Chapter 6: Summary and conclusions

1) The study of viruses have led to advancements in various fields of biology including molecular biology, bionanotechnology, vaccine development, anti-bacterial therapy, host biology, protein expression systems etc. Owing to their small genome size, viruses encode multi-functional proteins that play crucial roles in their life cycle. The present thesis deals with the use of SeMV VLPs as a nanocarrier for antibody delivery and biochemical characterization of a multifunctional viral protein GBNV NSs.
2) Bionanoparticles are taking over synthetic nanoparticles owing to their target specificity and biocompatibility with the host. Viruses are also being explored as a nanoparticle for drug delivery, imaging agents, protein scaffolds, three dimensional arrays etc. Plant viruses are particularly explored in this avenue as they are non-pathogenic in humans. Although a plethora of plant viruses and structural information on them are available, only a few have been explored as nanoparticles.
3) SeMV is a positive sense RNA virus infecting *Sesbania grandiflora* resulting in mosaic like symptom. It encodes for 4 ORFs and ORF3 encodes for the CP that forms the outer viral shell encapsidating the RNA genome. CP can self-assemble into VLPs with 30 nm sized particles, even when expressed in a heterologous system like E. coli. These high density particles can be purified via sucrose density gradient ultracentrifugation. The X ray crystal structure of native as well as recombinant CP revealed an icosahedral (532) symmetry with 20 hexamers and 12 pentamers. Each protomer of CP contains a jelly roll motif of β strands connected by loops and helices. Careful analysis of the structure revealed that both pentamers and hexamers have an 8 residue surface exposed loop (HI). In order to develop a chimeric VLP with an accessible domain, the midpoint of HI loop was chosen for insertion of a foreign domain.
4) Antibodies have gained importance as therapeutic molecules and are being currently administered for various diseases like cancer, autoimmune disorders etc. Intracellular antibody delivery is a major challenge in the medical field as antibodies cannot cross membrane barrier and might lose the activity once inside cells. Interestingly, there has been no plant virus based nanoparticle for antibody delivery in mammalian cells. Hence two antibody binding domains (B domain of

protein A and Z33) were genetically fused at the HI loop of CP. The resulting chimeras (SLB and SLZ33) also assembled into VLPs when expressed in E. coli and B/Z33 domain retained antibody binding affinity in the assembled VLPs. SLB showed 45 times higher IgG affinity as compared to protein A, indicating multiple functional B domains on the viral capsid.

5) In order to demonstrate VLP entry in mammalian cells, CP and SLB were labeled with Alexa fluor 488 and labeling did not affect the structural or functional integrity of the VLPs. Using confocal microscopy, it was demonstrated that CP and SLB can enter human cervix adenocarcinoma cells (HeLa), keratinized HeLa cells (KB), mus musculus melanoma cells (B16-F10), human mammary gland derived cells (BT-474), breast epithelial cancer cells (CB 704) and normal human mammary epithelial cells (HMECs 704). CP was also able to enter HeLa cells even in presence of non-specific proteins like BSA and polyclonal sera. No apparent cytotoxic effects due to CP and SLB entry into HeLa and BT-474 cells were observed.
6) Since SLB was able to enter cells, it was of interest to examine whether SLB can bind to antibodies and deliver them intracellularly. For demonstration of antibody delivery, three different kinds of monoclonal antibodies raised against abrin toxin (D6F10 antibody), intracellular tubulin (anti-α-tubulin antibody) and surface exposed HER2 receptor (anti HER2 antibody/Herclon) were chosen. SLB was not only successfully able to deliver all the three antibodies within mammalian cells but also these antibodies remained functional once inside cells.
7) Although, D6F10 cannot enter cells by itself, it can bind to abrin A chain and remains co-localized with abrin once inside cells. SLB mediated delivery of D6F10 reduced the abrin mediated protein synthesis inhibition as well as apoptosis better than abrin-D6F10 complex, indicating that D6F10 is functional inside cells and thus overcoming one of the caveat of administering D6F10 prior to abrin toxin exposure.
8) Tubulin is an abundant cytoskeleton element that is present throughout the cytoplasm. Anti-tubulin antibodies when delivered inside cells lead to depolymerization of tubulin, resulting in disruption of the tubular network and aggregation of tubulin monomers. Although there has been various ways of

delivering anti-tubulin antibodies, no virus nanoparticle has been demonstrated to deliver such antibodies. Hence, SLB mediated anti-tubulin antibody delivery was checked in HeLa cells. As expected, delivery of anti-tubulin antibody via SLB resulted in cytoplasmic appearance at low concentration/time point (≤ 2 hr) and aggregation pattern at higher concentration/time point (≥ 4 hr). Thus the anti-tubulin antibodies remain functional when delivered using SLB nanocarriers.

