naturally occurring water-soluble cationic polysaccharide that is generated by alkaline deacetylation of chitin. CH is a linear polysaccharide composed of β–(1-4) linked 2-acetamide-2-deoxy-D-glucose (a neutral sugar unit GlcNAc or A-unit) and 2-amino 2-deoxy-D-glucose (a cationic sugar unit GlcN or D-unit). CH can have a broad range of degree of deactylation (F_A), as well as variable chain length and molecular weight.

biopolymer found in all of the natural world. Chitin is the main component of insect cuticles, yeast, green algae and fungal cell walls (Einbu and Vårum, 2008), and is also found in crab and shrimp shells (Wang and Xing, 2007). On the other hand, chitosan itself is very rare in nature, being only found in the cell walls of certain fungi (Muzzarelli et al., 1986; Yen et al., 2009). There are three principal types of chitin called α, β, and γ. The α-chitin possesses anti-parallel chains, whereas β-chitin possesses parallel chains formed by in-sheet hydrogen bonds. The γ-chitin is made up from combinations of α and β chitin types with both parallel and anti-parallel chain arrangements (Franca et al., 2008; Yen et al., 2009).

PREPARATION OF CH NPS

The most common procedures for preparing CH-based NPs are, emulsion based solvent evaporation, ionotropic gelation, solvent diffusion, emulsification, as well as the microemulsion method (Garg et al., 2019; Zhang et al., 2019; Ansari et al., 2020). Some of the benefits associated with the above techniques include the use of less organic solvent and the need for a lower force. However, the size and surface charge of the CH NPs prepared *via* the above procedures, depend on the degree of deacetylation and the molecular weight (Mohammed et al., 2017). In addition, hydrophobic interactions, electrostatic interactions, and hydrogen bonding all govern the extent to which drugs will be entrapped inside the polymeric matrix. Moreover, the drug loading and drug release will need to be investigated for each application of CH NPs, considering the physiological environment at the administration site. In fact, both the ionic strength and the presence of enzymes or proteins will affect drug release and stability (e.g., the milieu of the eye versus the GI tract). It is fortunate that there are numerous techniques available to prepare the optimum CH NP formulation for each application (Mohammed et al., 2017).

Ionotropic Gelation

Calvo et al. were the first to describe the ionotropic gelation method for the preparation of CH NPs. Thereafter, additional experts in the field investigated and developed it further (Calvo et al., 1997). This procedure relies on electrostatic interactions between the amine groups of CH and a variety of polyanions with a negative charge, such as tri-polyphosphate (Divya and Jisha, 2018). First it is necessary to dissolve the CH in acetic acid in the presence or the absence of a stabilizing agent, such as a poloxamer. In the next step, the polyanion is added, and the NPs are spontaneously generated by mechanical shaking at room temperature. It is possible to modify the size as well as the surface charge of the particles by varying the CH-to-stabilizer molar ratio (Divya and Jisha, 2018). Researchers have found an overall increase in the particle compactness and size by increasing the CH concentration and the ratio of polymer-to-polyanion (Jonassen et al., 2012). Results indicated that NPs dispersed in a saline solution showed higher stability and a smaller particle size in the presence of sodium chloride, which may be caused by the monovalent sodium salt screening out the electrostatic repulsion between the positively charged amine groups on the CH backbone. The presence of salt may enhance the flexibility within the polymer chain as well as its stability (Ilium, 1998).

Polyelectrolyte Complexes (PEC)

A polyelectrolyte complex is formed when plasmid DNA is mixed with a cationic charged polymer such as CH, which self-assembles into nanostructures because the anionic charge on the DNA is neutralized by the cationic polymer (Divya and Jisha, 2018). Spontaneous formation of CH NPs occurs after adding the DNA solution to the CH dissolved in acetic acid solution with mechanical shaking at room temperature (Erbacher et al., 1998).

Reverse Micelles Preparation

Brunel et al. first described a method for the preparation of reverse micelles (Brunel et al., 2008). A key benefit was the

absence of poisonous organic solvents and cross-linkers. Moreover, it was possible to obtain ultra-fine NPs in a narrow size range by this technique (Divya and Jisha, 2018). The CH aqueous solution is poured into an organic solvent containing a surfactant with constant agitation to allow the formation of reverse micelles (Zhao et al., 2011).

Microemulsion Method

In the microemulsion procedure, CH is mixed with glutaraldehyde in an acetic acid solution and poured into a surfactant in an organic solvent such as hexane (Mohammed et al., 2017). Then the mixture is continuously shaken at room temperature overnight. This covalent cross-linking reaction results in the formation of CH NPs. In the next step, the organic solvent is removed via evaporation under vacuum (Mohammed et al., 2017). Excessive surfactant can be removed via precipitation with calcium chloride and centrifugation. The resultant NP suspension is finally dialyzed and lyophilized (Maitra et al., 1999). The CH NPs show a very narrow size distribution, which can be controlled by varying the glutaraldehyde concentration (Wang et al., 2008). The disadvantages of the microemulsion method are the need for an organic solvent, a prolonged reaction time, and a complicated washing-step (Nagpal et al., 2010).

Emulsification and Solvent Diffusion

In order to prepare an oil-in-water emulsion, an aqueous solution of CH and a stabilizer is mixed with an organic solvent by mechanical shaking accompanied by increased pressure homogenization (Niwa et al., 1993; El-Shabouri, 2002). Precipitation is caused by adding a large amount of water into the emulsion, and the NPs are formed with a size ranging between 300 nm and 500 nm. This procedure is a good choice to load hydrophobic molecules, with a high entrapment efficiency (EE%); however, a key caveat is the need for an increased shear force (Mohammed et al., 2017).

CHITOSAN AS AN ADJUVANT FOR ANTIVIRAL VACCINES

As the second most plentiful polysaccharide in nature, CH has seen widespread utilization in drug formulation and pharmaceutics, due to its non-toxicity, biodegradability, biocompatibility, and its ability to cross tight epithelial junctions (Ilium, 1998). CH can open tight intercellular junctions, thus allowing the loaded drugs to penetrate better into tissue and be taken up by target cells (Van Der Lubben et al., 2001a; Van der Lubben et al., 2001b; Van der Lubben et al., 2001c). CH NPs are also promising biomaterials to improve antigen delivery and the performance of vaccines. The association of antigens with chitosan-based nanoparticle systems, has shown that antigen uptake by mucosal lymphatic tissues is increased, resulting in more potent mucosal and systemic immune responses to different antigens (Bramwell and Perrie, 2006). Chitosan has mucoadhesive properties, which increases the long-term adhesion of the CH NPs, and therefore a longer time in contact with the bloodstream

capillaries, leading to greater uptake of the antigen protein or the plasmid DNA (Khatri et al., 2008a).

The respiratory mucosal surface acts as a primary immune defense barrier, and is the main site of influenza virus infection. Many studies have shown that mucosal vaccines could be used to induce both a systemic and a local mucosal immune response (Yuki and Kiyono, 2003). When vaccines are combined with an adjuvant, the induction of mucosal immunity is improved. Some adjuvants are toxins, such as *Escherichia coli* heat-labile toxin or cholera toxin (Glück et al., 2000). However these toxins can cause acute diarrhea and damage to the central nervous system (CNS) (van Ginkel et al., 2000). Therefore, the development of more effective and safer adjuvants is essential to improve mucosal immunization.

Recently, the use of self-assembled biodegradable CH NPs has emerged as an efficient vaccine delivery system (Uto et al., 2007). The interaction of NPs with antigens leads to improved antigen-specific acquired immune responses by increasing the uptake by antigen presenting cells, such as dendritic cells (DCs) and macrophages (**Figure 2**) (Akagi et al., 2005).

In addition, when NPs are taken up by DCs, it leads to up-regulation of costimulatory molecules, stimulation of cytokine production, and increased T-cell stimulation (Uto et al., 2007). Therefore, NP delivery systems have been proposed as efficient adjuvants for sub-unit vaccines. Nevertheless, the non-biodegradable nature of many types of NPs is a problem with the delivery of vaccines. Moreover, poly-gamma-glutamic acid (g-PGA), which is a capsular polymer secreted by *Bacillus subtilis*, is considered to be a safer alternative. (Poo et al., 2010). Moon et al. (2012) described a type of hybrid NPs composed of g-PGA plus CH. They showed that the hybrid NPs allowed intranasal immunization by an inactivated virus or by the model antigen, recombinant influenza hemagglutinin protein (rHA) leading to increased anti-HA immunoglobulin A (IgA) and IgG responses. This intranasal vaccination protocol protected mice against infection with a pathogenic dose of influenza type A H5N1 virus (Moon et al., 2012).

Mucosal immunization can lead to a mucosal immune response with or without a systemic immune response. The mucosal response was due to the production of secreted IgA (sIgA) antibody, without the production of systemic immunization (Nugent, 1998). However, it is accepted that oral vaccination shows higher efficiency. Degradation of antigens in the acidic stomach environment as well as enzymatic degradation in the intestinal tract contributes to the higher efficacy of oral vaccination (Gabor et al., 2002; Lavelle et al., 2004; Singh et al., 2004).

