Abstract

Gastroenteritis (GE) and its associated diarrheal diseases remain as one of the top causes of death in the world. Noroviruses (NoVs) are a group of genetically diverse RNA viruses that cause the great majority of non-bacterial gastroenteritis in humans. However, there is still no vaccine licensed for human use to prevent NoV GE. The lack of a tissue
culture system and a small animal model further hinders the development of NoV vaccines.

Virus-like particles (VLPs) that mimic the antigenic architecture of authentic virions, however, can be produced in insect, mammalian, and plant cells by the expression of the capsid protein. The particulate nature and high-density presentation of viral structure proteins on their surface render VLPs as a premier vaccine platform with superior safety, immunogenicity, and manufacturability. Therefore, this chapter focuses on the development of effective NoV vaccines based on VLPs of capsid proteins. The expression and structure of NoV VLPs, especially VLPs of Norwalk virus, the prototype NoV, are extensively discussed. The ability of NoV VLPs in stimulating a potent systemic and mucosal anti-NoV immunity through oral and intranasal delivery in mice is presented.

The advantages of plant expression systems as a novel production platform for VLP-based NoV vaccines are discussed in light of their cost-effectiveness, production speed, and scalability. Recent achievements from the first successful demonstration of NoV VLP production in plant expression system under the current Good Manufacture Practice (cGMP) regulation by the US Food and Drug Administration (FDA) are detailed.

Moreover, results of human clinical trials demonstrating the safety and efficacy of insect and plant-derived NoV VLPs are also presented. Due to the diversity of capsid protein among different NoV strains and its rapid antigenic drift, we speculate that vaccine development should focus on multivalent VLP vaccines derived from capsid proteins of the most prevalent strains. With the very recent approval of the first plant-made biologics by the FDA, we also speculate that plant-based production systems will play an important role in manufacturing such multivalent VLP-based NoV vaccines.

8.1 Gastroenteritis

Gastroenteritis (GE) is a worldwide health problem that affects people of all ages. As its name implies, GE is characterized by inflammation of the gastrointestinal tract and often associated with symptoms of diarrhea, nausea, vomiting, and abdominal cramping and pain. GE and its associated diarrheal diseases remain as one of the top causes of death in the world especially in developing countries and in young children with an estimated death toll of four to six million per year [1, 2]. GE is also the most common disease and the leading cause of morbidity in the developed world. For example, it is suggested that in the United States (USA), everyone is likely to suffer from viral GE at least once a year, 610,000 will be hospitalized, and at least 4,000 will die from this disease [1]. GE can be caused by a variety of pathogens including viruses, bacteria, and parasites and by ingestion of noninfectious toxins or medications, with viruses as the most common offending agents [1]. Norovirus (NoV) and rotavirus are the most common viruses that cause viral GE, while adenovirus, astrovirus, coronavirus, and parechovirus are also known to cause GE in humans [1–3].

8.2 Noroviruses

NoVs are a group of genetically diverse RNA viruses that belong to the genus of norovirus in the Caliciviridae family [4, 5]. They were first discovered and characterized in their prototype virus, the Norwalk virus (NV) in 1972 [6, 7]. Studies of NV revealed that NoVs are non-enveloped viruses with an RNA genome surrounded by a round capsid protein shell approximately 38 nm in diameter [8]. Classification of NoVs, however, has proved difficult and controversial until the recent development of molecular biology tools. Since there is no culture system available to grow these viruses in vitro, accurate serotyping based on neutralization is not possible [9]. Instead, classification of NoV had to rely on cross-challenge studies in
volunteers and immune cross-reactivity analysis with electron microscopy [10–13], which lacks accuracy and reproducibility due to the cross-reactivity of antibodies [14].

Recent development of sophisticated molecular methods including reverse transcription polymerase chain reaction (RT-PCR) has allowed a more reliable NoV classification based on the amino acid sequence of the major capsid protein [15]. In this classification system, NoVs are divided into five genogroups and 29 clusters with eight clusters in genogroup I (GI), 17 in GII, two in GIII, and one each in GIV and GV (Table 8.1) [15]. Within the five genotypes, GI and GIV strains are found to infect humans exclusively, and GII are found in both humans and pigs, while GIII and GV strains are animal viruses that infect cattle and murine species, respectively [15, 16]. Currently, strains in cluster 4 of GII (GII.4) are the most prevalent NoVs in human population [17, 18].

The genome of NoVs, which was first characterized in NV, contains a single-stranded positive sense RNA of 7.5–7.7 kb with three open reading frames (ORFs) and a poly A tail at its 3’ end [19]. ORF1 encodes a polyprotein that is processed by viral protease 3CLpro into the RNA-dependent RNA polymerase and approximately five other

### Table 8.1 Classification of noroviruses

| Genogroups | Animal hosts | Genotype clusters | Representative virus strains |
|------------|--------------|-------------------|-----------------------------|
| GI         | Human        | GI.1              | NV-USA93, Wtchest-USA, KY89-JPN |
|            |              | GI.2              | SOV-GBR93, C59-USA, FB258-JPN |
|            |              | GI.3              | DSV-USA93, LR316USA, VA98115-USA |
|            |              | GI.4              | Chiba-JPN00, Valetta-MLT, NO266-USA |
|            |              | GI.5              | Musgrov-GR00, AB318-USA, SzUG1-JPN |
|            |              | GI.6              | Hesse-DEU98, CS841-USA, WUG1-JPN |
|            |              | GI.7              | Wnchest-GR00 |
|            |              | GI.8              | Boxer-USA02 |
| GII        | Human and Pig| GII.1             | Hawaii-USA94, Miami81-USA, DG391-DEU |
|            |              | GII.2             | Msham-GR95, CF434-USA, SMV1-USA |
|            |              | GII.3             | Toronto-CAN93, MD102-USA, NLV2004-SWE |
|            |              | GII.4             | Bristol-GR93, Minerva, NT104-JPN |
|            |              | GII.5             | Hilingd-GR00, MOH99-HUN, NO306-USA |
|            |              | GII.6             | Seacrof-GR00, SU3-JPN, Miami292-USA |
|            |              | GII.7             | Leeds-GR00, GN273-USA |
|            |              | GII.8             | Amstmd-NLD99, SU25-JPN |
|            |              | GII.9             | VABeach-USA01, Idafall-USA |
|            |              | GII.10            | Erfurt-DEU01 |
|            |              | GII.11            | SW918-JPN01, SWVA34-USA, SW43-JPN |
|            |              | GII.12            | Wortley-GR00, U1GII-JPN, Pirna110-DEU |
|            |              | GII.13            | Faytvil-USA02, KSW47-JPN, |
|            |              | GII.14            | M7-USA03 |
|            |              | GII.15            | J23-USA02, Mex7076-USA |
|            |              | GII.16            | Tiffin-USA03, Fayett-USA, Tonto-USA |
|            |              | GII.17            | CSE1-USA03 |
| GIII       | Cattle       | GIII.1            | BoJena-DEU98 |
|            |              | GIII.2            | BoCH126-NLD00, BoNA2-GBR, BoCV95-USA |
| GIV        | Human        | GIV.1             | Alphatn-NLD99, FLD560-USA, SCDD624-USA |
| GV         | Murine       | GV.1              | Murine1-USA03 |

Table is generated based on results of phylogenetic analysis of capsid protein VP1 sequence in reference [13]
nonstructural proteins including p48, the nucleoside triphosphatase, p22, VPg, and 3CLpro [20, 21]. The two structural proteins, the major (VP1) and minor (VP2) capsid proteins, are encoded by ORF2 and ORF3, respectively [19, 22]. Structural analysis of NoV has revealed that each viral capsid is composed of 90 dimers of VP1 in a $T=3$ icosahedral symmetry [8, 23]. VP1 folds into two domains: a shell (S) domain that is responsible for initiating capsid assembly and icosahedral contacts and a protruding domain (P), containing two subdomains of P1 and P2, that enhance the stability of the capsid by providing intermolecular contacts between VP1 dimers [24].

In addition to providing the sole structural shell component of NoV capsid, VP1 has to perform other functions including immunogenicity and infectivity. It was suggested that the P domain may contain cellular receptor binding sites and viral phenotype or serotype determinants [23, 25, 26]. The P2 subdomain has the most variable sequence among different NoV strains and is found to protrude outside the capsid surface, suggesting that it is responsible for the immune recognition and cellular interactions [27, 28]. Studies of NV also indicate that the VP2 protein enhances the expression level of VP1 and stabilizes the VP1 in the viral capsid [24].

