

# **Mechanistic Insights of Host Cell Fusion of SARS-CoV-1 and SARS-CoV-2 from Atomic Resolution Structure and Membrane Dynamics**

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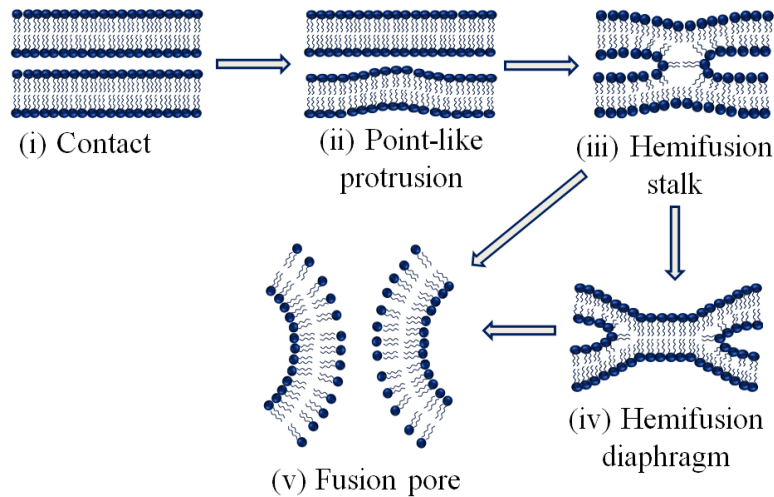
## **Abstract**

The emerging and re-emerging viral diseases are continuous threats to the wellbeing of human life. Previous outbreaks of Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) had evidenced potential threats of coronaviruses in human health. The recent pandemic due to SARS-CoV-2 is overwhelming and has been going beyond control. Vaccines and antiviral drugs are urgently required to mitigate the pandemic. Therefore, it is important to comprehend the mechanistic details of viral infection process. The fusion between host cell and virus being the first step of infection, understanding the fusion mechanism could provide crucial information to intervene the infection process. Interestingly, all enveloped viruses contain fusion protein on their envelope that acts as fusion machine. For coronaviruses, the spike or S glycoprotein mediates successful infection through receptor binding and cell fusion. The cell fusion process requires merging of virus and host cell membranes, and that is essentially performed by the S2 domain of the S glycoprotein. In this review, we have discussed cell fusion mechanism of SARS-CoV-1 from available atomic resolution structures and membrane binding of fusion peptides. We have further discussed about the cell fusion of SARS-CoV-2 in the context of present pandemic situation.

## Introduction

Membrane fusion, one of the most fundamental processes for the survival of eukaryotes, occurs when two closely apposed lipid bilayers merge into a continuous single bilayer and the inner contents are mixed with each other. Several cellular events such as endocytosis, exocytosis, cellular trafficking, compartmentalization, import of nutrients and export of waste, vesiculation, inter cellular communication, fertilization and many others involve membrane fusion, though they vary vastly in space and time. The fusion of synaptic vesicles is almost 10,000-fold faster than the fusion of vacuoles. Despite many diversities all fusion processes follow a common route that includes membrane contact (docking), membrane merger (stalk and transmembrane contact formation), and opening of pore to transfer the intracellular material (pore formation).

There are several models that describe the mechanism of membrane fusion, out of which the stalk model is the most accepted one [1]. The continuum model of membrane fusion or stalk model proposes that the fusion starts from a point-like membrane protrusion that transform into an hourglass like connection between two apposed monolayers [2]. This early hemifusion connection is called as the fusion stalk, which further expanded to form the hemifusion diaphragm [3]. The further expansion of the hemifusion diaphragm opens the pore either on the diaphragm or its perimeter [4,5]. Fusion pore can also be formed directly from stalk by avoiding the hemifusion state [6,7]. **Figure 1** provides a schematic description of the stalk model.



**Figure 1.** Schematic representation of different steps of membrane fusion as proposed in stalk model.

Though membrane fusion is an integral event for the survival of eukaryotes, enveloped viruses utilize this process to enter the host cell [8, 9]. The emerging and re-emerging viral pandemics prompted us to understand the mechanism of fusion for better intervention against viral diseases. The recent worldwide outburst of coronavirus-2 (CoV-2) causing severe acute respiratory syndrome (SARS), COVID-19, has created havoc in terms of morbidity and mortality. Middle East severe acute respiratory syndrome (MERS) coronavirus and SARS coronavirus-1 (SARS-CoV-1), which belong to the same family, *coronaviradae*, were confined in a certain part of the world [10]. SARS-CoV-2 has shown much higher infectivity, and has been spread all over the world.