Chowdhury et al. described the production of a mucosal influenza vaccine system, which showed broad cross-protection against both emerging and seasonal influenza A viruses. They loaded poly-γ-glutamic acid (γ-PGA)-chitosan NPs (PC NPs) with the strongly conserved matrix protein-2 (sM2), the mucosal adjuvant cholera toxin subunit A1 (CTA1), and a fusion peptide of hemagglutinin (HA$_2$), all of which have been demonstrated to be safe natural substances with the ability of being absorbed by mucosal membrane as a mucosal adjuvant (Chowdhury et al., 2017). Results of the study showed that mucosal administration of this sM2HA2CTA1/PC NP preparation could trigger both local
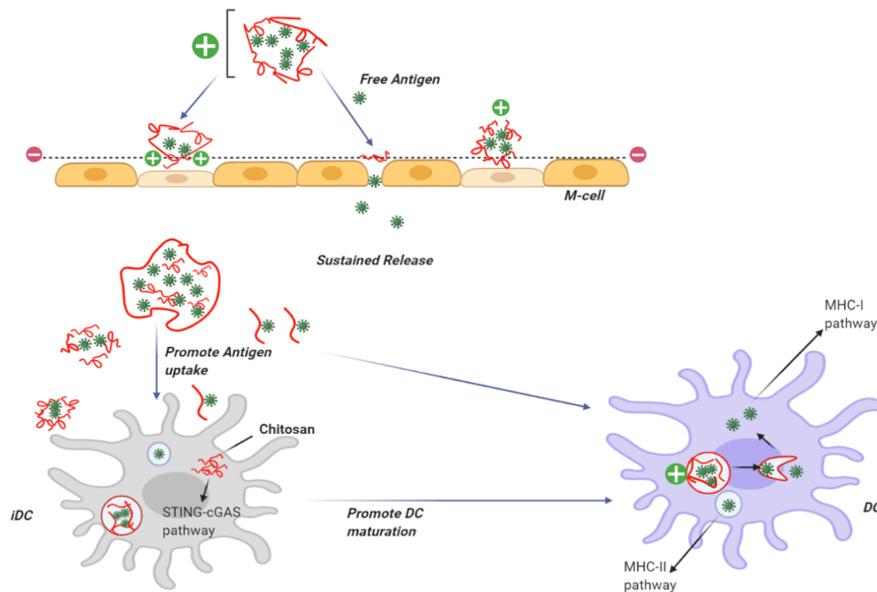
FIGURE 2 | Use of chitosan as an adjuvant for vaccine delivery. CH can be dissolved in acid to provide a positive charge on the particle surface. CH NPs containing antigens can adhere to the cell surface in the nasal mucosa giving a prolonged residence time. CH can open the tight junctions and allow the transfer of free antigens through the para-cellular route, as well as the transcytosis of the packaged antigens *via* M-cells. CH becomes insoluble at physiological pH, providing slow release of encapsulated antigens and uptake bydendritic cells DCs. Following uptake by DCs, CH activates the STING-cGAS pathway resulting in DC maturation. After the insoluble particles have been taken up by cells *via* endocytosis, CH becomes soluble at the acidic pH and the antigens escape from the lysosomes into the cytoplasm for cross presentation through the MHC-I pathway. Antigens taken up by DCs can berouted to the MHC-II pathway.

immunity (IgA& IgG) at the inoculation site, and remote systemic immunity. In addition, researchers confirmed that the inclusion of sM2 and HA2 triggered specific cell-mediated immune responses. The following challenge tests were carried out in BALB/c mice. IAV strains: A/Puerto Rico/8/34(H1N1), 10 MLD_{50} of A/EM/ Korea/W149/06(H5N1), A/Aquatic bird/Korea/W44/2005 (H7N3), A/Chicken/Korea/116/2004(H9N2), and A/Aquatic bird/Korea/W81/2005(H5N2). The sM2HA2CTA1/PC NPs showed protection against several lethal influenza sub-types, and the protection remained for up to 6 months following the vaccination. Therefore, sM2HA2CTA1/PC NPs may be an attractive option for a global influenza vaccine (Chowdhury et al., 2017).

Another study investigated the intranasal administration of NPs consisting of CH and poly-γ-glutamic acid (γ-PGA), and loaded with rHA antigen or an inactivated virus, for the induction of a high-degree of protective mucosal immunity in the respiratory tract (Moon et al., 2012). These researchers found that intranasal immunization with γ-PGA/CHNPs (PC NPs), containing either inactivated virus or rHA antigen, resulted in a good IgG response in the serum and an IgA response in the lung, with anti-HA neutralizing antibodies and influenza virus-specific cell-mediated immune responses. Therefore, PC NPs were capable of functioning as a potent mucosal adjuvant in comparison to cholera toxin (CT), which is a frequently used mucosal adjuvant. The mice were protected against challenge with a lethal dose of pathogenic influenza A H5N1 virus after intra-nasal administration of PC NPs loaded with rHA antigen, or the inactivated virus. It could be possible to combine PC NPs with a recombinant sub-unit influenza antigen manufactured on a large scale by a prokaryotic expression system (Moon et al., 2012).

However, low uptake of antigens by the gut-associated lymphoid tissue (GALT) leads to decreased effectiveness. Therefore a large dosage of antigen may be needed for effective immunity (Gupta et al., 2006). Mishra and colleagues (Mishra et al., 2014) tested hybrid NPs composed of CH plus *Lotus tetragonolobus* lectin (LTA) for mucosal immunization against the hepatitis B virus (HBV). In this study, the mucosal immunity was evaluated by examining salivary, intestinal, and vaginal secretions of IgA. LTA-conjugated CH-NPs evoked robust humoral and cellular responses, and could be used for oral mucosal immunization against HBV (Mishra et al., 2014).

In another study, Zhao et al. (2017) investigated NPs prepared from N-2-hydroxy-propyl trimethyl ammonium chloride modified CH (N-2-HACC) as well as N,O-carboxymethyl-modified CH (CMC) as carriers and adjuvants for vaccination. The modified CH NPs showed a higher stability and lower cytotoxicity than unmodified CH NPs. Both vehicles allowed steady release of antigens, following an early burst release. Moreover, in another study, N-2-HACC-CMC/NDV NPs administered intranasally, produced higher titers of IgA and IgG antibodies, and higher proliferation of lymphocytes, as well as increased levels of IL-2, IL-4 and IFN-γ. N-2-HACC-CMC may be a suitable delivery carrier and adjuvant for mucosal vaccines, as well as for transmucosal drug delivery (Zhao et al., 2017).

THE EFFECT OF CH AND ITS DERIVATIVES ON VIRAL INFECTIONS

CH is widely used in both human and veterinary medicine (Muzzarelli et al., 1998), therefore, its pharmacological properties, and especially its effect on the immune system have been studied in detail. Chitosan strongly modulates the functional activity of many immune cells, such as granulocytes and macrophages. After subcutaneous implantation, CH triggered chemotaxis of *Canis familiaris* L. macrophages, and increased nitric oxide production by macrophages *in vitro*. It also stimulated leukocytosis in the peripheral blood of laboratory dogs. The secretion of nitric oxide was mainly due to the N-acetylglucosamine residues in chitosan, which was more effective than N-acetylmannosamine or N-acetylgalactosamine (Chirkov, 2002).

The function of macrophages is critical for an effective immune system response. Macrophages are antigen-presenting cells, and their interaction with T-helper cells stimulates cellular and humoral immune responses. Stimulation of macrophage functional activity with CH can also be important for suppressing viral infection in animals. In this regard, it should be mentioned that when mouse alveolar macrophages took up chitosan or chitin NPs by phagocytosis, the generation of reactive oxygen species was increased. Likewise, mouse splenocytes secreted higher amounts of γ-interferon. Interferon is known to suppress virus replication by impairing the translation ability of genomic RNAs or early viral mRNAs (Shibata et al., 1997; Chirkov, 2002). Thus, the ability of CH to stimulate interferon synthesis by macrophages could be an additional antiviral mechanism. Chitosan inhibited the reproduction of *Chlamydia trachomatis* (an obligate intracellular parasite) within HeLa cells, mainly by suppressing the uptake of parasites by neighboring cells (Pawar et al., 2008). Chitosan enhanced antiviral immune responses by increasing the production of IgG and IgA antibodies against influenza A (Texas H1N1) and influenza B (Panama) viruses (Chirkov, 2002).

Sulfated chitosan derivatives have been synthesized to specifically inhibit retrovirus replication. Studies have shown that N-carboxymethyl chitosan-N,O-sulfate could inhibit the synthesis of virus-specific proteins, and decrease HIV-1 replication in cultured T-cells, as well as the replication of Rausher murine leukemia virus in cultured mouse fibroblasts. It was found that sulfated N-carboxymethyl chitosan prevented the interaction of the viral glycoprotein gp120 (the key viral coat protein) with its receptors on T-lymphocytes. This activity lessened the entry of the virus into CD4+ cells. In addition, the modified CH competitively inhibited the activity of virus specific reverse transcriptase, most probably through interference with the enzyme binding to its polyA-oligo-dT template. The chitosan derivative showed virtually no cytotoxicity towards these cell cultures (Chirkov, 2002; Kim, 2013). The highest activity was found with a chitosan derivative sulfated at the O2 and/or O3 positions within the glucosamine residues. This derivative was effective in inhibiting the replication of HIV-1 in MT-4 lymphocytes. The anionic polymer probably bound to the positively charged V3-loop in the gp120 molecule *via* an electrostatic interaction, preventing the fusion of the virus and the cell membrane (Chirkov, 2002). It was noted that, although the ability of anionic chitosan derivatives to inhibit retroviral infection was overall similar to the effects of sulfated polysaccharides, such as heparin, dextran sulfate, etc., the effectiveness and specificity depended on the position of the sulfate groups in the glucosamine residue.