### 8.3 Transmission and Infection Cycle of NoVs

NoVs are one of the leading causes of GE in people of all age groups and are responsible for more than 95% of viral GE in adults [29]. With the development of new diagnostic methods, it is now recognized that the impact of these viruses had been significantly underestimated as they cause far more outbreaks and infections than previously realized [30, 31]. NoVs require an extremely low infectious dose, as challenge studies with NV have suggested that the probability of infection with a single virus particle is approximately 50% among susceptible human populations [32]. They can be transmitted by several routes including fecal-contaminated food or water, direct person-to-person contact, indirect exposure through droplets, or contaminated objects of infected persons [33].

The high environmental stability of NoVs further facilitates their spread among human populations. For example, NoVs are stable at a wide range of temperatures from subzero to 60°C and at pHs even below 2.7 and are resistant to treatment with chlorine (0.5–1.0 mg/L), ethanol, quaternary ammonium compounds, or detergent-based cleaners [34, 35]. As a result, NoVs are highly contagious, spread rapidly, and their outbreaks commonly occur in various social places and settings where people share common food and water sources or are in close physical proximity, such as cruise ships, schools, military units, nursing homes, daycare centers, hospitals, restaurants, and catered events [29, 36–39].

Viral shedding may start before symptoms even occur and continue after they have disappeared. This prolonged shedding period (several weeks) of NoVs further enhances the secondary attack rate and usually results in large-scale outbreaks [38, 40–42]. Consequently, these outbreaks often lead to the infection of thousands of people and the closure of facilities and businesses [43, 44]. For example, NoV has been identified as one of the most common pathogens for hospital ward closures [45]. Outbreaks of NoV GE are distributed worldwide and year-round, although as its old name “winter vomiting disease” implies, there are more outbreaks during the winter season of the year [46]. It was suggested that this seasonality is due to climatic conditions that promote a closer person-to-person contact and favor the survival of the viruses [47]. Further studies demonstrated that various NoV strains have different seasonal outbreak periodicities as GII strains mostly occur in winter, while GI viruses are more evenly spread over the year [48–50].

Due to the routes of transmission, young children, elderly nursing home residents, students, military personnel, travelers, and immunocompromised people are particularly vulnerable to NoV infections. Recent studies indicate a possible genetic basis of susceptibility to NoV infection. For example, susceptibility in a human
population is found to be associated with an individual’s ABH histo-blood type, mucosal cell-expressed carbohydrates, secretor status, and strain binding preferences of histo-blood group antigen (HBGA) receptors [26, 51–55]. Furthermore, P2s of different viral genotypes have been found to have specific affinity for certain ABO HBGA, with GI NoVs preferentially binding to group antigens A and O, and GII to group antigens A and B [56, 57]. Consequently, it is suggested that HBGA may serve as putative receptors for NoVs [54, 55, 58–60]. These findings may help to explain the phenomena of “asymptomatic infections” in certain individuals who develop NoV-specific antibody response and shed virus, but do not display any typical symptoms of disease after NV infection [52]. In addition to acute GE, chronic NoV infection has also been documented in immunocompromised patients [61, 62].

The life cycle of NoV has not been fully understood due to the lack of an in vitro cell culture system and a small animal model of infection. However, a study by Asanaka and colleagues has demonstrated that an expressed NV genomic RNA has the ability to replicate, to be transcribed into NV subgenomic RNA, and subsequently translated into VP1 in mammalian cells [63]. Moreover, the expression of VP1 was found to generate virus particles filled with NV genomic RNA in this particular cell [63]. These results clearly demonstrate the ability of mammalian cells in supporting the replication of NoV genomic RNA and the assembly of viral RNA into virus particles. Thus, the failure of NoV replication in mammalian cell cultures is not due to the lack of host factors to support intracellular expression of NoV RNA. Instead, the problem may lie in the steps of viral binding to cellular receptors, virus entry into cells after receptor binding, or uncoating of virus particles for releasing genomic RNA into the cytoplasm.

After ingestion of viral particles, symptoms of vomiting and diarrhea usually appear after a 12–48-h incubation period. These symptoms persist during the course of the illness (12–72 h) and are often accompanied by nausea, abdominal cramps, and occasionally by malaise, chills, muscle aches, headaches, and low-grade fever [34]. The molecular and cellular mechanism of how diarrhea and vomiting are induced by NoV has not been fully revealed. Furthermore, the target cell(s) for human NoVs has yet to be conclusively identified. However, it has been observed that NoV starts to multiply within the small intestine upon infection and cause histopathologic lesions in the jejunum and reversible broadening and blunting of the jejunal villi in both symptomatic and asymptomatic individuals [64]. Moreover, cytoplasmic vacuolization and infiltration with a unique CD8+ lymphocyte population in the epithelium have also been observed [65, 66]. It is speculated that these changes may be involved in the blunting of the villi. While it is still not possible to visualize NoV particles in biopsies from challenge studies, in vitro binding experiments suggested that viral binding occurs mostly at the villi level [67].

Although NoV-caused acute GE is usually mild and self-limiting and can be treated with hydration fluids, instances of necrotizing enterocolitis and mortality due to dehydration do occur. It has been estimated that NoVs cause up to 200,000 deaths in young children in developing countries and 300 deaths in the US per year [68–70]. Furthermore, the shear frequency and scale of NoV outbreaks have become a major burden on the health-care system and caused tremendous economic loss in the developed world [45]. Since the emergence of a new NoV variant GII.4 in 2002, the number of outbreaks has increased significantly, and large epidemics have occurred worldwide almost every 2 years [71, 72]. As a result, NoVs have been considered as emerging pathogens. Furthermore, because of the recent realization of their prominence in causing GE and their potential of causing rapid and large water-, food-, and possibly airborne outbreaks with as few as 10–100 viral particles, NoVs have been also classified as class B biodefense pathogens [73]. Currently, there are no effective drugs or vaccines available for treating or preventing NoV infections. Therefore, development of vaccines and therapies for these viruses is urgently needed.
8.4 Current Diagnostic Methods and Therapeutics for NoV GE

Various diagnostic methods have been developed for NoV [74]. While serum antibodies to NoV can be readily detected, this method has little clinical relevance due to the cross-reactivity of antibodies. Since there is no culture system available for NoV, the detection of virus in stool samples has become the preferred method of diagnosis. Traditionally, NoV infection was diagnosed by detecting the virus by immune transmission electron microscopy (TEM) [11]. TEM offers the advantage of direct visualization of any potentially responsible virus particles in stool samples. However, it does have the disadvantage of requiring sophisticated and expensive equipment and highly specialized technicians for its operation.

Moreover, the concentration of intact virus particles in the samples has to be at least $10^5$–$10^6$ particles/ml in order to be detected. TEM can provide rapid diagnostic results for individual samples, usually within 3 h of sample delivery [75]. However, since operators have to spend substantial amount of time in examining each specimen, TEM is not a high-throughput assay for rapidly processing a large number of samples. TEM relies on the identification of viral particles with characteristic NoV morphology, therefore, does not allow reliable speciation within the NoV genus, which lowers its specificity and may hinder diagnosis. These challenges may have contributed to the historic underestimation of NoV epidemics.

Several enzyme-linked immunosorbent assays (ELISAs) that detect NoV antigens were later developed for NoV diagnosis [76, 77]. Antigen-based ELISAs are high-throughput, relatively easy to operate and can rapidly provide diagnostic results within one working day [78]. Consequently, they are usually the method of choice in situations where large numbers of specimens are required to be screened rapidly and economically. Studies have shown that NoV antigen-detecting ELISAs have high specificity (94–96 %) but poor sensitivity (40–60 %), most likely due to the antigenic diversity of NoV strains [4, 79]. As a result, they are useful for specific detection of certain NoVs, but not sensitive enough for applications where a detection of a broad spectrum of NoVs is desired. The employment of pooled antibodies against multiple antigens or multivalent antibodies against a wider range of recombinant viral antigens has, however, improved the sensitivity of ELISAs [80].

With the cloning of the NV genome in 1990 and the development of molecular biology methods, RT-PCR with stool samples (which detects the viral RNA) has increasingly become the popular assay for NoV diagnosis [81–83]. Similar to ELISA-based assays, RT-PCR is rapid and robust, because it can process large numbers of samples simultaneously and results can be obtained within a working day. However, it requires RNA extraction from fecal samples and needs expensive equipment and skilled workers to operate. Therefore, RT-PCR is more labor intensive and less economical than ELISAs. Using primer pairs designed for either highly conserved regions or strain-specific regions of the genome, RT-PCRs are versatile and offer both high specificity comparable to ELISAs and much higher sensitivity. The high sensitivity of this assay allows the detection of NoV in samples in which the concentration of virus is too low to be detected by other methods. These include clinical specimens with low viral load and environmental samples such as food and water [83].