9) Most of the therapeutic antibodies are dominated by those against surface exposed receptors. Herclon is one such therapeutic antibody used in treatment of breast cancer patients over-expressing the human epidermal growth factor (HER2). HER2 is known to activate various signaling cascades (MAPK, PI3K) promoting cell proliferation and inhibiting apoptosis. Herclon binds to the surface exposed epitope of HER2, localized in the membrane and inhibits further signaling cascade by HER2, resulting in inhibition of cell proliferation. SLB mediated delivery of Herclon was analyzed using confocal microscopy. Indeed SLB, and not CP, was able to deliver Herclon intracellularly. The functionality of this delivery when analyzed by anti-proliferation assay showed that although Herclon by itself can cause 25 % cytotoxicity by 48 hr at 2.5 µg/ml while SLB mediated Herclon delivery resulted in 83 % cytotoxicity. Although CP and SLB by themselves do not cause any adverse effects, preincubation with Herclon resulted in 4 fold enhanced toxicity as compared to Herclon alone. However, at a 90 hr time point, the cytotoxic effects were reversed with Herclon alone resulting in 88 % cytotoxicity and SLB mediated Herclon delivery resulting in 20 % cytotoxicity. These results indicate that the Herclon delivered by SLB starts degrading once inside the cell for a long period of time, hence reducing the effective concentration of Herclon. This confirms that internalized antibodies are more effective than those targeted to the surface proteins. Thus SLB can effectively deliver any antibody inside mammalian cells and also retaining their functionality, overcoming one of the major challenges in antibody mediated therapy.

10) Most viruses encode helicases or hijack host helicases to unwind complex structures in their genome. However, no RNA helicases have been so far identified from negative strand RNA viruses. It was earlier identified that Non structural protein (NSs) of *Groundnut Bud Necrosis Virus* (GBNV) (Tospovirus

genus) is a multifunctional protein with Poly A stimulated NTPase, 5' α phosphatase and bidirectional DNA helicase activities. However, GBNV is a RNA virus and hence in the present study, the RNA helicase activity of NSs was explored. NSs was successfully able to unwind duplex RNA (with 14 bp complementarity) in a Mg^{2+} and ATP dependent manner in a bipolar direction. Alanine mutation of the lysine 189 residue of the walker A (K189A NSs) motif abolished the helicase activity.

11) In an attempt to identify the domains involved in helicase activity, NΔ124 NSs and CΔ80 NSs were created with an intact walker A and probable walker B motifs. NSs and K189A NSs majorly exist as dimers in solution while NΔ124 NSs and CΔ80 NSs exist as monomers, although there is no major change in their secondary structure of all four proteins. The ATPase activities of both the mutants were unaffected. However, NΔ124 NSs lost DNA as well as RNA helicase activity suggesting that the N terminal 124 aa residues could be essential for substrate binding and duplex unwinding, while CΔ80 NSs retained partial RNA unwinding activity. Thus a change in the oligomeric state could also account for loss in helicase activity. Thus NSs acts as a dual helicase in its dimeric form.

12) Plants possess elegant but complex mechanisms to defend themselves against infectious pathogens. One such mechanism against viruses is the post transcriptional gene silencing (PTGS), wherein presence of dsRNA triggers a set of proteins cleaving dsRNA into small interfering RNA (siRNA), culminating in sequence specific degradation of viral mRNA. However, viruses encode suppressors to this pathway (Viral suppressor of RNA silencing, VSRs), ensuring efficient viral life within the host. GBNV NSs was known to act as a suppressor of PTGS pathway. GBNV NSs was earlier known to act as a VSR. Since it also possess RNA helicase activity, the role of ATPase or helicase in suppressor activity was analyzed using transient GFP based suppression assay in *Nicotiana benthamiana* and *N. benthamiana* 16c (GFP expressing transgenic plants). It was observed by GFP fluorescence, northern blot analysis and western blot analysis of infiltrated leaves that both NSs and K189A NSs was able to suppress PTGS pathway, indicating that the ATPase or helicase do not have any role in its suppressor activity.