Amino groups (NH2) and hydroxyl groups (OH) are functional groups that mediate the biological activity of CH (Chen and Zhao, 2012). The molecular weight and degree of deacetylation of CH also play an important role in defining the biological activity (Tong and Chen, 2013). The number of amino groups affects the antibacterial and antioxidant activity (Klaykruayat et al., 2010; Xiao et al., 2011). Je and Kim (Je and Kim, 2006) found that aminoethyl modified chitosan provided an antioxidant effect against hydroxyl and superoxide anion radicals. Moreover, the antibacterial effects of aminoethyl-modified chitosan were seen in the inhibition of *Escherichia coli* (Meng et al., 2012). Chitosan also showed antiviral effects against viral infections in plants and phage infections in bacteria (Pospieszny et al., 1991; Chirkov, 2002; Kulikov et al., 2006). One study suggested that aminoethyl-modified CH exerted its antiviral activity by stimulation of the immune response (He et al., 2019).

Topical microbicides have been developed to prevent infection by human papillomaviruses (HPV). The main goal of these microbicides is to block the interaction between HPV virions and heparan sulfate proteoglycan (HSPG) molecules that act as receptors on the surface of the host cells (Shafti-Keramat et al., 2003). Heparin, as well as some heparin-like polysaccharides including dextran sulfate and carrageenan, have shown the ability to prevent HPV binding to the cell surface (Lembo et al., 2008). Various types of CH have also demonstrated this property (Wang et al., 2012). Sulfated CH shows a range of biological activities, including antitumor, antioxidant, anticoagulant and antiviral properties (Kulikov et al., 2006; Artan et al., 2010; Yang et al., 2013). Sulfated chito-oligosaccharide exerts an inhibitory effect on viral entry, as well as preventing virus-cell fusion through the inhibition of HIV-1gp120 binding to the CD4+ cell surface receptor. (Wang et al., 2012). Therefore, CH and its sulfated derivatives could be useful as new antiviral agents (Wang et al., 2012). To further investigate the antiviral properties of sulfated chitosans, a study by Gao et al. (2018) investigated the anti-HPV effects of 3,6-O-sulfated CH (36S). They suggested that the anti-HPV effect of 3,6-O-sulfated CH could be due to targeting the viral capsid protein, and also to regulation of the host PI3K/Akt/mTOR pathways (Gao et al., 2018).

Recently, it has been reported that the wide use of current anti-influenza drugs has led to the emergence of drug-resistant viruses (De Jong et al., 2005; Yen et al., 2013). Therefore, it is very important to discover new treatment approaches. Zheng and colleagues (Zheng et al., 2016) showed that the innate immune system could be stimulated by nasal administration of chitosan, and this was effective enough to protect BALB/c mice from infection with the H7N9 virus, which is also highly pathogenic to humans

(Zheng et al., 2016). Su et al. (2009) reported that high-molecular-weight chitosan at high concentrations resulted in the inactivation of murine norovirus MNV-1. Li et al. synthesized a sialyl lactose-CH derivative by grafting a lactoside aldehyde-functionalized aglycone onto the CH amino groups, followed by enzymatic sialylation using sialyl-transferase. The glycosylated CH bound the influenza virus surface hemagglutinin protein with high affinity to suppress virus binding to host cells (**Figure 3**) (Li et al., 2011)

CHITOSAN VEHICLES FOR ANTIVIRAL DRUG DELIVERY

Drug delivery systems (DDS) are used for controlled and sustained drug release as well as targeted delivery to specific cells and tissues (**Figure 4**). The primary advantage of these controlled-release systems is to lessen the side effects of drugs by widening the therapeutic range between the lowest effective and toxic concentrations. This approach can enhance patient satisfaction by decreasing the number of injections, reducing the overall dose, and increasing the specificity for the diseased site. Nonetheless, DDS still suffer from some limitations, like poor biocompatibility as well as possible toxicity of the matrix itself, or its bio-degradation products. The administration route may be problematic, for example surgical operation may be needed for implantation, and their cost may be high in comparison with conventional formulations (Bernkop-Schnürch and Dünnhaupt, 2012; Hu et al., 2013).

CH has biological properties such as being hemostatic, bacteriostatic, anti-cholesteremic, anti-carcinogenic, as well as fungistatic (Saikia et al., 2015), These activities have made it a popular candidate in biomedical applications like DDS. Since CH is a naturally occurring biopolymer, its biocompatibility and lack

of toxicity are viewed as advantages. CH has been approved by the FDA as a hemostatic dressing called HemCon™ for battlefield and civilian medical uses (Wedmore et al., 2006). Both low and high molecular weight CH will undergo metabolism as well as enzymatic degradation within the body, leading to removal by renal clearance (Funkhouser and Aronson, 2007). Notably, the degradation rate is dependent on the degree of deacetylation. CH can be dissolved in acid, but then becomes insoluble at physiologic (7.4) or basic pH. The presence of two hydroxyl groups on each structural unitas well as the primary amine groups, allows easy chemical modification, and facile physical interaction with metallic or metal oxide NPs, organic or inorganic compounds, and polymers (Luo and Wang, 2014; Abd Elgadir et al., 2015; Saikia et al., 2015). CH is preferred in the formulation of DDSs, because of its cationic nature allowing interaction with anionic drugs or compounds, its mucoadhesive properties, greater permeation into tissue, ability to allow cellular transfection of nucleic acids, and its suppression of multi-drug efflux pumps (Ngo et al., 2015).

Acquired immune deficiency syndrome (AIDS) is caused by infection with the HIV virus, and has caused the deaths of millions of people worldwide (das Neves et al., 2010; Sadri Nahand et al., 2020). A new therapeutic approach using a combination drug regimen, is called highly active anti-retroviral therapy (HAART) (das Neves et al., 2010). Five different types of antiretroviral drugs have been used to treat AIDS (Wong et al., 2010), each of which functions at different stages of the viral lifecycle (Gupta and Jain, 2010). For example, a protease inhibitor targets the viral protease that cleaves the GAG-POL precursor responsible for producing the viral enzymes that are required for viral proliferation (Tritch et al., 1991). Currently, the FDA has approved eight protease inhibitors, of which saquinavir has shown the highest activity.
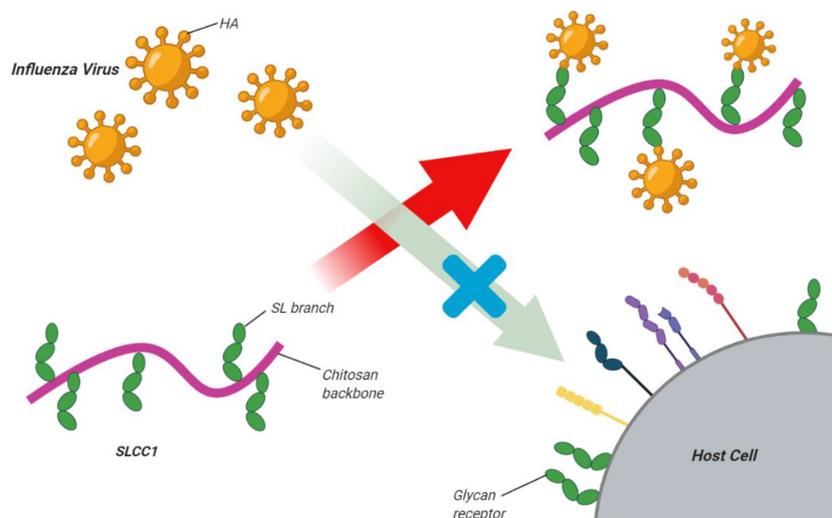
FIGURE 3 | Sialyl lactose-CH (SLCC1) derivative for inhibition of influenza virus. Li et al. synthesized a sialyl lactose-CH derivative *via* graftinga lactoside aldehyde-functionalized aglycone onto the CH amino groups followed by enzymatic sialylation using sialyl-transferase. The glycosylated CH bound the influenza virus surface hemagglutinin protein with high affinityto suppress virus binding to host cells. This figure adapted from (Li et al., 2011).
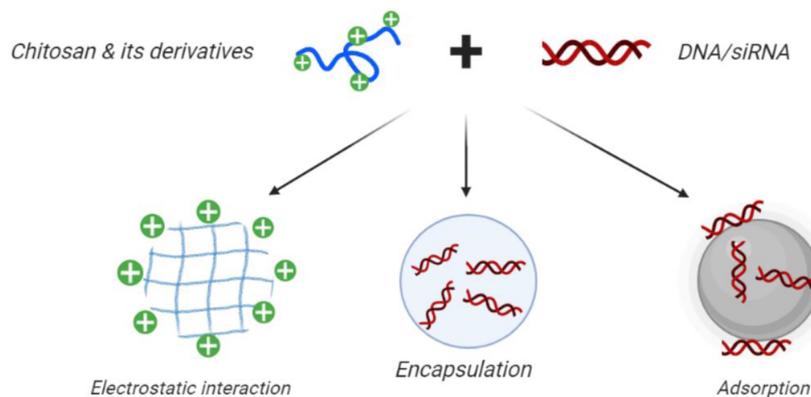
FIGURE 4 | Use of CH in gene therapy and gene silencing. Chitosan nanoparticles can be loaded with plasmid DNA, antisense oligonucleotides, or small interfering RNAs for targeted gene silencing. Positively charged CH can readily form polyelectrolyte complexes with negatively charged nucleic acids. This figure adapted from (Santos-Carballal et al., 2018).