Another advantage of RT-PCR is that, when combined with subsequent nucleotide sequencing, it allows genotyping to trace the source of outbreaks [84]. On the other hand, the high sensitivity of the assay may produce false-positive results from nonspecific amplification or potential sample cross-contamination. This challenge has been addressed by designing more specific primers to avoid amplification of nonspecific RNAs and by implementing more stringent procedures to prevent cross-contamination of samples. Due to the extraordinary sequence diversity among viruses in this genus, there is no single universal primer pair that can detect all strains of NoV. However, several primer pairs have been developed that can detect more than 90 % of all
strains in GI and GII [85]. The robustness of RT-PCR-based diagnostic methods is being further optimized by employing faster and more sensitive assays such as real-time quantitative RT-PCR, which aims to rapidly detect even low copy number NoV in a large number of stool samples during outbreaks [86]. These studies demonstrated that TaqMan based real-time PCR offers more sensitive real-time diagnosis of NoV in both sporadic cases and the outbreak setting, and its results are easy to interpret and available within the working day [87].

Overall, TEM, ELISA, and RT-PCR-based methods all have their advantages and challenges. TEM allows detecting all responsible viral agents, but is expensive and low throughput. In contrast, ELISA is high throughput, economical, and easy to use, but requires improvement in sensitivity. RT-PCR combines the advantage of both sensitivity and specificity and enables molecular epidemiological studies. The three methods detect different components of the virus and therefore are complementary to each other.

For a particular diagnostic application, three factors should dictate the choice among TEM, ELISA, and RT-PCR. They include (1) the type of diagnosis such as epidemic investigations vs. clinical diagnosis, (2) the sensitivity and specificity of each assay, and (3) the speed, robustness, and ease of use of the assay. It is also crucial to remember that the heterogeneous and complex nature of stool samples and their storage conditions may often cause changes in the integrity of virus particles and/or RNA and consequently affect their reactivity in enzymatic and immunological assays. For example, the low sensitivity of ELISA-based methods will result in a significant number of false-negative samples if they are used as the only diagnostic method for clinical samples. Instead, they should be used only as “screening” assays for clinical diagnosis to take advantage of their speed and availability to be performed at a location close to the patient. However, the high specificity of ELISAs makes them an appropriate assay in outbreak investigations because the availability of multiple samples improves their reliability to confirm the cause of an outbreak [79, 80].

Nevertheless, RT-PCR should be used in conjunction with ELISAs in both clinical diagnosis and outbreak investigations to confirm positive findings and assess negative samples. In contrast, if the goal of the diagnosis is to track down the point-source of infection in epidemiology studies, RT-PCR followed by DNA sequencing should be the method of choice due to its high sensitivity and specificity [88, 89]. TEM has been accepted as a “gold standard” for the diagnosis of NoV. However, as more comparative results become available, it has been proposed that either RT-PCR or a positive result in two of the three assays discussed above should be regarded as the “gold standard” [78, 79, 90]. Regardless, it is recommended that at least two of the three methods among TEM, ELISA, and RT-PCR should be combined to achieve a reliable clinical diagnostic result, while all three methods are applicable for epidemiological investigations for NoV outbreaks depending on the number of samples available [79].

Currently, there is no specific and effective vaccine or therapy available for preventing or treating NoV gastroenteritis [34]. General clinical management includes oral administration of rehydration solutions containing essential electrolytes and sugar to treat diarrhea. Parenteral fluid and electrolyte replacement may be required for patients with significant dehydration symptoms or those who cannot tolerate oral fluids replacement [4]. Current efforts are focused on the development of effective vaccines and possible attachment inhibitors for anti-adhesion therapy through glycomimetics [91].

8.5 Vaccine Development for NoV

8.5.1 Immunology of NoV Infection

The immunological knowledge of NoV is mostly obtained from human challenge studies and natural outbreaks due to the lack of small animal models. These studies showed that infected volunteers did develop immunity after a NoV challenge [13, 92, 93]. However, immunity to one strain did not provide complete protection from
challenge of heterologous strains, and symptomatic individuals could often be reinfected when exposed to the same NoV strain 2–4 years later, indicating their immunity seems to be strain or genogroup specific and short lived [13, 92–94]. Challenge studies did not yield conclusive results regarding long-term immunity, which may be confounded by preexposure of volunteers to various circulating NoV strains [71]. Observations of repeat infections in adults suggest the scarcity of long-term immunity against these viruses [38, 84]. However, other studies showed that close to 50 % of the genetically susceptible subjects were not infected by NoV challenge, which support the possibility of long-term immunity [95]. Furthermore, community cohort studies indicate that the duration of symptoms were generally decreased with age, suggesting the development of at least partial protection against NoV [38, 93].

While no vaccine is currently available to prevent NoV GE in humans, these studies support the feasibility of developing vaccines that can induce protective immunity and reduce the disease burden of these viruses.

**8.5.2 Virus-like Particles as an Effective Vaccine Against NoV**

The lack of a tissue culture system also impedes the development of vaccines against NoV. Fortunately, the discovery of the spontaneous assembly of expressed VP1 into virus-like particles (VLPs) that are morphologically and antigenically similar to the native viruses has facilitated vaccine development [73]. VLPs combine the best traits of whole-virus and subunit antigens for vaccine development. VLPs are non-infectious, therefore, safer than inactivated or attenuated virus due to the lack of viral nucleic acid genome. At least in theory, the immunogenicity of VLPs can even be enhanced over that of the native virus by excluding immunosuppressive viral proteins in their composition. Moreover, an inactivation process is unnecessary for VLP production. Consequently, no unintended epitope modification would occur, further ensuring the VLP’s immunogenicity. Importantly, VLPs can induce potent cellular and humoral immune responses without adjuvants and are more effective vaccines than other subunit antigens because their architectures mimic infectious viruses. VLPs can be produced by recombinant technology in heterologous expression systems without requiring the ability to support viral replication [31]. This is particularly important for NoV because no such culture system has been developed to support the growth of these viruses [96].

The particulate nature and the dense repetitive array of epitopes on their surface make VLP far more immunogenic than other subunit vaccines. VLPs can effectively induce T-cell-mediated immune responses through interaction with antigen-presenting cells (APCs), especially dendritic cells (DCs). Specifically, VLPs can mimic the natural viral infection process by being specifically recognized and taken up by DCs and subsequently processed and presented to cytotoxic T cells to trigger their activation and proliferation [97]. Studies have demonstrated that viruses and corresponding VLPs have a particle size ideal for DC and macrophage uptake to initiate antigen processing [98, 99]. Thus, the particulate nature of VLPs favors their targeting to relevant APCs for optimal induction of T-cell-mediated immune responses.

VLPs can also be presented efficiently to B cells and induce strong antibody responses. Like live viruses, the quasicrystalline surface of VLPs, with its arrays of repetitive epitopes, presents a prime target that vertebrate B cells have evolved to specifically recognize [100]. This recognition triggers the cross-linking of surface membrane-associated immunoglobulins (Ig) on B cells [101–103] and leads to their proliferation and migration, T helper cell activation, antibody production and secretion, and the generation of memory B cells [101]. Thus, VLPs can directly activate B cells at much lower concentrations than other subunit antigens and induce high titer and durable B-cell responses in the absence of adjuvants.

Since NoV is an enteropathogenic virus, a potent vaccine should also induce NoV-specific gut mucosal immunity such as through oral delivery. In general, oral delivery of subunit protein
vaccines for gut immunity may not be effective due to the possibility of denaturation and degradation of antigens by stomach acid and digestion enzymes, poor transport to the gut-associated lymphoid tissue (GALT) for antigen processing and presentation, and potential stimulation of systemic immune tolerance [102]. However, VLPs’ compact and highly ordered structures allow them to be more resistant to degradative enzymes in the digestive tract than other protein vaccines. The resemblance of VLPs to authentic viral particles may also present a “danger signal” that overcomes the perception of gut antigens as benign and thus prevents the development of immune tolerance [55]. Both of these characteristics are especially true for VLPs of NoV because their cognate viruses are natural gastrointestinal pathogens. Moreover, they are also naturally recognized and efficiently transported into GALT [4]. Thus, the challenges of oral vaccine delivery can be potentially overcome by the unique structure of VLPs that allow them to elicit a potent gut immune response.

These inherent advantages of VLPs have made them one of the most successful recombinant vaccine platforms. For example, five VLP-based vaccines for hepatitis B virus (HBV) and human papillomavirus (HPV) have been commercially licensed, and all have demonstrated excellent safety profiles and long-term protection against infection in humans [31]. These successes and the potential of evoking a gut mucosal immune response upon oral delivery have encouraged the preclinical and clinical development and testing of VLP-based vaccine candidates for NoV.