13) Since NSs also possess RNA helicase as well as 5' α phosphatase activities, the ability of NSs to unwind siRNA duplexes and also remove terminal 5' phosphate crucial for recognition by host proteins involved in PTGS pathway, was tested. It was found that NSs could not unwind siRNA duplex and also failed to remove the terminal 5' phosphate that are crucial for siRNA. NSs binds to these siRNA duplexes and thus acts as a VSR by sequestering siRNA and not by its ATPase or helicase activities. These results are in confirmation with transient GFP based suppression assay *in vivo*. However, this study is in contrast with *Tomato spotted wilt virus* (TSWV) NSs and *Watermelon Silver Mottle Virus* (WSMV) NSs, where the mutation of lysine to alanine resulted in complete or partial loss of VSR activity by NSs. This signifies that the homologous proteins belonging to the same genus can have varying ability to suppress PTGS pathway.

14) Viruses solely depend on host translational machinery for production of viral proteins. Each virus has cleverly evolved to seize host translational factors by diverse mechanisms and one such method is coding for translational enhancers (cis acing RNA elements as well as proteins). So far in Tospoviruses, only NSs and N protein of TSWV were shown to enhance translation by 2-3 fold using viral RNA template in transfected BHK cells. Using wheat germ extract in an *in vitro* translation assay, NSs was demonstrated to enhance translation by ~10 fold of viral as well as non-viral RNA templates. Since GBNV NSs can translate non-viral RNA template, it is possible that it can enhance translation of mRNAs that are poorly translated. This study is in contrast with earlier report on TSWV NSs that is hypothesized to enhance translation of only viral RNA template.

15) For analyzing the domains involved in translation enhancement by NSs, K189A NSs as well as deletion mutants (NΔ124 NSs and CΔ80 NSs) were tested in an in vitro translation assay using wheat germ extract. Interestingly, K189A NSs and NΔ124 NSs also enhanced translation, but at higher concentrations while CΔ80 NSs showed only ~3.8 fold enhancement even at a higher concentration. This signifies that NSs acts a translation enhancer independent of its ATPase or helicase activities and perhaps the C terminal 80 residues are crucial for translation enhancement.

16) It was also observed that when translation assay was performed using viral 5' untranslated region (UTR) containing RNA template, abortive translation product was formed. The nature of the abortive translation is currently unknown. Deletion of 14 and 46 nucleotides from the 5' UTR resulted in enhanced abortive translation suggesting that 5' UTR alone is not sufficient for correct translation of viral proteins. These results are in stark contrast with many viruses which are known to possess cis acting RNA elements that enhance viral translation. This could be a strategy employed by host for prevention of viral RNA translation. It is also plausible that presence of concomitant 3' UTR can rescue such abortive translation products.
17) Addition of NSs in trans to in vitro translation assay using 5' UTR containing RNA template, lead to enhancement of translation of both products (expected 52 kDa as well as the abortive translation product), highlighting the fact that NSs can enhance translation irrespective of the presence of 5' UTR and absence of 3' UTR. It is possible that NSs interacts with host factors (probably via its C terminus) and results in translation enhancement of RNA templates.
18) Expression analysis of NSs (fused with mcherry) *in planta* revealed that it majorly remains dispersed in the cytoplasm unlike membrane localized punctate spots observed in GFP fused *Impatiens necrotic virus* (INSV) NSs. The apparent difference could be due to the tag attached to NSs as RFP fused INSV NSs did not show any punctate patterns.
19) Thus GBNV NSs is a multifunctional protein with an independent RNA helicase, PTGS suppression as well as translation enhancement activities. Such multifunctional proteins could serve as good targets for anti-viral therapy as they play vital role in multiple stages of viral life cycle.
20) The present study has bought the focus on two aspects of virology-development of plant virus based nanocarriers and analysis of multifunctional role of viral proteins. The future aspects that can be explored include:
    - Demonstration of SLB mediated antibody delivery *in vivo*.
    - Mechanism of CP or SLB entry into mammalian cells.
    - Mechanism of helicase activity by NSs.
    - Mechanism of translation enhancement by NSs.