Nevertheless, saquinavir has been shown to be limited by its poor bioavailability (Li and Chan, 1999), that has been largely ascribed to a group of MDR1 (multidrug resistance 1) drug efflux pumps. P-gp mediates anti-HIV drug efflux because of their similarity to the natural substrates of P-gp mediated efflux systems (Kim A. E. et al., 1998; Kim R. B. et al., 1998). Rapid efflux of the drugs causes a lower intracellular concentration of the drug within the cells. Another mechanism ssuggested for the lower bioavailability of saquinavir is its metabolism in the liver and small intestine, that results in rapid clearance (Thummel et al., 1997). The poor bioavailability of saquinavir necessitates the use of higher doses that could encourage the emergence of drug resistance (Schapiro et al., 1996). The use of saquinavir can have many side effects like nausea, dizziness, arrhythmia, etc. Hence, nanotechnology-based delivery vehicles could be used to increase the potency of saquinavir. Ramana et al. (2014) investigated a chitosan-based nanodelivery strategy for saquinavir. In this study, the CH NPs were loaded with saquinavir and characterized by transmission electron microscopy (TEM) and differential scan calorimetry. They also measured the encapsulation efficacy, swelling properties, zeta potential, and dimensions of the NPs. The cellular uptake of CHNPs was assessed by confocal microscopy and flow cytometry. The anti-viral effectiveness was studied in cell culture. The researchers reported a 75% efficiency for saquinavir encapsulation, and a cell targeting efficiency > 92%. Saquinavir loaded CH carriers resulted in better control of viral proliferation compared to soluble drug alone, with two different HIV strains (NL4-3 and Indie-C1) and two different types of T-cells (CEM-CCR5 and Jurkat). This study demonstrated that better drug loading combined with increased cell targeting efficiency led to effective control of viral replication in the targeted T-cells (Ramana et al., 2014).

Herpes simplex virus type 1 (HSV-1) is an endemic highly transmissible virus, which is mainly transmitted by mouth-to-mouth contact. It is projected to infect up to 3.7 billion people under 50 years old (D'Affronte and Platia, 2020). In fact, HSV-1 primary infection usually occurs in childhood as an asymptomatic infection, but can develop into herpetic gingiva-stomatitis with recurrent bouts of oral labial lesions (*e.g.*, fever blisters, cold sores, or herpes labialis). HSV-2, which is primarily transmitted through sexual contact, causes genital herpes which affected about 417,000,000 people globally in 2012 (Roizman, 2006; D'Affronte and Platia, 2020). Different anti-herpes drugs like famciclovir, valacyclovir or acyclovir have been approved, and are used for treating acute symptomatic herpetic infections (Andrews et al., 2006; Le Cleach et al., 2014). Moreover, recurrent orolabial mucocutaneous herpes will usually be treated with topical anti-viral agents to accelerate wound healing and reduce symptoms like pain, tingling, itching, and burning (Spruance and Kriesel, 2002). In addition, acyclovir (9-[(2-hydroxyethyl)methyl])-9H-guanine) is the treatment of choice for HSV infection. However, it should be administered several times a day to be effective, because of its short half-life as well as imperfect adsorption. Although acyclovir is used as a topical treatment (Zovirax), due to its hydrophilic nature and its poor solubility in both aqueous and fatty solvents, it does not penetrate well into the stratum corneum. New topical formulations of acyclovir have been designed to penetrate through the epidermis to reach the basal layer where the virus exists, at sufficient concentrations to inhibit replication (Prabhu et al., 2012). Donalisio and colleagues (Donalisio et al., 2018) investigated the effects of acyclovir-loaded CH nanospheres prepared by nanoemulsion templating for topical treatment of herpes-virus infections. The novel CH nanospheres were characterized by dynamic light scattering (DLS), TEM, and *in-vitro* drug release studies. A Franz cell was used to study the drug penetration through porcine skin *ex vivo*. Viral inhibition studies were done on Vero cells infected with HSV-2 and HSV-1 strains. The chitosan NS-loaded with acyclovir had a spherical shape about 200 nm in diameter and a 40.0 mV negative zeta-potential. The drug loading capacity was nearly 8.5% and 30% of the acyclovir was released from the nanospheres within 6 h. Ex vivo skin penetration was better than with acyclovir 5% cream. The acyclovir-NS complex exhibited more potent anti-viral activity

compared to free acyclovir against HSV-1 as well as HSV-2 strains. Furthermore, the acyclovir-loaded NS did not display any anti-proliferative activity or any sign of cytotoxicity against host cells (Donalisio et al., 2018).

In another study, Calderón et al. (2013) prepared microparticles (MPs) as well as NPs composed of CH cross-linked with tripolyphosphate to deliver acyclovir. The system showed biocompatibility, bioadhesive properties, and potential as a penetration enhancer through the skin. The amounts of acyclovir diffused within 24 hours were as follows: 30, 40, and 80 μg for ACV alone, ACV + MP solution, and ACV loaded NPs respectively. Moreover, CH-based particles caused less tissue damage and only moderate irritation as judged by the slug mucosal irritation (SMI) assay, suggesting that ACV-NPs could be used to develop an anti-viral formulation.

In another study, Hasanovic et al. (2009) created a skin delivery system for aciclovir based on CH–tripolyphosphate NPs with good chemical stability. In this study, NPs were spontaneously formed by ionotropic gelation with tripolyphosphate. Two distinct dimensions of aciclovir-loaded NPs were characterized for their zeta-potential, particle size, and polydispersity index. A standard diffusion test with the use of a Franz diffusion-cell indicated good skin permeability dependent on the size of CH NPs, and good acyclovir loading. If the chitosan content of the NPs was higher, more acyclovir penetrated through porcine skin. Differential scanning calorimetry showed a significant reduction in the average transition temperature, showing that the NPs interacted with two layers (skin and fat). In addition, the chemical stability of acyclovir was considerably enhanced by NPs incorporation. After five weeks of photo-oxidation, the acyclovir content in the nanoparticles was remarkably higher than with a pure aqueous solution. The present study demonstrated that the incorporation of aciclovir within the CH-tripolyphosphate NPs substantially enhanced the chemical stability. Skin diffusion was better with acyclovir CHNPs, particularly those with a high CH content (Hasanovic et al., 2009).

Applications of CH for drug delivery have been limited due to its low tissue penetration as well as its poor solubility at pH> 5.6. N,N,N-trimethyl CH was prepared via amine functionalization, and showed improved chemical stability, solubility, biological adsorption, porosity, and non-antigenic properties (Hagenaars et al., 2009a). Therefore N-TMC has been proposed as a DDS for vaccines (**Figure 4**), drugs, biomolecules, antimicrobials, and as a scaffold matrix for skin, bone and nerve regeneration (Balmayor et al., 2012; de Britto et al., 2012; Radhakumary et al., 2011; Tsai et al., 2011).

In another study, Dehghan and colleagues (2014) investigated chitosan nanospheres to deliver an influenza vaccine. In this study, whole influenza virus combined either with CpG oligo-deoxynucleotide (CpG ODN) or with Quillaja saponin (QS) as adjuvants, and incorporated into CH nanospheres. Three doses of the dry powder nanosphere vaccine were administered nasally into rabbits on days 0, 45, and 60, with an additional booster dose on day 75. The cellular and humoral immune responses were examined, showing an increased titer of hemagglutination

inhibition (HI) antibody in both groups in comparison with controls. CH nanospheres (WV+CpG) and CH (WV+QS) were more effective that virus alone, and CpG alone (p<0.001). The CH(WV+CpG) group showed the maximum response with rabbit serum IgG titer. The CH (WV+CpG) resulted in higher induction of sIgA titers compared to CH (WV+QS) (p<0.001), and the CpG adjuvant had a major contribution to the secretion and stimulation of IL-2 and IFN-g cytokines (3-fold & 3.5-fold increase). The CH (WV+CpG) vaccine induced the best cellular and humoral immune responses against the influenza virus following nasal administration (Dehghan et al., 2014).