8.5.3 Characterization of NoV VLPs

VLPs of NoVs were first produced in insect cells using baculovirus vectors [23, 104] and then in plants using tobamovirus [101] and geminivirus [102] vectors and in mammalian cells using the Venezuelan equine encephalitis (VEE) replicon system [105]. These studies demonstrated that expression of the major capsid protein VP1 alone can drive the self-assembly of VLPs that morphologically and antigenically resemble native virus particles (Fig. 8.1). VLPs generated by all three expression systems are similar to each other.

The structure of NoV VLPs is exemplified by the VLP of NV capsid protein (NVCP). Studies of insect cell-baculovirus-derived NVCP VLPs by cryo-electron microscopy and X-ray crystallography reveal that the NV capsid is a 38 nm icosahedral arrangement of 180 copies of the 58 kDa capsid protein VP1 organized into 90 dimers in a $T=3$ symmetry [104]. While all dimers are formed from two identical NVCP monomers, two different dimer configurations are required to correctly form the complete assembled capsid [106, 107].

As in native NV particles, the NVCP also folds into two distinctive domains in VLPs, with S domain forming the inner core of the shell and P domain protruding out from the capsid [108]. Similarly, the P2 subdomain is also the most surface exposed region in NV VLPs and may contain HBGA and neutralizing antibody binding sites and determinants of strains specificity [27, 56, 108–111]. The similarity between VLPs of NV and other NoVs including GII.4 viruses has been demonstrated [112].
8.6  Preclinical Development of NoV VLPs

8.6.1  Insect Cell-Baculovirus Vector Produced NVCP VLPs

As discussed earlier, it is desirable for NoV vaccines to elicit gut mucosal immunity. Oral delivery is a feasible strategy for NVCP VLPs to induce such immunity as evolutionary selection for enteric infection has allowed them to be stable in the oral-gastrointestinal environment and efficiently transport to GALT for antigen processing and presentation [113]. Consequently, various oral doses of NVCP VLPs in the range of 5–500 μg were given to mice to examine their ability to elicit a systemic and mucosal antibody response.

It was shown that four oral doses of as little as 5 μg NVCP VLPs without any adjuvant triggered serum NV-specific anti-IgG response in the majority (8/11) of VLP-fed iCD1 outbred mice [106]. Systemic IgG response was observed after two oral dosages, and the highest titer was induced by four doses of 200 μg VLPs. Moreover, mice in the 200 μg dosage group developed NV-specific intestinal IgA in a level up to 0.1 % of total IgA. Inclusion of the mucosal adjuvant cholera toxin (CT) did not significantly change the number of positive responders of serum IgG or intestinal IgA, but significantly enhanced the amplitude of serum IgG response, especially for higher doses of VLPs [106]. Thus, NVCP VLP is clearly a potent oral immunogen and can induce both systemic and gut mucosal antibody responses.

The success of oral delivery of NVCP VLPs encouraged the exploration of their delivery through alternative mucosal routes. A study by Guerrero and colleagues demonstrated that intranasal (IN) delivery was more effective than oral delivery at provoking NVCP-specific serum IgG and intestinal IgA responses by low doses of VLPs [114]. For example, IN delivery of two 10 μg doses of insect cell-derived NVCP VLPs in the presence of a mucosal adjuvant (mutant E. coli heat-labile toxin LTR192G) elicited anti-NVCP titers equivalent to that of two dosages of 200 μg orally delivered adjuvanted VLPs [114].

In addition to intestinal IgA in fecal samples, a strong anti-NVCP IgA response was also detected in vaginal washes. Furthermore, these mucosal IgA responses were long lasting and could be detected a year after the IN immunization [114]. These data not only demonstrate that NVCP VLP is a potent mucosal antigen in stimulating systemic and local mucosal antibody responses, but also indicate its ability in eliciting antibody response at distal mucosa. These findings clearly demonstrated the ability of insect cell-derived NVCP VLPs in eliciting systemic and mucosal B-cell responses to potentially neutralize NV and inhibit its infection and also suggest their application as carriers of heterologous epitopes to combat sexually transmitted infections (STI). For example, chimeric NVCP VLPs that are decorated with epitopes of STI pathogens can potentially induce the production of neutralizing IgAs in the reproductive mucosa.

8.6.2  Mammalian cell-VEE Replicon Produced NoV VLPs

VEE, an alphavirus, has been developed as a replicon vaccine vector. To express a specific vaccine, the coding sequence for the antigen of interest is cloned in place of the VEE structural gene just downstream from the 26S promoter in VEE replicon cDNA to drive its high expression levels [105]. Cotransfection of the cDNA replicon construct with another construct carrying VEE structural genes into mammalian cells will allow the recombinant viral RNA to pack in the VEE viral capsid to form virus replicon particles (VRPs) [57, 105, 115]. These VEE VRPs can infect mammalian cells and accumulate large amounts of vaccine proteins [116]. Because the VEE structural genes are provided in trans for VRP formation and are not part of the recombinant genome, the infection of cells by VEE VRPs is a one-hit event [116].

To produce NVCP VLPs, the gene for NV VP1 was cloned in VEE replicon cDNA and expressed in mammalian cells [105, 115, 117, 118]. VEE-VRP/mammalian cell-derived VLPs can then be purified and delivered through
parental or mucosal routes as immunogens. In addition, the recombinant VRPs can be used as vaccines themselves to produce VLPs in vivo. Therefore, this system can be used in two ways to induce immunity and potentially be advantageous, as VRPs can be directly used to infect permissive mammalian host target cells in which a large quantity of NVCP VLPs are assembled for antigen presentation to B and T cells [117].

For example, direct subcutaneous inoculation of two doses (10^7 infectious units per dose) of recombinant NVCP-VRP through the footpads in mice elicited strong systemic IgG and intestinal IgA responses against NV VLP, as well as heterotypic responses to VLPs of another GI NoV [117]. Furthermore, the serum and intestinal immune responses in mice that were inoculated with two doses of NVCP-VRPs are substantially stronger than those in mice that were given two oral doses of either 75 or 200 μg of VEE-derived NVCP VLPs [117].

Multivalent NoV vaccine candidates have also been produced in the VEE-VRP system and tested in mice [115, 119]. These studies demonstrated that inoculation of a cocktail of three or four VRPs that express strain-specific VLPs in mice not only induced strong serum antibody response against all inoculum strains but also elicited receptor-blocking heterotypic antibody response against novel strains [115]. Moreover, coadministration of a panel of eight human NoV VLPs that cover more than 95% of all NoV infections revealed that inclusion of VLPs from both genogroups (GI and GII) in the vaccine cocktail did not detract from either genogroup-specific response, but induced receptor-blocking antibodies against intergenogroup strains [119].

Compared with the insect cell-baculovirus system, the VEE VRP system can be cumbersome and more expensive due to the need of cotransfection and BSL-3 facilities for VRP production. However, parenteral VEE VRP inoculation has been shown to target DCs and stimulate potent humoral and cellular immunity including protection at mucosal surfaces from infection [113, 120, 121]. Furthermore, VRPs have been shown to possess inherent adjuvant activity most likely due to stimulation of immune cells by a single round of viral RNA replication in mammalian cells [119, 122]. Overall, these advantages may allow VEE VPR to be a superior system over the baculovirus system when VRPs are directly used as vaccines for the induction of potential protective immunity against NoV.

### 8.7 Production and Immunogenicity of Plant-Derived NoV VLPs

#### 8.7.1 Plants as Production Platform for NoV VLPs

Even though NoV VLPs or VRPs have shown promising results as NoV vaccine candidates, production systems based on insect and mammalian cell cultures have several limitations that may hamper the commercial development of these vaccines [54]. For example, the coproduction of baculovirus particles with NoV VLPs in the baculovirus/insect cell system may create problems in VLP purification, immunogenicity, and regulatory approval. Since residual baculovirus may alter the overall immunogenicity of NoV VLP preparation and raise safety concerns, they and their infectivity have to be removed or inactivated by cumbersome and expensive purification processes or chemical treatments. These procedures not only increase the overall production costs but may also impair the quality of the resulting VLPs [31].