There is no vaccine for MERS and SARS, and now the whole world is eagerly waiting for COVID-19 vaccine. The fusion between the host cell and virus being the first step of infection process, it is important to understand the fusion mechanism. This review discusses the mechanistic insights of the fusion of SARS-CoV-1 and SARS-CoV-2 from their atomistic structure, and membrane interaction.

### **Membrane fusion: Gateway of viral infection**

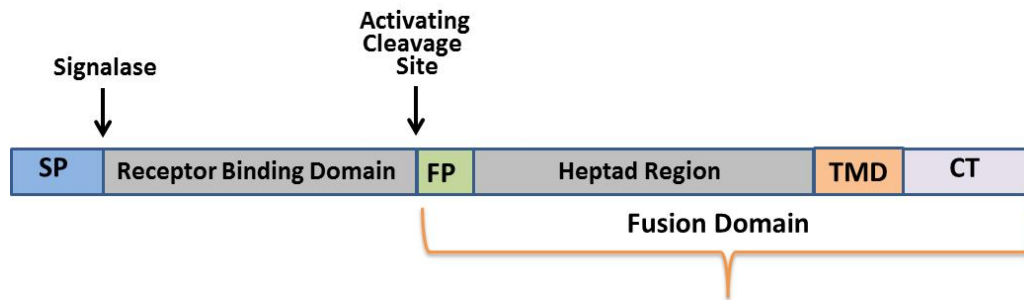
Enveloped viruses exploit membrane fusion for their entry to the host cell. The enveloped viruses cover their genetic material with the host cell derived lipid bilayer, which

houses the fusion protein that is instrumental in the fusion between virus and host cell [11]. The receptor binding domain of the fusion protein finds its interaction partner on the cell surface and the interaction between these two allows the virus to dock on the host cell surface. Several viruses such as human immunodeficiency virus and coronavirus fuse at the cell surface to transfer the genetic material in the host cell, whereas viruses like influenza enters the host cell through endocytosis and then fuses with the endosome upon pH trigger [12]. Therefore, the viral fusion with the host cell is considered as the first step of viral infection.

The fusion protein presents on the viral envelope is called fusion machine as it orchestrates the fusion process between the virus and host cell. Interestingly, there is no sequence homology among the fusion proteins of different viruses however, all the fusion proteins share several common features [13]. Now we have a decent understanding on the fusion mechanisms aided by three dimensional structures of fusion proteins and peptides of several viruses with the help of NMR spectroscopy, cryo-electron microscopy and x-ray crystallography.

### **Fusion protein**

In recent years, the fusion proteins are considered as the target for antiviral intervention due to our growing understanding on the proteins and protein complexes that mediate fusion between virus and host cell. The amino acid sequences of fusion proteins are remarkably different among the viruses, but all initiates membrane fusion through trimer formation, and a common pathway of membrane dynamics [13]. The trimeric glycoproteins of class I virus generally contain a signal peptide, a receptor binding domain, a fusion domain, and a cytoplasmic tail (**Figure 2**).