CH and its derivatives have been used for organ-specific targeted drug delivery. For instance some systems can target the liver by relying on passive accumulation of DDS by the reticulo-endothelial system (RES), or else by active targeting of the liver by recognition of the ligand-modified DDS by specific hepatic receptors. One study reported the synthesis of lactosaminated N-succinyl-CH, and investigated its potential as a liver-specific drug carrier (Kato et al., 2001). This carrier bound to the asialoglycoprotein receptor (ASGP-R) and accumulated in the liver. The lactose-conjugated CH modified with PEG formed polyionic complex micelles to deliver the anti-inflammatory drug called diammonium glycyrrhizinate to treat acute HBV infection (Yang et al., 2009). Another study from Lin et al. (2008) described the modification of CHNPs by conjugation to glycyrrhizin. Conjugation was achieved by the oxidation of glycyrrhizin using sodium periodate to form aldehyde groups, that could then react with CH amino groups (Lin et al., 2008). In-vitro investigations showed the localization of the glycyrrhizin conjugated CH NPs in hepatocytes, with an uptake 4.9 times greater than that of non-hepatic parenchymal cells. The dose of NPs and the incubation time governed the cellular uptake, that was mediated by ligand-receptor interactions (Singh et al., 2018).

Another group used a double emulsification procedure to prepare cationic PLGA-CH NPs with HBsAg passively absorbed onto the surface, for site-specific delivery of IFN-α to hepatocytes. The HbsAg-coated (99m)Tc labeled PLGA-CHNPs showed remarkable recovery of liver function compared with unmodified PLGA NPs (Giri et al., 2011). The size of the NPs as well the hydrophobicity affected the cell-mediated and mucosal immune responses. The HBsAg-modified NPs could be administered via pulmonary delivery. Hydrophobic particles > 500 nm gave the best improvement in secreted IgA, IFN-γ, and interleukin-2 levels compared to hydrophilic particles < 500 nm. Larger hydrophobic particles were taken up into rat alveolar macrophages to increase the immune response (Thomas et al., 2011). Polymeric NPs formulated for HBV gene silencing using the biodegradable polymer PLGA were more effective when the cationic polymer CH was incorporated into the matrix. The plasmid DNA loading efficiency and the cellular internalization were improved. In this regard, Zeng et al. (2011) showed improved HBV silencing with CH–PLGA system, than the plain plasmid DNA (pDNA) or simple PLGA NPs. The CH–PLGA system did not show any adverse effects (Zeng et al., 2011). **Table 1** lists various chitosan nanoparticles that have been used as drug delivery systems for viral infections.

CHITOSAN VEHICLES FOR ANTIVIRAL VACCINATION

Influenza occurs in pigs (swine flu) caused by the influenza A-virus (IAV) of the Orthomyxoviridae family. Influenza is an economically significant disease in the pig farming industry (Dykhuis-Haden et al., 2012; Crisci et al., 2013). Swine IAV (SwIAV) infection causes an acute febrile respiratory illness, which is frequently followed by secondary bacterial infections (Dykhuis-Haden et al., 2012). Moreover, the SwIAV virus modifies its genetic diversity via repeated antigenic drifts or shifts. The main circulating strains of SwIAV in pigs are H1N1, H1N2, and H3N2 (Vincent et al., 2008). Since pig respiratory tract epithelial cells have receptors for human IAVs as well as avian IAVs, pigs may become infected with IAV strains from various hosts. SwIAV infections allow genetic recombination as well as adaptation of new influenza strains that may infect humans and animals, and even cause epidemics (Ito et al., 1998). Commercially available swine flu vaccines are based on multivalent whole inactivated virus (WIV) vaccines given intramuscularly (IM). (Vincent et al., 2017). These WIV vaccines protect against homologous virus infection, however they cannot induce sufficient heterologous immunity against the continually evolving IAVs caused by point mutations (Van Reeth and Ma, 2012; Vincent et al., 2017).

In addition, when the IM pathway is utilized for WIV vaccines, it will not produce an effective mucosal immune response, that is really necessary to provide cross-protective immunity against different types of IAV (Tamura and Kurata, 2004; van Riet et al., 2012). An intranasal (IN) vaccine, which targets the respiratory tract mucosal immune system, could be an improvement over IM influenza vaccines employed in pigs. Additionally, nasal mucosal vaccination results in the induction of a powerful protective immune response in the respiratory tract, and also improves immunity at the distal mucosal and systemic sites (Neutra and Kozlowski, 2006; Kim and Jang, 2017). Various kinds of NPs have been studied for IN delivery of antigens in influenza vaccines. For instance, IN immunization in a mouse model using liposome-based DNA and influenza nanovaccines induced cellular, mucosal and humoral immune responses (Wang et al., 2004). Furthermore, poly (lactic-co-glycolic) acid (PLGA) NPs loaded with highly-conserved H1N1 influenza virus peptides and administered IN triggered epitope-specific T-cell responses, as well as protective immunity in pigs (Hiremath et al., 2016). A ferritin-based IN influenza nanovaccine increased mucosal IgA secretion, T-cell responses and provided homo-subtype and hetero-subtype protection in mice (Qi et al., 2018).

Hepatitis virus B (HBV) is a severe chronic infectious liver disease that can cause cirrhosis, hepatocellular carcinoma, and even mortality if left untreated. It is estimated there are 4.7 million clinical cases of acute HBV annually (Beisel et al., 2020). Considering that effective drug treatments for HBV are lacking, parenteral vaccines involving recombinant DNA encoding HBsAg are used to protect nearly 95% of the recipients (Kwon and Lee, 2011). However the need for injection is expensive and causes patient dissatisfaction. Thus, a different administration route like intranasal (i.n.) using an appropriate DDS may be an alternative (Illum, 2003). The nasal mucosa has plentiful nasal-associated lymphoid tissue (NALT), a larger surface area, the presence of dendritic cells, as well as less enzymatic degradation compared to the oral route (Kang et al., 2009; Slütter et al., 2010). Finally, nasally administered drugs may have higher concentration, no first-pass effects, better permeation, and more patient compliance, without any side effects (Al-Qadi et al., 2011; Taranejoo et al., 2011).

Antigens encapsulated inside NPs and administered intranasally showed a higher uptake, a more controlled release of the antigens that can pass through the nasal membranes to reach the vasculature with higher immunogenicity and a systemic immune response (Kyd et al., 2001; Costantino et al., 2007). Moreover, DDS greater than 100 nm in size administered intranasally have a long residence time and mucoadhesive properties according to their surface charge, preventing polymer bio-degradation (Slütter et al., 2010).

Hepatitis-B virus surface antigen (HBsAg) was loaded into N, N,N-trimethyl chitosan NPs (N-TMC NPs) for controlled intra-nasal delivery as reported by Subbiah et al. (2012). The NP size was tunable by modifying the N-TMC content, giving 66 ± 13 nm for 0.25 wt%, and 76 ± 9 nm for 0.5 wt.%. A HBsAg loading of 380 and 760 μL/mL resulted in 143 ± 33, 259 ± 47 nm-sized spherical N-TMC NPs with a loading efficiency of HBsAg-antigen of 90%-93% and 96%-97% respectively. In-vivo immunological studies were conducted on 6-8 week old female BALB mice over 43 days, showing the high stability and efficiency of this HBV vaccination (Subbiah et al., 2012).

Recent approaches to treat hepatitis B have attempted to clear active HBV infections by suppressing viral replication. Some nucleoside or nucleotide analogs have been used as anti-viral drugs, to efficiently suppress HBV replication. In addition, lamivudine (LA) targets the host cell nucleus and inhibits the reverse transcriptase enzyme in order to terminate HBV replication (Asselah et al., 2005). There are some limitations of anti-viral drugs, such as non-specific biodistribution in-vivo, enzymatic metabolism, and issues in transportation across biological membranes (Langer, 1998). These limitations reduce the therapeutic benefit, require larger doses, and lead to side effects well as drug resistance. Moreover, by utilizing pharmaceutical engineering, the delivery of anti-viral drugs to their molecular targets will increase efficiency and decrease adverse effects. Earlier investigations revealed that the molecular activity of anti-HBV drugs largely takes place in subcellular organelles of the host cells, in addition to inhibiting HBV binding to its receptors (Zoulim and Perrillo, 2008). Thus, nanotechnology-based vehicles that can target sub-cellular organelles are an example of molecular targeted therapy (Torchilin, 2008). One example is stearic acid-grafted CH oligosaccharide polymeric micelles, that could target specific sub-cellular organelles (You et al., 2007; You et al., 2008).

Hydrophobic drugs are loaded into the hydrophobic core of the micelles via hydrophobic interactions, and possibly by metal-ligand coordination bonds (Nishiyama et al., 2001) or

TABLE 1 | Chitosan nanoparticles used as drug delivery systems for viral infections.