The production cost with the VEE/mammalian cell system is significantly higher than that of insect cell cultures. In addition, it also requires heavy capital investment to construct a BSL-3 manufacturing facility [123–126]. Both insect and mammalian cell-based production systems also share challenges in scalability as new fermentation tanks and facilities have to be built for larger-scale production. These challenges may hinder the full realization of the health-benefit potential of NoV VLPs especially in the developing world. As a result, plants have been explored as a safe, cost-effective, and scalable production platform for NoV VLPs.
Plants are considered as an alternative protein vaccine production system because they can produce high levels of vaccine protein at low cost, and biomass production does not require expensive investment in cell culture facilities or facility duplication for scale-up production [124, 127]. As a result, the flexibility and capital efficiency for plant biomass generation and scale-up are far more superior to current fermentation-based technologies [128, 129]. In addition, plants possess eukaryotic processing machinery for proper posttranslational modification and assembly of proteins and have low risk of introducing adventitious pathogens to humans [124, 127]. Despite these potential advantages, earlier production of protein vaccines using stable transgenic plants resulted in slow and low levels of target protein accumulation [54, 126]. The long time frame (several months to a year) to generate transgenic plants, the lack of strong promoters and the position effects from the random insertion of the transgene are responsible for these problems that reduce the cost-saving benefit of plants as a production system [102].

The challenges of VLP production speed and yield has been overcome by the development of plant virus-based transient plant expression systems [130, 131]. For example, our group and others have reported that the cloning and high-level transient expression of plant-derived VLPs can be achieved quickly in 1–2 weeks of vector infiltration with the MagnICON system, which is based on a tobacco mosaic virus (TMV) RNA replicon system or a geminiviral DNA replicon system derived from bean yellow dwarf virus (BeYDV) [126, 132–134]. These improvements in the speed and yield of VLP expression also provide plant expression systems, an additional advantage in versatility for producing VLP vaccines against NoV and other viruses that have rapid antigenic drift and multiple genogroups and strains with unpredictable epidemics around the world.

An effective NoV vaccine needs to be produced in the shortest achievable time frame after strain identification in order to halt the spread of the new strain, preferably by low-cost platforms that allow affordable vaccine manufacturing in locations including the developing world. A platform based on transient plant expression is likely to address such cost and time issues and provide the critical versatility that allows the rapid production of strain-specific vaccines to control potential NoV outbreaks in a timely manner.

### 8.7.2 Plants as Delivery Vehicle for NoV VLP-Based Vaccines

VLP vaccines produced from insect or mammalian cell cultures are purified products that require expensive downstream processing, cold storage, and transport temperatures [102]. The successful induction of systemic and mucosal antibody responses by oral delivery of insect cell-produced NoV VLPs suggests that oral immunization may also be achieved by ingesting edible plants parts containing NoV VLPs [124]. This is an attractive approach as it may reduce the need for the costly purification steps and may circumvent logistic challenges to allow implementation of immunization programs in regions where refrigeration and other medical supplies are limited.

This approach is most likely to be successful with NoV VLPs as NoV naturally infects the gastrointestinal system and, therefore, is resistant to denaturation and digestion in the oral-gastrointestinal tract [31, 107]. Moreover, their resemblance to the native virus allows them to be efficiently sampled by the “M” cells of the gut epithelium and transported into GALT for antigen processing and presentation [102]. Thus, VLPs produced in edible plants represent a novel and cost-effective approach to establishing gut mucosal immunity by oral delivery [135–137].

As a vaccine is required to have a defined dosage unit, this strategy may face regulatory hurdles for commercialization in developed countries [102, 123, 124]. However, this strategy may eventually offer a feasible option for commercial vaccine delivery by oral route, especially as more consistent VLP accumulation per unit of plant tissue is being achieved by the new generation of expression vectors.

Overall, current plant expression systems offer advantages far beyond the traditional proper
eukaryotic protein modification and assembly, low cost, high scalability, and increased safety. For example, they allow VLP production at an unprecedented speed to control potential epidemics or pandemics.

8.7.3 NoV VLPs Derived from Stable Transgenic Plants

Our laboratory has investigated the expression and assembly of VLPs in plants and successfully produced several non-enveloped and enveloped VLPs including ones based on NVCP, HBV core antigen (HBcAg), and enveloped protein of West Nile virus [31]. VLP based on NVCP is one of the most investigated VLPs in plants and has been successfully expressed in many plant species including tobacco, potato, *Nicotiana benthamiana*, tomato, and lettuce by our group and collaborators [103, 129, 132, 133].

As for other vaccine proteins, NVCP was first expressed in transgenic tobacco and potato plants [103]. It usually took several months to generate and select transgenic tobacco and potato plants that expressed NVCP. Expression of NVCP in these transgenic plants was approximately 10 μg/g fresh tissue weight, which is rather low compared with the insect or mammalian cell-based expression system [101]. However, assembled virion-sized icosahedral VLPs that resemble insect cell-derived VLPs or native NV particles were observed in transgenic tobacco leaves and in potato tubers [101]. In another experiment, the VP1 gene of NV was codon optimized and expressed in transgenic tomato plants [138]. Comparative studies indicated that VLP yield and assembly varied depending on the codon usage, host plant species, and possibly the target tissue of NVCP accumulation [101]. For instance, NVCP expression level was low, and only 25–50 % of them was assembled into VLP in potato tubers, while at least ten-fold higher expression and more efficient VLP assembly were achieved in tomato fruits [101].

To achieve gut mucosal immunity against NV, four doses of 4 g uncooked potato tuber containing 40–80 μg NVCP were fed to mice at days 1, 2, 11, and 28. This immunization regime induced specific serum IgG and intestinal IgA responses in mice [101]. When four doses of 50 μg purified NVCP VLPs were orally delivered to mice in the same immunization regime, serum anti-NV response was four-fold higher than that of mice fed with NVCP-expressing-tuber [101]. The humoral immune response can be further enhanced to 16-fold higher when adjuvant (CT) was co-delivered [101]. Perhaps, the enrichment of assembled VLPs in the purified samples is responsible for the enhancement of humoral immunity because only ~50 % of the NVCP in the potato tubers was assembled into VLPs [31]. It is also possible that NVCP was not efficiently released from the potato tissue to be delivered to GALT for antigen processing and presentation.

Tomato fruits present a more feasible plant material for developing oral NoV vaccines as they are more palatable than raw potato tubers, and their production and processing have been well established by the food industry [31]. Oral delivery of four doses of 0.4 g freeze-dried tomatoes (containing 40 μg VLP) stimulated strong serum anti-NVCP IgG and intestinal mucosal IgA responses in more than 80 % of mice [139]. Furthermore, 100 % of mice developed strong systemic and mucosal antibody responses when a higher dosage (0.8 g per dose) of transgenic tomato was used [138].

The same study also showed that NVCP fed in freeze-dried tomato was more immunogenic than that in freeze-dried potato tubers [138]. The less oxidative environment or the unique tissue structure in tomato fruits may allow better stability or more efficient release of VLPs and, in turn, better immunogenicity. It was also noticed that ingestion of NVCP air-dried tomatoes elicited more potent serum IgG and intestinal IgA responses than that NVCP freeze-dried tomatoes [138]. It is possible that the freeze-drying process altered the assembly status of VLPs in tomato fruits. Alternatively, air-drying may result in better VLP stability by preserving the architecture of VLPs and the tissue structure so that VLPs are better protected from the digestive enzymes in the oral-gastrointestinal tract or...
more efficiently released from the tissue for uptake by GALT. Overall, these data support the development of oral vaccines for NoV in tomato plants.

8.7.4 NoV VLPs Produced by Transient Expression with Plant Virus-Based Vectors

The major challenges up to this point for NoV VLP production in plants were the slowness of obtaining VLP-expressing plant lines and poor VLP yield. One of the strategies to overcome these challenges was the use of transient expression systems based on vectors developed from plant viruses. In these systems, transgenes are not integrated into one of the plant genomes, but instead, they are present in the plant nucleus transiently while being transcribed, and later, the transcripts are transported into the cytoplasm, and the transgenic proteins are translated [140, 141].

These systems are focused on production speed and yield and gain the flexibility of nuclear gene expression with the speed and expression amplification of viruses. For example, the MagnICON transient expression system based on replication-competent TMV and potato virus X (PVX) allows high levels of recombinant protein production within 7–10 days of vector delivery [141, 142]. Indeed, our results show that the MagnICON system allows us to produce fully assembled NVCP VLPs at a level of 0.8 mg/g of fresh leaf weight (FLW) within 12 days of infiltration in N. benthamiana plants, at least an 80-fold greater production than in transgenic tobacco and tomato [133].

When delivered through the oral route, the partially purified VLPs (100 μg per dose) from transiently expressed plant material provoked potent and balanced systemic IgG1/IgG2a response in the absence of any adjuvant [133]. Significant NVCP-specific vaginal and fecal mucosal IgA responses were also detected in 100 % of immunized mice by the same immunization regime [133]. Moreover, a significant enhancement in NVCP-specific immunity was achieved by the inclusion of adjuvant CT in the oral immunization [103]. Thus, the “destructed” viral vector-based transient expression system has enabled us to overcome the challenges associated with transgenic plant systems and rendered a robust plant system for NoV production.