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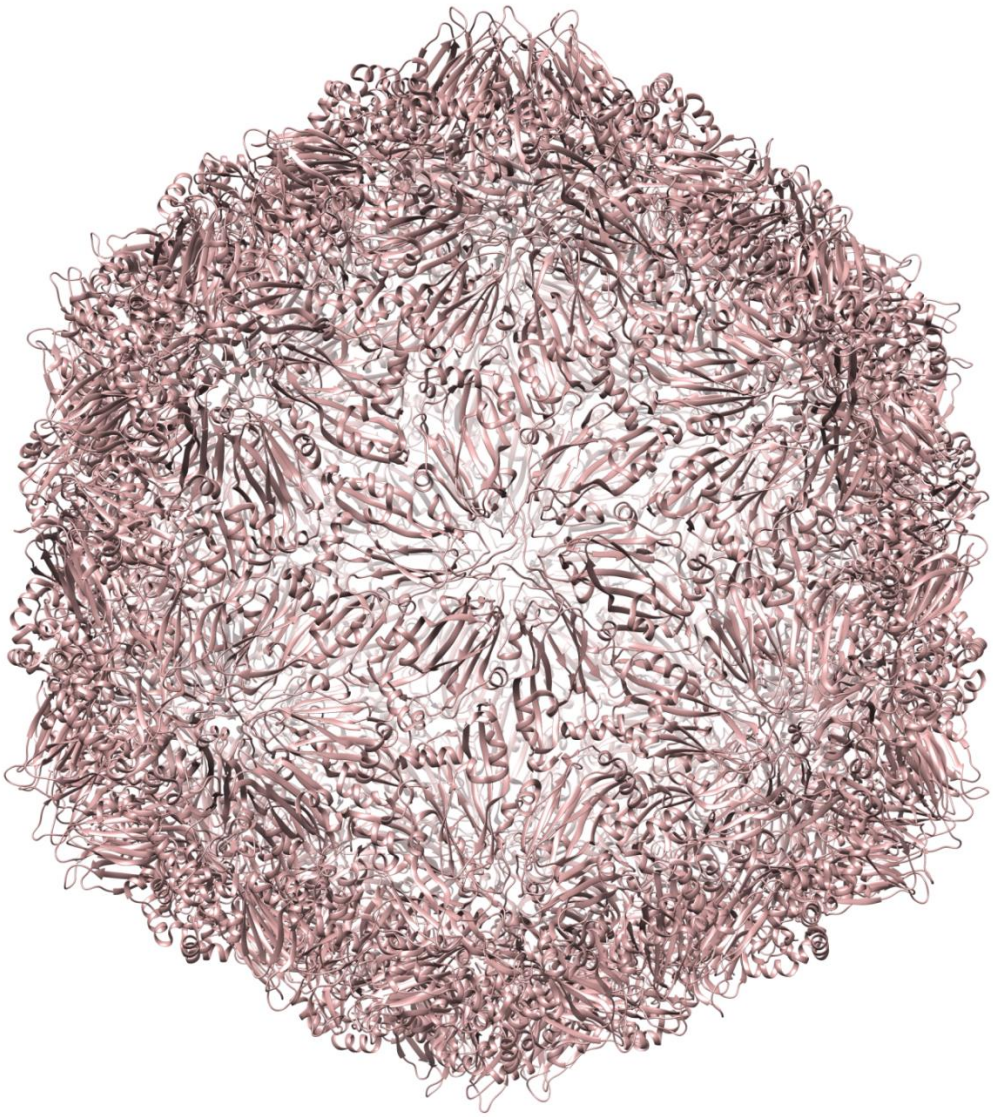

SeMV CP structure generated by using Chimera software

Extraordinary claims require extraordinary evidence."

— Carl Sagan