Chitosan NP composition	Size	Target virus	Model (*In vitro/In vivo*/Human)	Type of cell line	Findings	Ref
CH/AL-BV	434.6 ± 22.1 nm	Porcine reproductive & respiratory syndrome virus (PRRSV)	*In vivo*		Increased level of PRRSV-specific IgG as well as viral neutralizing antibody. Less severe interstitial pneumonia signs were reported on the microscopic evaluation and lung gross examination. Decreased viral burden in serum and tissue (lung and bronchial lymph nodes)	(Lee et al., 2018)
C-IV	222.1 nm	Viral hemorrhagic septicemia virus (VHSV)	*In vitro*		Increased anti-VHSV antibody titer following vaccination. Increased immune gene transcripts (IgT, IgM, MHC-I, pIgR, IFN-γ, MHC-II, & Caspase3).	(Kole et al., 2019)
CNPs	571.7 nm	Swine influenza A virus (SwIAV)	*In vitro* and *in vivo*	MDCK	Reduced nasal viral shedding and lung virus titers. Induced the secretion of cytokines, increased IAV-specific mucosal antibody response, and systemic specific cell mediated immune response against IAV.	(Dhakal et al., 2018)
CS/TPPr-VP28	50-80 nm	White spot syndrome virus	*In vivo*		Induced expression of immune-related genes	(Taju et al., 2018)
siRNA/CN	278 nm	Influenza virus	*In vitro* and *in vivo*	Vero	Inhibition of influenza virus replication, antiviral effects.	(Jamali et al., 2018)
CN - CpG	65–250 nm	Influenza	*In vivo*		Stimulation of humoral and cellular immunity, increased weight gain, and decreased mouse mortality.	(Sadati et al., 2018)
CN/CMD/PEI	100-150nm	HIV	*In vitro*	RAW264.7 and HEK293	Improved siRNA delivery with good cell viability. Reduced the RNA and protein expression of HIV-1 tat peptide	(Iranpur Mobarakeh et al., 2019)
CH-NP	295 ± 26 nm	HBV	*In vitro*	M cells	Elicited strong humoral and cellular immune responses.	(Mishra et al., 2014)
pFNDV-CS	199.5 nm	Newcastle virus	*In vitro*	293T	Improved immune response and prolonged release of plasmid DNA	(Zhao et al., 2014a)
CN- S	211 ± 11.50nm	HIV	*In vitro*	Jurkat cells	Better drug loading Increased cell targeting efficiency. Effective control of viral proliferation and rapid growth of targeted T cells.	(Ramana et al., 2014)
CS-TGA	240 - 252 nm	HIV	*In vitro*	VK2/E6E7 &End1/ E6E7	Prevention of HIV transmission Superior biophysical properties Maximized RT of a topical microbicide.	(Meng et al., 2014)
CS-M	130-500 nm	Foot and mouth disease virus	*In vitro* and *in vivo*	BHK-21	Improved immunological parameters. Enhanced protection.	(Nanda et al., 2014)
Nac-6-IOPs	NA	Coxsackie-adenovirus	*In vitro* and *in vivo*	K562	High transduction efficiency in cells and organs. Binds to K562 cells	(Bhattarai et al., 2008)
O-2'-HACC		Newcastle virus	*In vivo*		Induced strong cellular, humoral and mucosal immune responses	(Dai et al., 2015)
N-2-HFCC/ CMC	252.2 ± 32.68nm	Newcastle virus	*In vivo*		Increased lymphocyte proliferation and serum antibody titer. Increased IFN-γ and IL-2.	(Jin et al., 2017)
c-ChonS nanoPECs	NA	HIV-1	*In vitro*	PBMCs	Non-cytotoxic to PBMCs Dose-dependent decrease ofHIV-1 infection.	(Wu et al., 2016)
CH-PLGA-DNA and CH-Tre-Inactivated	500 nm	Type A foot-and-mouth disease	*In vivo*		Increased mucosal, systemic, and cell-mediated immunity.	(Pan et al., 2014)
f-TFV CS NPs-Gel	545.1 ± 69.17nm	HIV virus	*In vitro*	L929	Controlled release of tenofovir. Prevented HIV transmission.	(Timur et al., 2018)
CS-PCL	125.64 ± 6.51 nm	Influenza A virus (A/California/07/2009) H1N1	*In vivo*		Mucosal & systemic humoral& cellular immune response.	(Gupta et al., 2011)
CS/TPP-HA	358.67 ± 5.13nm	Hemagglutinin (HA)-split influenza virus	*In vivo*		Increased number of IFN-γ-secreting cells in spleen. Markedly reduced influenza morbidity.	(Sawaengsak et al., 2014)
N-TMC NPs	66 ± 13, 76 ± 9 nm	Hepatitis B virus	*In vivo*		Treated hepatitis B & allergic rhinitis.	(Subbiah et al., 2012)
C-RGVP	200-400 nm	Hepatitis B virus	*In vivo*		–	(Kim and Kang, 2008)
HBsAg CH NP	00-250 nm	Hepatitis B surface antigen	*In vivo*		Increased anti-HB secreting cells. Generated robust durable immunity.	(Lugade et al., 2013)

(Continued)

TABLE 1 | Continued

Chitosan NP composition	Size	Target virus	Model (*In vitro/ In vivo*/Human)	Type of cell line	Findings	Ref
mPEG-PLA-PEI	88.9nm	Hepatitis B virus	*In vitro*	PLC/PRF/5	Increased Tfh cells (BAFF-R (+) B-cells and CD138 +plasma cells). siRNA delivery	(Wang et al., 2010)
CH nanoemulsion	120-160 nm	Hepatitis E virus	*In vitro*	HeLa & THP1	Good cell uptake and release No cytotoxicity	(Rani et al., 2018)
pFDNA-CS/ PLGA-NPs	699.1 ± 5.21 nm	Newcastle disease virus	*In vitro* and *in vivo*	293T	Controlled release of plasmid DNA. Induced strong cellular, humoral, & mucosal immune responses.	(Zhao et al., 2014b)
CH-NS/MS & HTCC-NS/MS	60 nm	Human corona viruses, HCoV-NL63 &HCoV-OC43 Mouse hepatitis virus (MHV)	*In vitro*	LLC-MK2, HCT 8 & LR7	Genipin used to cross-link CH Could be used for concentration of virus from samples or purification of water supply	(Ciejka et al., 2017)
PLGA-CHS NS	60 nm	Hepatitis B virus	*In vitro*	HepG2.2.15	Able to treat viral hepatitis in-vivo.	(Zeng et al., 2011)
CTS-Fe$_3$O$_4$ NPs	NA	HCV	*In vivo*		Increased antibody production and T-cell activity.	(Yang et al., 2011)
NDV/La Sota-N-2-HACC-NPs	303.88 ± 49.8nm	Newcastle disease viruses	*In-vitro* & *in-vivo*	293-T	Increased strong humoral, mucosal and cellular immune response.	(Zhao et al., 2016)
C- QCH4-dsRNA	400nm	Yellow head virus	*In vitro*	Sf9	Effective dsRNA carrier	(Theerawanitchpan et al., 2012)
CH-O-isopropyl-5'-O-d4T monophosphate	166.8nm	HIV	*In vitro*	MT4	Prevented leakage of cargo from the NPs before reaching targeted viral reservoir.	(Yang et al., 2010)
D- CN	382 ± 18nm	HIV	*In vivo*		Improved delivery system	(Al-Ghananeem et al., 2010)

electrostatic interactions (Kabanov et al., 1996). However, lamivudine (LA) has only very low hydrophobicity, which can be increased by chemical modification. For instance, lamivudine stearate (LAS) was synthesized by esterification of LA with stearic acid by Li et al. (2010). After esterification, the octanol: water distribution coefficient (log P) of LA was increased from -0.95 to 1.82. Moreover, g-CH oligosaccharide modified with stearic acid (CSO-SA) formed micelles that could be loaded with LAS showing rapid internalization and accumulation in tumor cells. CSO-SA with a 3.79% amino substitution degree (SD) showed a critical micellar concentration (CMC) of 0.032 mg/ml, and the micelles of a 1 mg/ml CSO-SA concentration showed a mean diameter of 460.8 nm with a narrow size distribution and zeta potential of +29.7 mV. After LAS loading, the micelle size decreased and the zeta-potential increased. The LAS-loaded CSO-SA micelles (CSO-SA/LAS) showed a pH dependent release of LA, with a greater release rate at pH 7.4 compared to pH 6.2. CSO-SA/LAS demonstrated lower cytotoxicity and higher cellular uptake of LA by HBV transfected tumor cells (HepG2.2.15) compared to free LA. The antiHBV activity of CSO-SA/LAS *in vitro* showed higher inhibition of antigen expression and DNA replication, in comparison to LAS and LA alone (Li et al., 2010).

Dhakal et al. (2018) assessed the immune response and cross-protective efficiency of inactivated SwIAV vaccine encapsulated in chitosan NPs and delivered *via* the IN route to pigs. Killed or inactivated SwIAV H1N2 (δ-lineage) virus antigens (KAg) were encapsulated in the CH NPs to form CNPs-KAg which was administered twice as an IN mist to nursery pigs. Vaccinated and control animals were challenged with a SwIAV H1N1 (γ-lineage). The pigs vaccinated with the CNPs-KAg showed elevated serum IgG antibodies and more mucosal IgA secretion in the nasal swabs, broncho-alveolar lavage (BAL) fluids as well as lung lysates. Protection was achieved against homologous (H1N2), heterologous (H1N1), and hetero-subtype (H3N2) influenza A-virus strains. Before the challenge, they found higher levels of cytotoxic-T lymphocyte proliferation, antigen specific lymphocyte proliferation, and IFN-γ secretion from peripheral blood mononuclear cells, in pigs immunized with CNPs-KAg compared to controls vaccinated with KAg alone. When CNP-KAg vaccinated pigs were challenged with the heterologous virus, the microscopic and macroscopic pulmonary lesions were reduced. Notably, titers of SwIAV in nasal swabs and BAL fluid were substantially reduced in pigs vaccinated with CNPs-KAg, but not in the KAg control group. Also, an increased frequency of helper T memory cells and an increase in IFNγ secretion in bronchial lymph nodes were observed. The SwIAV CH nanovaccine delivered by the IN route triggered IgA and cross-protective mucosal cellular immune responses in the respiratory tract, leading to lower virus titers in the nose and lungs. They suggested that the chitosan-based nanovaccine for influenza might be useful for commercial pig farms, and could be tested for human influenza vaccination (Dhakal et al., 2018).