To further optimize the transient expression systems for commercial production of NoV VLP vaccines, we developed another robust expression system based on geminiviral BeYDV DNA replicon vectors and commercially available lettuce [129, 143]. There are two major advantages of this transient expression system: the noncompetitive nature of BeYDV DNA replicon vector and the use of commercially produced lettuce. In contrast to the MagnICON system, which can produce proteins with maximally two different hetero-subunits, the geminiviral vector allows the production of VLPs with up to at least five hetero-subunits [143].

Lettuce is cultivated readily and rapidly produced in large quantities in well-established commercial greenhouses. Unlike tobacco and related species of Nicotiana such as N. benthamiana, lettuce is a palatable plant and can be consumed raw for oral delivery of NoV VLPs. When NVCP was expressed in N. benthamiana plants with BeYDV replicon vectors, assembled VLPs were accumulated up to 0.4 mg/g FLW in leaves [132]. Our data also indicate that the BeYDV replicon system allows a high level of NVCP VLP expression and assembly in lettuce as those driven by the MagnICON system in N. benthamiana [129].

Actually, VLP accumulated to its highest level at day four after vector introduction, indicating that this expression system can produce similar levels of VLPs in lettuce but in a much shorter time frame than the MagnICON system in tobacco [129]. Importantly, this study is one of the first demonstrations of using commercially produced lettuce for rapid and high-level production of VLPs [129]. This represents a significant milestone for the eventual commercial production of NoV VLP-based vaccines, as this would allow our production system to have access to unlimited quantities of low-cost material from existing commercial sources. Coupled with the almost unlimited nature of
lettuce production, the high efficiency and scalability of our BeYDV replicon vectors provide us a new NoV VLP production platform that is low cost, robust, safe, and amenable to large-scale manufacturing.

### 8.8 Production of Plant-Derived NoV VLP Vaccines Under Regulatory Conditions

Despite the successes in expressing NVCP VLPs and a variety of other subunit vaccines in plants and the recent improvement of vaccine production levels by transient expression systems, no plant-derived vaccine has been licensed yet for human use. This lack of commercial success lies in several technical and regulatory barriers that remain to be overcome. These challenges include the lack of scalable downstream processing procedures, the uncertainty of regulatory compliance of production processes, and the lack of demonstration to date of plant-derived vaccines that meet the required standards of regulatory agencies in identity, purity, potency, and safety [123, 124, 127].

While immunization by ingesting plant tissue still presents a viable approach to orally delivering plant-produced VLPs, product regulatory concerns have necessitated the development of downstream processing technologies to produce purified VLP vaccines with a defined unit dosage [123]. Therefore, for the promise of plants as an alternative for vaccine production to become a reality, the technology must be able to produce sufficient quantities of VLPs at a relevant scale and with product qualities that meet all required standards of regulatory agencies such as US Food and Drug Administration (FDA).

### 8.9 Establishment of Facilities for Plant Production of NoV VLPs Under FDA Regulation

To overcome these remaining challenges, the regulatory compliance of facilities, materials, and procedures for production of NoV VLPs under current Good Manufacture Practice (cGMP) has to be addressed first. Our group has led the effort in this area and established the first cGMP-compliant bioprocessing facility in academia for the production of NoV VLPs in plants [126].

As shown in Fig. 8.2, the purposefully built facilities in our university permit biomass generation and bioprocessing of plant-derived NoV VLP vaccines under cGMP Quality Management System (QMS). For example, separated rooms for buffer preparation, incoming plant samples, chromatography, and sterile fill are purposefully designed in the central bioprocessing suite (Fig. 8.2a).

As indicated by the arrows in the same figure, the suite is operated under differential pressure with the sterile fill room being a Class 100 environment and designed to have a separated unidirectional flow of human workers and biological material, so that the products meet the requirements of both US and European Union (EU) regulatory agencies for manufacturing of human pharmaceuticals. As a part of the QMS, the quality control (QC)/Quality Assurance (QA) laboratory is adjacent to the bioprocessing suite for product testing and quality management (Fig. 8.2a). A 3,600 ft² biosafety level 2 (BSL-2) greenhouse facility has also been built for plant biomass production under cGMP (Fig. 8.2b).

The BSL-2 greenhouse is equipped with advanced technology for containment of plants or pathogens, which is necessary to maintain the integrity of the plant-derived VLPs and to minimize the risk of accidental genetic contamination of the external environment.

The established bioprocessing facilities and QA/QC laboratories have also been certified for producing human vaccines, which laid a solid foundation for the cGMP compliance of VLP production procedures and the final VLP vaccine product.

### 8.9.1 A. tumefaciens and N. benthamiana Master and Working Banks

As part of the cGMP compliance of upstream processes, biomaterials including relevant
Agrobacteria strains and the N. benthamiana plant line have to be qualified by the QMS. This process starts with the establishment of master and working banks of A. tumefaciens strains that containing the NVCP expression vectors and a seed bank of wild-type N. benthamiana for growing biomass for transient expression. To generate plant material for cGMP-compliant production of NVCP VLPs, three A. tumefaciens master banks, each containing the expression cassette for VLP production in TMV 3’ module, TMV 5’ module, and integrase of the

Fig. 8.2 cGMP production facilities for NVCP VLP vaccine. (a) The design of the central bioprocessing suite. The arrows indicate the unidirectional flow of in-process materials (green), purified final product (red), and people (yellow). (b) The BSL-2 greenhouse facility for plant biomass generation and NVCP expression. (c) The Plant Biopharmaceutical Center that houses the central bioprocessing suite, the QA/QC laboratory, and the Process Development laboratory (Adapted with kind permission from Springer Science + Business Media: Plant Cell Reports, Lai and Chen [126], Fig. 1)
MagnICON system, [141] have been established in the bacterial strain of GV3101 [126]. The identity of the A. tumefaciens host strain and associated functional genes and vectors are fully validated. These banks have been shown to be genetically stable over a 4-year period [126]. A master seed bank for wild-type N. benthamiana was also established. Our experiment has shown that this seed bank has a germination rate of 99% and produced uniform seedlings with typical N. benthamiana morphology even after a 4-year storage at 4 ± 2 °C (Fig. 8.3) [126]. These results collectively demonstrated that it is feasible to establish master and working banks of A. tumefaciens strains and N. benthamiana seeds that meet the qualification standards of identity and stability for cGMP production of NoV VLPs.

8.9.2 Biomass Production and Infiltration at Pilot Scale

In this part of the upstream process, the goal is to optimize conditions that permit the maximal production of leaf biomass and VLPs per square meter of green house space. Studies by our group and others demonstrated that temperature, light source and intensity, plant age, and incubation time after leaf infiltration all have significant impacts on biomass generation and VLP accumulation [126]. For example, N. benthamiana grown under nature light produces more biomass, but accumulates much less NVCP VLPs than plants grown under artificial light (Fig. 8.4a). Furthermore, leaves grown under natural-light also produce more solid debris during VLP extraction, causing problems for VLP purification [126]. Our data also indicate that a 16-h light/8-h dark cycle at 25 °C is the optimal condition to generate biomass under such artificial lighting [126]. When N. benthamiana plants were grown under these conditions and sampled at various ages for NVCP VLP expression, it was founded that 5-week-old plants provided the optimal material for VLP production, because they already accumulated an adequate amount of biomass and produced the highest level of VLPs (Fig. 8.4b). Younger plants produce insufficient biomass, and older plants are too tall for infiltration and start to produce more secondary metabolites that complicate VLP purification.
Consequently, 5-week-old *N. benthamiana* optimally balance the combined need for biomass generation, filtration operation, and VLP accumulation. Our data demonstrated that under these optimized conditions, biomass for producing sufficient amount of purified NVCP VLPs for a Phase I clinical trial can be generated in 5.6 square meters of greenhouse space in a 5-week period.[126] The horizontal space requirements for this type of production could be significantly reduced by vertically stacking layers of growth trays vertically.

