Summary

Summary of the Thesis Work

HA is a potential candidate for targeting influenza virus; if we can block the functioning of this protein we can derail the viral entry process. Considering the potential of HA, main objective of this project was to explore the possibility of druggable site(s) in glycosylated form of HA trimer. To achieve this goal, the major hurdle was the absence of a fully glycosylated HA trimer molecule. Though servers are available which can glycosylate the molecule with different kinds of sugars, we employed SAXS technique to derive parameters to steer computational glycosylation of HA. For this study we considered five different subtypes of HA protein: H1N1, H3N2, H5N1, H9N2 and H14N5. SAXS data analysis revealed that all the subtypes of HA used herein are monodisperse and fit for further studies. On analyzing the scattering data for the five subtypes at different pH it was found that lowering down of pH results in oligomerization of HA which leads to the formation of high order oligomers. This phenomenon was observed in all the five subtypes of HA proteins used here. It has been reported that HA trimer undergoes large scale structural transition at pH equivalent to the endosomes (4.9 – 5.9) resulting in protrusion of the fusogenic hydrophobic patch which inserts itself into the target membrane for enabling membrane fusion (Ruigrok, Wrigley *et al.* 1986). In absence of a target membrane the fusogenic segment self associates and form rosette like assemblies (Ruigrok, Wrigley *et al.* 1986). We also observed rosette like association at low pH which is likely to be due to the protrusion of fusion segment that associates due to its hydrophobic nature to form oligomers. The detection of higher order assembly was done by comparing the scattering profiles of HA subtypes at different pH values. Midpoint of transition for all the subtypes was observed around pH 5.5. Using the structural parameters calculated from SAXS experiments for the five functional subtypes, we derived D_{max}, R_g and mass percentage values by comparing the SAXS data at pH 7.4 with the non glycosylated models of all HA subtypes under study. The parameters obtained were used for modulating *in silico* glycosylation done through Glyprot server. After successfully optimizing the glycosylation protocol, we generated a glycosylated model for consensus sequence based homology model representing sequences from 2000 to 2009. The scattering data for H3N2 was taken as reference for

modeling because of the similarity of the consensus sequence with the H3N2 sequence. This model was then used for drug site identification and peptide screening. By systematically parsing, analyzing and aligning the sequences we found segments which were conserved, non-glycosylated, non-disordered and surface exposed. On locating these stretches onto the structure we found that the segments projected in the form of a concave pocket with tunnel like architecture. On further analysis it was evident that the pocket was druggable and could be appropriate for small molecule screening/designing. Interestingly the pocket was found in trimer head and was involved in maintaining the trimer head integrity as suggested by normal mode analysis. Based on the pocket location and mode of activation of HA we hypothesized that if we can block/lock this pocket, HA trimer will not be able to respond to low pH resulting in loss of viral fusion efficacy. To provide a proof of concept to our hypothesis we designed a computational peptide library. The peptide library was docked onto the pocket and top leads and their variants were checked for the potential of the molecules to block pH dependent structural transition in HA using SAXS. Scattering data for HA at different pH values in the presence of peptides revealed six peptides (PYDVPDYE, PYDVPDYA, PGDFNDYE, TKRSQQTVIP, KEFSEVEGR and KEFNNLEKR) which were able to make HA ineffective in H1N1, H3N2, H5N1, H9N2 and H14N5 subtypes. Based on our results we propose that these peptides have strain independent behavior and can neutralize a broad spectrum of flu viral subtypes.

The major findings of this study are:

- We can derive information from SAXS data and use it for modeling a glycoprotein. During the progress of this work, we also tested similar protocol for another important protein, tissue plasminogen activator and found that the protocol worked well and we were able to model the full length structure and explain its functionality (Rathore, Rehan *et al.* 2011).
- We could model the fully glycosylated structure of HA and through computational analysis we found a potentially druggable pocket which has not been discovered earlier. Based on the pocket location and its properties we propose that any molecule interacting with the pocket and blocking its motion will make HA functionally ineffective.

- We have developed a SAXS based protocol to test computationally designed peptides and tested their efficacy *in vitro*. With this work we were able to identify peptides which can block HA opening in solution.

Remaining Questions and Future Prospects

Though this study was an attempt to understand the glycosylated form of HA and identify druggable pocket in it, we encountered some other questions during the work. One of the important questions is what will be the fate of the potential peptides in cell/animal model? What will be the half life and localization of the peptide molecules? How much dose of the peptides will be required to lower down viral titer? Can we link two or more peptides to obtain better results? Do these peptides react to other subtypes in a similar way?

Future experiments related to this work can take two routes: one is structural or biophysical and another is cell biological. To further confirm the docking modes high resolution or biochemical techniques can be applied to HA-peptide complex. Crystallographic studies can provide insights into the binding of peptides and their variants at pH 7.4. Low pH crystallographic studies in relation to binding of peptides to HA will be difficult because low pH structure of full length HA has not been solved yet. So to study the binding of peptides to HA one can employ isothermal titration Calorimetry (ITC), florescence correlation spectrometry (FCS) and surface plasmon resonance (SPR) *etc.* Another approach could be to apply FRET (Forster resonance energy transfer) to estimate the distance between labeled peptide and pocket residues. For FRET the major challenge will be to label pocket residues specifically. Apart from this the binding of peptides to the identified pocket can be tracked by estimating the distance of the labeled peptides from the surface of HA expressing cells. This experiment will give an approximate idea of where the peptide is hitting. Toxicity of the peptides can be estimated by adding the peptides to the cell lines and monitoring differences in cell survivability in increasing amount of peptides. This will also help in studying the health and behavior of uninfected cells in the presence of peptides Fate of these peptides and approximate life time can be studied in animal model system(s). Further the efficacy and dosage of peptides to clear virus from infected cells can be determined by comparing viral titer in the presence and absence of peptides. Furthermore, peptides can be used to raise antibodies in animals and then the binding affinity of the antibodies with HA can be

tested. If the peptides work well in *in vivo* model systems (like ferrets, mouse or chicken), pharmacokinetic parameters can be optimized. Additionally, by applying protocol similar to Fleishmann *et al.* the pocket identified in this study can be used to engineer and develop biomolecules which can cross react with different subtypes of HA *(Fleishman, Whitehead et al. 2011)*.

Extension of this project could be to identify small molecules from variety of available libraries, comparing the features required for having binding potency we can design a new scaffold or modify existing molecules. Another interesting thing will be to link two or more peptides and check efficacy of the fusion peptides or to graft the peptides to small proteins which can interact with the pocket. Altogether a lot can be explored using this study as a template and newer insights can be obtained.

"A thinker sees his own actions as experiments and questions, as attempts to find out something. Success and failure are for him answers above all."

— Friedrich Nietzsche