Table 2 lists some chitosan nanoparticles that have been used for improving vaccination against viral infections.

TABLE 2 | Chitosan nanoparticles investigated for vaccination against viral infections.

Chitosan NP composition	Size	Target virus	Model (*In vitro/ In vivo*/Human)	Type of cell line	Findings	Ref
GLPC	431.21 ± 0.90 nm	Respiratory system virus	*In vivo*		Antibacterial activity Antioxidant activity Effective anti-inflammatory activity	(Singh et al., 2018b)
CN/IL-12	NA	HPV-16	*In vitro* and *in vivo*	TC-1	Combined vaccination inhibited tumor progression compared with chitosan or IL-12 alone. Co-formulation of chitosan and IL-12 resulted in higher IFN-γ and IL-4 and decreased IL-10 generation.	(Tahamtan et al., 2018)
AIV-CN	149.5 nm	Avian influenza virus	*In vivo*		Increased% phagocytic activity and phagocytic index.	(Mohamed et al., 2018)
N-2-HACC and CMC	251.8 ± 10.2 nm and 122.4 ± 4.2 nm	Newcastle virus	*In vivo*		Induced cellular, mucosal and humoral immune responses as an adjuvant Induced higher titers of IgA and IgG antibodies. Promoted the proliferation of lymphocytes. Induced more IL-2, IL-4, and IFN-γ.	(Zhao et al., 2017)
IBV-CS	286nm	Avian infectious bronchitis virus	*In vivo*		Induced strong mucosal immune responses. Induced both humoral and cell-mediated immune responses. Switched from IgA isotype to IgG.	(Lopes et al., 2018)
HACC/CS and SCS	320.03 ± 0.84, 156.2 ± 9.29 nm	Newcastle virus	*In vivo*		Lower humoral immunity and higher cellular immunity.	(Yang et al., 2020b)
CS-TPP	NA	Infectious pancreatic necrosis virus (IPNV)	*In vitro* and *in vivo*	CHSE-214	Promoted gene transcription of Mx-1, IFN-1, IgT, CD4 and IgM. Decreased VP4 transcripts. Higher levels of circulating antibodies.	(Ahmadivand et al., 2017)
PC NPs	200nm	Enterovirus 71	*In vitro* and *in vivo*	Vero and Raw264.7	Increased virus-specific humoral immunity (IgG, IgG2a & IgG1) and cell-mediated immune response (IFN-γ & IL-4).	(Pathinayake et al., 2018)
c-poly(I:C) NPs	368 ± 1.3 nm	VHSV-G	*In vivo*		Significantly protected against VHSV.	(Kavaliauskis et al., 2016)
γ-PGA-PC NPs	NA	Influenza A virus strains	*In vivo*		Induced systemic immunity (IgG & IgA). Increased level of sM2- or HA2-specific cell mediated immune response. Provided cross-protection against lethal influenza sub-types.	(Chowdhury et al., 2017)
TMC	225.3 ± 23nm	Influenza virus	*In vivo*		Induced Th1 cellular and humoral immune responses. Increased IgG, IgA & IFN-γ.	(Dabaghian et al., 2018)
Plasmid DNA loaded-CN$_S$	300–400 nm	Hepatitis B virus	*In vitro*	HeLa	Induced systemic and mucosal humoral and cellular immune responses.	(Khatri et al., 2008b)
CS-γ-PGA	265.3 ± 7.28 nm	HBV virus	*In vivo*		Long-term protection against HBV	(Wang et al., 2018)
C-PAP-phMGFP	NA	Yellow head virus	*In vivo*		Increased phagocytic activity.	(Khimmakthong et al., 2013)
PC NPs	900nm	Influenza A H5N1 virus	*In vivo*		Increased protective mucosal immunity in respiratory tract. Induced anti-HA IgA response in the lung. Induced IgG response in sera including anti-HA neutralizing antibodies.	(Moon et al., 2012)
H1N1-TMC/NP	39.4 ± 1.5 nm and 141.3 ± 1.9 nm	Influenza A (H1N1)	*In vivo*		Increased mucosal sIgA & serum IgG. Produced IL-1β, IL-6, IL-2, and IFN-γ. Stimulated macrophages and spleen lymphocytes.	(Liu et al., 2015)
GC- E2- NPs	304 nm	HIV-1	*In vivo*		Antiviral activity against HIV-1	(Ariza-Sáenz et al., 2017)
BCG-CWCs-CS-NPs	372.0 ± 11.2 nm	Dengue virus	*In vitro*	THP-1	Increased DC markers (CD86, HLA-DR & CD80). Induced production of cytokines and chemokines Increased THP-1 cellular uptake. Stimulated immature dendritic cells (iDCs).	(Hunsawong et al., 2015)
pRSC-NLDC145.gD-IL21 DNA/CHNPs	NA	Herpes simplex virus	*In vivo*		Alleviated symptoms of recurrent and primary herpes simplex virus keratitis. Targeted corneal DCs. Induced strong humoral & cellular immune responses.	(Tang et al., 2018)

(Continued)

TABLE 2 | Continued

Chitosan NP composition	Size	Target virus	Model (*In vitro*/ *In vivo*/Human)	Type of cell line	Findings	Ref
pVAX(HBc)DNA-CH NPs	271.1 ± 6.5nm	Hepatitis B Virus	*In vivo*		Enhanced immunogenicity. Increased antibody production, IFN-γ secretion, antigen-specific cell lysis.	(Jiang et al., 2007)
CN$_S$	200 nm	Hepatitis B virus	*In-vitro* & *In-vivo*	RAW 264.7	Delivered imiquimod& recombinant HBV surface antigen Increased proinflammatory cytokines TNF-α & IL-6. Enhanced IgG over time and specific immunological memory. Balanced cellular &humoral responses	(Vicente et al., 2013)
CH (WV+CpG)	581.1 ± 32.6nm	Influenza virus	*In vivo*		Increased hemagglutination inhibition (HI) antibody titer Increased sIgA and IgG titers Stimulated IFN-γ & IL-2 secretion	(Dehghan et al., 2014)
N,N,N-trimethyl chitosan (TMC)	200–220 nm	Influenza virus	*In vivo*		Increased IgG1, IgG2a/c and IgG responses Improved immunogenicity and protected against viral challenge.	(Hagenaars et al., 2009b)
Chitosan-pJME/ GM-CSF NPs	108.3 nm	Japanese encephalitis virus	*In vivo*		Increased splenic DC activity & cell mediated immunity.	(Zhai et al., 2015)
Chitosan-JEV DNA vaccines	200 nm	Japanese encephalitis virus	*In vivo*		Activated DCs in hair follicles & epidermis.	(Huang et al., 2009)
pCAGG-ChIL2 CNPs	100 and 200 nm	Newcastle disease virus (NDV)	*In vivo*		Increased hemagglutination inhibition antibody titer& serum IFN-γ.	(Zhang et al., 2010)

CHITOSAN VEHICLES FOR DELIVERY OF RNA-BASED THERAPEUTICS

As mentioned earlier, nucleic acid-based drugs have been introduced as a treatment for various conditions. Nucleic acid-based drugs can be divided into two main groups called double-stranded RNA-mediated interference (RNAi) and antisense oligonucleotides (ASO) (Chery, 2016). RNAi is used for post-transcriptional gene silencing, mediated by ribonucleases in combination with other complexes and enzymes that cleave the targeted messenger RNA into small segments (Agrawal et al., 2003). Antisense oligonucleotides bind to the respective target nucleic acids by Watson-Crick base pairing, which inhibits or alters gene expression through splicing modification, target degradation, steric hindrance, etc. In contrast to small molecules drugs, that work by binding to proteins with frequent off-target toxic side effects (Sharma et al., 2014), RNA-based drugs are much more specific by targeting individual nucleic acids. Nucleic acids based drugs have more specificity, lower toxicity, and higher activity. Two major diseases, spinal muscular atrophy (SMA) and Duchenne muscular dystrophy (DMD) are good examples of those conditions, which have received approval for treatment by these approaches (Kole et al., 2012). Although RNA-based drugs enjoy enormous potential, some potential challenges should be considered. For example, RNAs have been proven to be innately unstable. Hence, delivering nucleic acids in quantity to the target tissues is difficult because of nuclease-mediated degradation in the circulation and clearance by renal excretion (Moreno and Pêgo, 2014). Moreover, some other issues such as toxicity as a result of over-activation of the immune system, as well as off-targeting effects should be investigated (Davidson and McCray, 2011).