Methods for efficient introduction of *Agrobacterium* into leaves by vacuum infiltration were also developed.[126] As illustrated by the expression of the GFP and NVCP VLPs, plants infiltrated based on these methods under scale-up conditions allowed efficient infiltration of vector-carrying *Agrobacteria* in the entire leaf area (Fig. 8.4c) and accumulated the highest level of NVCP VLPs in leaves 7 dpi (Fig. 8.4a, b). Moreover, the yield and temporal expression pattern of NVCP VLPs under these scale-up conditions are similar to that of our previous smaller

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**Fig. 8.4** Optimization of biomass and NVCP VLP accumulation. (a) Biomass and NVCP yield under natural and artificial light. *N. benthamiana* plants were growth either under natural or artificial light for 5 weeks. Leaf biomass (green square) and NVCP expression level (red column) were measured by weighing and ELISA, respectively. (b) Temporal pattern of biomass yield and NVCP expression. Plants were grown under artificial light for 5 weeks, and biomass production (green square) and NVCP accumulation (red column) were examined. For both (a) and (b), mean ± standard deviation (SD) of samples (*N* > 10) from three independent infiltration experiments is presented. (c) and (d) visualization of GFP expression in agroinfiltrated leaves. *N. benthamiana* plants were infiltrated either with GV3101 cultures that carry the three MagnICON vectors for GFP expression (c) or with infiltration buffer as a negative control (d). Leaves were examined and photographed 7 dpi under UV light. One representative of at least three independent experiments is shown (Adapted with kind permission from Springer Science + Business Media: Plant Cell Reports, Lai and Chen[126], Fig. 3)
bench-scale experiments [126, 133]. These results clearly indicate that the upstream process for NV VLP production in plants is scalable and can be further scaled up for commercial production.

8.9.3 Downstream Processing of NoV VLPs

While eating unprocessed or partially processed NoV VLP-containing plant tissue remains a viable option for vaccine delivery, purified VLPs with a well-defined unit dosage are most likely to be the first generation of successful vaccine candidates for commercialization due to regulatory requirements [123, 124]. Therefore, it is absolutely necessary to have a robust and scalable downstream process that can effectively recover and purify VLPs in place in order for plant-produced NoV VLP vaccines to become commercial reality. An optimized downstream process should be able to preserve the integrity of VLPs and produce highly purified VLPs with a high recovery rate and a minimal number of processing steps.

It should also be scalable, economical, and compliant with the FDA’s cGMP regulations. The most common practice for NoV VLP purification is density gradient ultracentrifugation [106, 108, 133, 144]. While these methods are useful for characterizing VLP size and assembly [107, 133, 144], they are not practical for commercial VLP manufacturing as they are difficult to scale-up and time-consuming [143, 145]. They may also be problematic for preserving the integrity of VLPs because the hyperosmotic gradient agents and severe centrifugation force can often shear VLPs [146]. To meet the demand for more scalable and robust methods of NoV VLP processing, a new trend has started toward more sophisticated methods like filtration and chromatography [31]. For example, our group has recently developed a robust and scalable downstream processing scheme for recovering NVCP VLPs from plants (Fig. 8.5).

In this three-step process, plant extracts are processed by low pH precipitation, ultrafiltration (UF/DF) with tangential flow filtration (TFF) membranes, and ion-exchange (IEX) chromatography [126]. We showed that low pH and UF/DF eliminated most plant host proteins and IEX chromatography purified NVCP VLPs to >95 % purity (Fig. 8.6, Lane 5) [126]. Production runs with various scales of plant biomass demonstrated that this downstream process is highly scalable and can consistently yield highly pure NVCP VLPs with high recovery rates [126]. In contrast to gradient centrifugation that is laborious and time-consuming, the new method is robust and more scalable and can be completed within ≤12 h instead of several days [126, 143]. Importantly, this new process is fully cGMP compliant and has produced cGMP grade VLPs for a human clinical trial [31].

New technologies such as microarrays have also been employed to further optimize NoV VLP purification from plants [143]. For example, our lab and its collaborators have identified peptide ligands with specific affinity to NVCP VLPs from a microarray. When conjugated to chromatography beads, these affinity ligands allow the recovery of highly purified NVCP VLPs from N. benthamiana plant extracts [31]. This approach is advantageous over the traditional affinity chromatography because our affinity ligands are entirely synthetic and therefore insensitive to ligand denaturation or degradation.

These advantages along with the rapid ligand discovery process and the low cost of peptide production would allow its application to large-scale NoV VLP manufacturing.

8.9.4 Quality Control of Plant-Derived NoV VLPs

One of the remaining challenges for the commercialization of plant-derived vaccines is the lack of examples that meet the required standards of regulatory agencies in identity, purity, potency, and safety [123, 124, 127]. As part of the efforts to overcome this challenge, our laboratory has identified and developed analytical assays to monitor the in-process samples and to ensure that
the final VLP product meets preset specifications for the release of human pharmaceuticals in identity, purity, concentration, tertiary structure, functionality, and in concentrations of host contaminating molecules essential for the cGMP compliance (Table 8.2). Our analyses showed that the identity and assembly of the final VLP product were confirmed and its purity, concentration, appearance, residual host DNA concentration, and stability all conformed to the insect cell-derived reference standard. These results have provided the first example of plant-derived NoV VLPs from a scale-up process that meets the predetermined release specifications.

In summary, production studies by our laboratory have successfully developed upstream process for plant biomass generation, infiltration and robust NoV VLP accumulation, and a novel downstream process for efficiently recovering VLPs from plant tissue. Moreover, these processes have been successfully operated under cGMP regulations and produced high-quality VLPs that meet all preset release specifications in identity, purity, potency, and safety. Hence, these studies provide the first precedent in an academic setting of producing a plant-derived vaccine at scale and under cGMP regulations and are an important step for plant-produced NoV VLP vaccines to become a commercial reality.

Ongoing research by our group and collaborators is evaluating the systemic and mucosal immunity of the cGMP-purified VLPs when co-delivered mucosally with various adjuvants. Preliminary data suggest that mucosal immunization with several adjuvants evoked stronger serum IgG and mucosal IgA responses than with VLP alone. We anticipate that the purified NVCP VLPs from our cGMP plant production runs and the adjuvants defined in our current studies will be used in a new Phase I human clinical trial in the near future.
8.9.5 Human Clinical Trials with NoV VLPs

The successful demonstration of strong systemic and mucosal immunogenicity in preclinical studies has led to several clinical trials to examine the safety and immunogenicity of NoV VLP-based vaccines in humans (Table 8.3).

Insect cell-derived NVCP VLPs were initially tested in two Phase I trials [144, 147]. In the first trial, 20 antibody-positive adult volunteers were given two oral doses (days 1 and 21) of 100 or 250 μg of VLPs formulated in water without adjuvant. It was observed that serum IgG responses were dose dependent and 100% of vaccinated subjects who received 250 μg of VLPs increased their NV specific titers at least four-fold [147]. Fifteen out of 18 participants responded in serum IgG titers after the first VLP dose and showed no increase after the second dose [147]. Importantly, no side effects were observed in vaccinated volunteers [147].

In the second trial, the safety and immunogenicity of NVCP VLPs were further tested in 36 adult seropositive healthy individuals between 18
and 40 years of age with two oral doses of increasing amounts (250, 500, and 2,000 μg) of antigens without adjuvant using the same immunization regime [144]. Significant increase in the number of NV-specific IgA antibody-secreting cells (ASC) was observed in all vaccinated volunteers and approximately 30–40 % of volunteers developed salivary, fecal, or genital fluid IgA antibody. An increase of NV-specific IgG titers was also detected in 90 % of participants

| Production system | Number of volunteers | Formulation | Number of doses and dosage range | Delivery route | Immunogenicity |
|-------------------|----------------------|-------------|----------------------------------|----------------|---------------|
| Insect cell/baculovirus | 20 | Water, no adjuvant | Two doses of 100–250 μg at days 1 and 21 | Oral | Serum IgG and IgA responses were dose-dependent and NV-specific titers increased at least four-fold in 100 % of volunteers in the 250 μg group. 83 % responded in serum IgG titers after the first VLP dose and showed no increase after the second dose [147] |
| Insect cell/baculovirus | 36 | Water, no adjuvant | Two doses of 250, 500, and 2,000 μg at days 1 and 21 | Oral | Increase of NV-specific IgG titers was detected in 90 % of participants in the 250 μg group. No further increase in the rates of seroconversion or IgG titers for the 500 and 2,000 μg groups. Increase of NV-specific IgA ASC numbers in 100 % of vaccinated volunteers was observed and 30–40 % of volunteers developed salivary, fecal, or genital fluid IgA antibody [144] |
| Insect cell/baculovirus | 28 | Dry power with chitosan, MPL as adjuvant | Two doses of 5, 15, and 50 μg at days 0 and 21 | IN | Serum IgG and IgA responses were dose dependent with their titers increased 4.7 and 4.5 folds, respectively, for the 50 μg group. 53 % of subjects developed rises in NV-specific IgA ASCs [148] |
| Insect cell/baculovirus | 61 | Dry power with chitosan, MPL as adjuvant | Two doses of 50 and 100 μg at days 0 and 21 | IN | 63 and 79 % of vaccinated subjects in the 100 μg group increased IgG and IgA titers by 4.8- and 9.1-folds, respectively. The IgG and IgA titers of the 100 μg group are higher than the 50 μg group, but not statistically different. All vaccinated individuals in the 50 and 100 μg groups developed IgA ASCs. Homing molecules to mucosal and peripheral lymphoid tissues were detected on these ASCs [148] |
| Transgenic potato | 20 | Raw potato tuber a | Two or three doses of 215–751 μg in 150 g potato tuber a at days 0 and 21 or at days 0, 7, and 21 | Oral | 20 and 30 % of vaccinated volunteers developed NV-specific serum IgG (12-fold rise) and fecal IgA (17-fold increase), respectively. 95 % of subjects developed NV-IgA ASCs [149] |

IN intranasal, ASCs antibody-secreting cells

a Each 150 g of raw potato tuber contained between 215 and 751 μg variable amounts of NVCP antigen
who received 250 μg of VLPs [144]. However, further increase in VLP doses (500 and 2,000 μg) did not increase the rates of seroconversion or IgG titers [144].