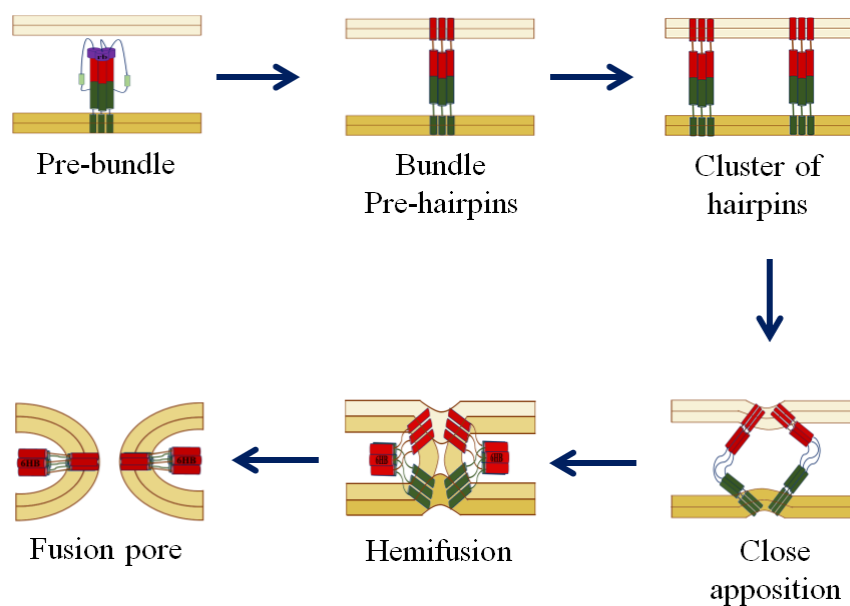


**Figure 2.** The schematic representation of viral fusion protein.

The signal peptide is being cleaved by the signal peptidase but remains in contact with the lipid and subunit of peptidase complex before it is released in the cytosol. The signal peptide of HIV fusion protein, gp160 is known to interact with a calcium binding cytosolic protein, calmodulin [14]. However, the direct role of signal peptide in membrane fusion is not yet clear. The receptor binding domain (RBD) is extremely critical as it provides host tropism and zoonotic transmission of the virus [15]. There are specific receptors on the host cell surfaces, which are being recognized by the receptor binding proteins to dock on the host cell surface. The CD4 receptor binds to the RBD of HIV (gp120) [16], whereas the RBD of influenza hemagglutinin (HA1) binds to the cell surface sialic acid to anchor on the host cell [17]. The spike protein (S1) of coronaviruses utilizes host angiotensin converting enzyme (ACE) to find the host cell for infection [18]. Any small alteration in the RBD leads to inefficient binding resulting in reduced infectivity of the virus [19]. Interestingly, binding of virus glycoprotein with cell surface proteins other than the specific receptor does not promote entry of the virus to the cell [20].

The interaction between RBD and the cell surface receptor induces dramatic conformational changes in the fusion protein leading to exposure of the fusion domain [21]. Generally, fusion domain (or transmembrane domain) contains four distinct regions, i.e., fusion peptide (FP), heptad region (HR), transmembrane domain (TMD), and cytoplasmic tail (CT). The role of CT is not well established in membrane fusion. A 20-25 amino acids long N-terminus of fusion domain plays a crucial role in fusion, and known as the fusion peptide [17]. Mutations in this stretch of amino acids have been shown to block fusion mediated viral infection for many viruses [22-24]. Interestingly, fusion peptide itself is capable to induce