The ease of chemical modification of chitosan, as well as the possibility to tailor its structural and functional properties, are due to the plentiful hydroxyl and amino groups in the CH chains (Singh et al., 2014). The amino groups are responsible for the positive charge formed at pH < 6, that equates to the pH of tumoral extra-cellular medium. Nevertheless, the cationic charge of CH disappears at the physiological pH of the blood, resulting in lower NP stability, and poor efficiency in siRNA complex formation. Stability is a key concern for *in vivo* gene silencing applications, along with interactions with various serum proteins after systemic delivery (Ragelle et al., 2013). Nonetheless, because positively charged CH is a natural polymeric structure, it has been widely explored for the delivery of nucleic acids (Zhang et al., 2013). A key advantage is the strong electrostatic interactions with the negatively charged RNA, which creates stable polyplexes. In addition, the siRNA binding activity of CH is clearly higher than other natural polysaccharides, that commonly possess negative or neutral charges. For instance, another natural polysaccharide, hyaluronic acid required to be to be chemically modified to become a cationic polymer in order to be applied in the delivery of nucleic acids (Serrano-Sevilla et al., 2019).

In one study, Wang et al. used nano-precipitation and solvent evaporation procedures to prepare four types of cationic NPs for delivery of siRNA to target HBV virus infections. Poly(D,L-lactide-co-glycolide) (PGLA) or mPEG-PLA was attached to either PEI or CH as a surface coating, and the size and size distribution of the NPs were measured by laser scattering, surface charge by zeta potential, and surface chemistry by X-ray electron spectroscopy (XPS). The mPEG-PLA-PEI NPs showed the best siRNA transfection efficiency and the highest inhibition of the expression of HBV surface antigen (Wang et al., 2010).

Another research group created CH/siRNA NPs as a possible treatment option against influenza virus infections *in vivo* and *in vitro* (Jamali et al., 2018). They formulated a siRNA sequence against influenza nucleoprotein, incorporated with CH polymer as a siRNA/chitosan NP complex. They used dynamic light

scattering to measure the NP zeta-potential and size. Fluorescence microscopy was used to visualize the uptake of the labeled siRNA into Vero-cells. Flow cytometry was used to analyze and quantify the NP-mediated knockdown of the enhanced green fluorescent protein (EGFP) gene in Vero cells. The CH/siRNA NPs were efficiently taken up by Vero cells, and inhibited the replication of influenza virus. Nasal delivery of the siRNA using the CH-NP complex showed anti-viral activity, resulting in significant protection of BALB/c mice from a lethal influenza challenge. They concluded that CH NPs equipped with siRNA had the potential to control influenza virus infections (Jamali et al., 2018).

MiRNA-based therapeutics are being investigated to restore the expression of down-regulated miRNAs, or inhibit the expression of unwanted mRNAs (Chaudhary et al., 2018). For example, McKiernan et al. explored the treatment of cystic fibrosis using a miRNA-based nanomedicine. These researchers utilized a nano-delivery system composed of cytosine and PEI for targeting of miR-126 (McKiernan and Greene, 2015). Moreover, Deng et al. described the delivery of a combination of miR-34a plus doxorubicin (DOX) to treat breast cancer employing hyaluronic acid-CH-NPs (Deng et al., 2014). Louw et al. used CH-mediated delivery of miRNA-124 to reduce the number and activation state of microglial cells in spinal cord injuries in rats (Louw et al., 2016).

Chitosan-based nanocarriers are expected to be useful for the delivery of small interfering RNAs, microRNAs, or antisense oligonucleotides, as a molecular targeted therapy in the near future.

CONCLUSIONS

Nanomedicines are being increasingly used for drug delivery, with many advantages, including tissue targeting, controlled release, improved permeability and solubility of drugs, greater effectiveness, improved safety, and lower toxicity. Naturally occurring materials are often preferred for the construction of these nanomedicines, compared to synthetic polymers, inorganic materials, or carbon-based nanomaterials. The bio-pharmaceutical properties of these natural nanomedicines, include low toxicity, and improvements in cell uptake, biodistribution, metabolism, and excretion. The possible long-term accumulation of non-biodegradable nanoparticles in tissue and organs, has led to some concerns about possible carcinogenicity and genotoxicity. Metallic NPs (gold and silver), carbon-based NPs (carbon nanotubes and graphene), and inorganic NPs (silica and titania) have all been faced with these worries and concerns. On the other hand, there have been many naturally occurring biodegradable materials that have been used to prepare various types of nanostructures, including proteins, peptides, lipids, and polysaccharides such as cellulose. Nevertheless, we believe that chitosan is one of the most promising types of naturally occurring materials in the nanomedicine arena. This preference is based on the well-known lack of toxicity of chitosan, as illustrated by the fact that chitosan is a food ingredient and is widely consumed by

human beings as a health-food supplement. Another very important property of chitosan is its mucoadhesive ability that allows CH NPs to be administered by transmucosal routes, such as intranasal, intraocular, intravaginal, intratracheal or intrapulmonary etc. This ability is highly relevant when it comes to consider viral diseases, which often gain entry to the human body via a mucosal route.

Viral infections are a universal challenge to the human race that affect the health and economic well-being of millions of people, and cause disability, suffering, and death throughout the world. Treatment of viral infections has been challenging because viruses hijack the host cells in order to proliferate, and killing the virus often means killing the host cell as well. Some antiviral drugs have been developed based on nucleoside analogs and inhibitors of viral-specific enzymes. However, drug resistance often emerges due to the ability of viruses to undergo relatively facile mutations. Drug resistance is considered to be a significant threat to public health, leading to an increase in mortality and medical expenses. Infectious virus particles (virions) attach to specific receptors on susceptible cells leading to their entrance, and establishment of a viral infection. Viruses can spread within the body by local invasion, or by long distance transport via the bloodstream, lymphatics, or neuronal pathways. Cell-to-cell transmission of infectious viruses involves the direct export of the infectious particles out of the cell into the extra-cellular environment. The virus particles can be transported along nerve cells and spread to epithelial cells.

In addition to anti-viral drugs, vaccines are an important therapeutic approach that can not only prevent the development of a viral infection, but can also decrease the severity of the infection once it has become established. Vaccines have been proved to be highly effective against many viral infections, but many vaccines are poorly effective or even completely ineffective. The reasons for this lack of universal efficacy are manifold, and some are still under investigation. For this reason many groups have studied the use of various NPs to deliver different kinds of vaccines, whether they be whole inactivated viruses, recombinant viral antigens, or DNA and RNA sequences. Moreover, the NPs themselves can act as an adjuvant, by increasing the uptake of the vaccine by antigen presenting cells. Additional adjuvants can be combined with the vaccine inside the NPs to further improve the immunogenicity.

Chitosan-based NPs have an intrinsic positive charge, which can interact with the negative charge present on call membrane and on mucosal surfaces accounting for its mucoadhesive properties. This is useful because delivering the vaccines across the mucosal surface allows the induction of specific mucosal immunity characterized by secretion of IgA antibodies as well as IgG and IgM. Moreover the mucosal-associated lymphoid tissue (MALT) can be activated by vaccines encapsulated in CH NPs and delivered to the mucosal surface, particularly by the intranasal administration route.

Chitosan-based nanocarriers show many advantageous properties, such as nanoscale dimensions, high surface area to volume ratio, as well as the ability to tailor the surface charge and attach targeting ligands. Moreover, the chitosan matrix is able to

incorporate a wide variety of different types of cargo, including antiviral drugs, proteins, peptides, nucleic acids, and even whole inactivated viruses. One relatively new approach to treating viral infections is based on gene silencing. Gene silencing uses small interfering RNAs, microRNAs, or antisense oligonucleotides, all of which can be loaded into CH NPs. The idea is to recognize the 3'-untranslated region of the viral mRNA to allow it to be degraded by the RNA-induced silencing complex (RISC). Chitosan nanostructures are also able to cross biological barriers such as the blood-brain barrier (BBB). CH NPs can increase drug or gene delivery to the site of the viral infection and also improve the cellular uptake.

As mentioned earlier, CH-based carriers enjoy increasing popularity due to their many advantages. Nonetheless, they still suffer from several limitations, like poor solubility at the physiological pH, premature release in the cytoplasm, as well as questions about the stability of the complexes following cellular uptake. Therefore, it is necessary to discover further improvements in CH-based nanocarriers. Researchers have employed chemical modification of CH using PEG (PEGylation) to improve CH solubility, although excessive PEGylation can decrease the density of the positive charges on CH, and lower its ability to bind to nucleic acids. Furthermore, chemical modification made to the siRNA itself may enhance the stability of the NPs, although this approach can make the siRNA activity less efficient. The addition of other components with a negative charge has been proposed to increase the stability of the CH NPs to improve gene silencing. CH NPs often show a higher encapsulation efficiency (EE%), and the ability for sustained release after uptake into the cells. Additional experiments are warranted in animal models of diseases such as viral infections and unwanted fibrosis. However, future researchers must optimize the CH modification procedures for full realization.

AUTHOR CONTRIBUTIONS

HM and MH contributed in conception, design, statistical analysis and drafting of the manuscript. HB, FB, SM, ZS, JS, MN, HB, BB, MA-K, and MG contributed in data collection and manuscript drafting. All authors contributed to the article and approved the submitted version.

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