While the results from the first two trials were encouraging, however, the maximal serum IgG titers elicited by oral immunization were lower than those after experimental NV infection. Consequently, two additional Phase I studies were conducted to investigate whether the immunogenicity of NVCP VLPs can be further enhanced by using mucosal adjuvants [148]. Insect cell-produced NVCP VLPs were formulated in a dry power containing a TLR4 agonist adjuvant, monophosphoryl lipid A (MPL), and the mucoadherent chitosan and delivered by IN route to healthy subjects [148].

Study 1 was a stepwise dosage escalation trial with 5, 15, and 50 μg of NVCP VLPs. Study 2 was a dose comparison study of the two highest dosages (50 and 100 μg) VLPs. These studies indicate that IN delivery of NVCP VLPs in a dry power formula with MPL and chitosan was well tolerated and no vaccine-related serious adverse events occurred [148]. In study 1, dose-dependent serum IgG and IgA responses were observed with their titers increased 4.7- and 4.5-fold, respectively, for the 50 μg group [148]. In study 2, the 100 μg group developed higher titers of IgG and IgA (4.8- and 9.1-fold increase, respectively) than that of the 50 μg group, but the differences were not significant in statistical terms [148].

All vaccinees in the 50 and 100 μg groups developed IgA ASCs. Furthermore, expression of homing molecules targeting to mucosal and peripheral lymphoid tissues was detected in these ASCs. Compared with the previously described oral nonadjuvanted VLPs, IN delivery of MPL-adjuvanted VLPs induced a higher number of these ASCs [148]. While the correlates of protective immunity against NV disease are still unknown, these mucosally primed ASCs in combination with the serum IgG and IgA antibodies may contribute to protection.

A Phase I/II trial has been initiated to assess safety and immunogenicity of NVCP VLPs administered by the IN route, followed by a live virus challenge to determine the effectiveness of this approach in preventing or limiting NV infection in humans [103].

To demonstrate the safety and efficacy of plant-derived NoV VLPs, a Phase I clinical trial was conducted with potato-produced NVCP VLPs. In this trial, two or three doses of 150 g uncooked NVCP-transgenic potato tubers (215–751 μg VLPs per dose) were orally ingested by 20 human subjects on days 0 and 7, or on days 0, 7, and 21 [149].

All but one of the volunteers responded with a rise in NV-specific IgA ASCs, while the majority responded following the first dose. Four of the volunteers developed NVCP-specific serum IgG, and six developed specific intestinal IgA with a mean titer rise of 12- and 17-fold, respectively [149]. The incidence rates of nausea, vomiting, mild cramps, fever, or diarrhea were similar among volunteers who ate recombinant or control tubers, indicating that the ingestion of VLP-producing potato tubers appeared to be safe [149].

Together, these results indicate the immunogenicity and safety of using edible VLP-containing plant parts as oral vaccines of NoV in humans. However, the overall antibody response was not as strong as that obtained by orally delivered purified NVCP VLPs (250 μg per dose) produced in insect cells [144]. Perhaps, the variable effective VLP dosage due to inconsistent NVCP content and poor VLP assembly in potato tubers may cause this weak antigenicity. The potency of the plant-derived VLPs may also be further reduced by their poor release from the potato tissue in the gut lumen. In light of this and the results from recent human clinical trials with insect cell-derived VLPs, in the near future, we are planning a new human clinical trial with purified NVCP VLPs from our cGMP runs and the adjuvants defined in our current studies (see Quality control section above) [126].

Overall, these clinical studies have demonstrated that NoV VLP-based vaccine candidates produced in insect cells and plants are safe and immunogenic in humans. Since the model of NV infection in human volunteers has been established recently [40, 150], the efficacy of adju-
vanted NVCP VLP vaccines through IN and oral delivery should be further examined in this human challenge model.

8.10  **Strengths and Challenges of VLP-Based NoV Vaccines**

For uncultivable NoV, capsid protein-based VLPs possess the best properties in immunogenicity, safety, stability, and manufacturability as vaccine candidates to prevent NoV GE. Like other VLP-based vaccines, NoV VLPs have been shown to induce potent cellular and humoral immune responses without adjuvants due to their resemblance to infectious viruses. Since NoV naturally causes infection in the gastrointestinal tract, the VLPs are stable at low pH and resistant to digestive enzymes. As a result, they can be administered orally and elicit a potent mucosal antibody response and systemic response in mice and humans. For example, oral delivery of NVCP VLPs induced robust production of anti-NV serum and intestinal antibodies and interferon-γ in peripheral blood mononuclear cells [144].

Similarly, potent systemic and mucosal immune responses were provoked in mice and humans by IN delivery. The ability to induce NoV-specific gut mucosal immunity by mucosal delivery provides VLPs a distinguish advantage as vaccines for these enteropathogenic viruses. Moreover, the safety of NoV VLPs have been demonstrated in several human clinical trials. The stability of NoV VLPs also favors them as commercial vaccines as they can be lyophilized or stored at 4 °C in simple buffers for many years without degradation [73]. Significantly, studies by our group and others have demonstrated that NoV VLPs can be robustly produced by recombinant technology in plants at large scales with low cost [31].

The development of a novel downstream process and the successful production of NVCP VLPs that meet all regulatory release specifications in identity, purity, potency, and safety by our group provide an additional step for NoV VLP vaccines to become a commercial reality [126]. These successes have demonstrated the potential and feasibility of VLPs as NoV vaccines and suggest they can serve as an excellent model for developing effective strategies of mucosal immunization with non-replicating antigens.

There are still remaining challenges for the development of efficacious NoV vaccines. For example, the question of whether VLP-based vaccines can protect humans against a live NoV challenge still remains unanswered. Moreover, it is difficult to predict the level of protection that new vaccine candidates can provide due to the lack of a complete understanding of the immune correlates of protection. This problem is further intensified by the lack of culture systems to cultivate NoV in vitro and the lack of small animal models of NoV pathogenesis. Furthermore, the lack of complete cross-protection among diverse genotypes and genogroups of viruses and the rapid evolution of new variant strains further hamper the development of efficacious vaccines that are protective against multiple NoV strains.

Overall, the potent systemic and mucosal immunogenicity, the robust and low-cost cGMP manufacturability in plants, and the safety profile in human clinical trials all support the further development of VLP-based vaccines for the prevention of NoV-related GE. Due to the diversity of capsid protein among different NoV strains and its rapid antigenic drift, vaccine development should focus on multivalent VLP vaccines that are derived from capsid proteins of the most prevalent strains. Furthermore, vigilant epidemiological surveillance must be coupled with NoV vaccine production to identify this moving target and to include the most prevalent circulating strains in the formulation for optimal protection. New data from ongoing and planned human clinical trials, particularly challenge trials, should shed new light on the efficacy of VLPs in preventing or limiting NoV infection in the next few years. They may also provide clues for the immune correlates of protection. This knowledge will facilitate the development of efficacious NoV vaccines.

A lingering criticism of plant-based production platforms has been the absence of approved human products in the USA after 25 years of
active research and development [125]. Excitingly, this last barrier has been overcome by the recent approval of a plant-produced glucocerebrosidase (commercial name: ELEYSO™) by the FDA for treating Gaucher disease, heralding a new era in the field of plant-made pharmaceutics [31]. We speculate that plant-based production systems will offer a superior scalability, safety, time and cost-saving benefits for NoV VLP manufacturing.

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