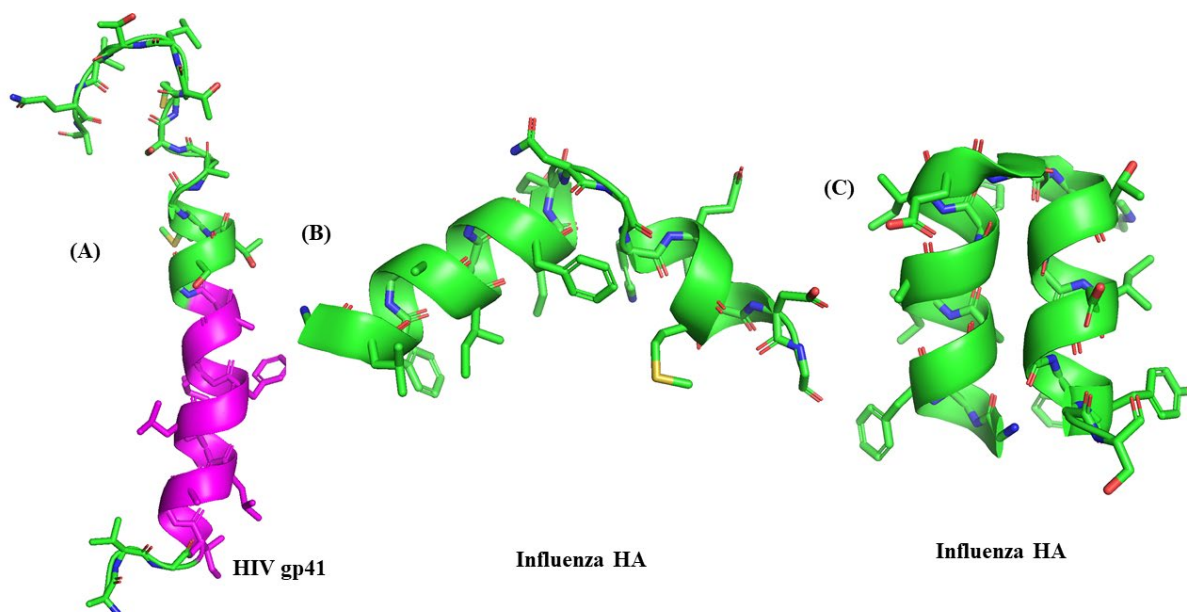
fusion between lipid vesicles, and several putative mechanisms have been proposed on how fusion peptide promotes membrane fusion [19, 25-28]. The  $\alpha$ -helical trimeric heptad region plays a crucial role in inducing fusion by the formation of six helix bundle. The six helix bundle formation, a trademark of class I viral fusion protein, brings two apposing membranes close to each other [18]. The heptad region is further divided into two regions, portions close to the N-terminal (or near the fusion peptide) and the C-terminal (or near transmembrane domain) are termed as fusion peptide proximal region (FPPR) and membrane-proximal external region (MPER), respectively [29]. The interaction between FPPR and MPER promotes the formation of six helix bundle. The transmembrane domain is a stretch of 20-25 hydrophobic amino acids that remains anchored to the viral envelope. It is hypothesized that the fusion peptide (partitioned in the host membrane) interacts with the transmembrane domain (anchored in the viral envelope) to facilitate pore formation [30]. **Figure 3** summarizes the conformational changes of the fusion protein in different steps of the fusion process.



**Figure 3.** Schematic representation of conformational transitions of fusion protein in different intermediates of membrane fusion.

## **Fusion Peptides (FPs): An Overview**

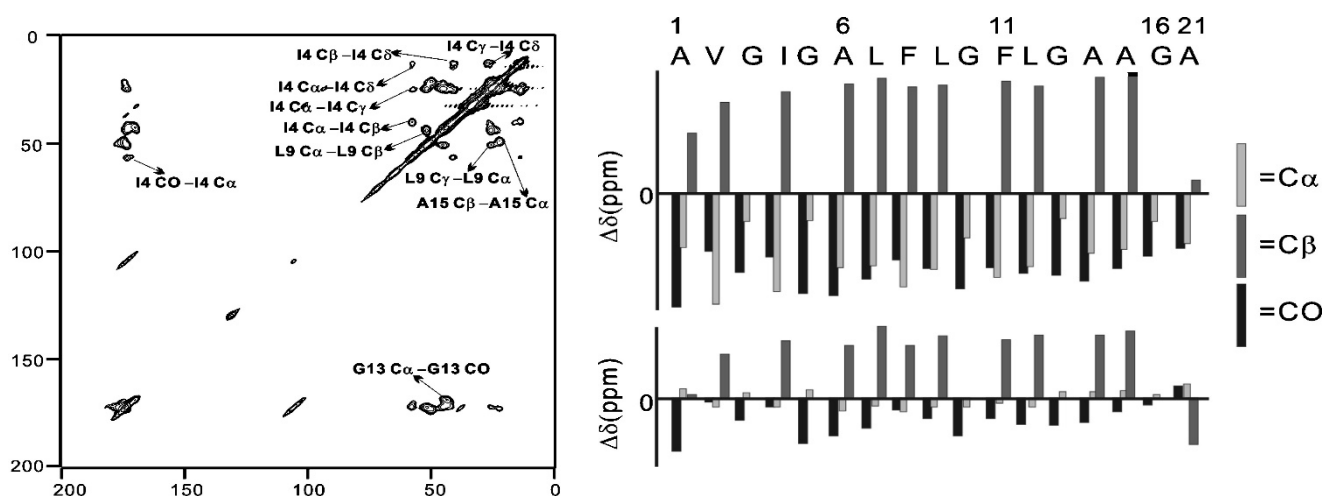
The ability of viral fusion proteins, of all three classes, I, II and III, to accomplish membrane fusion critically involves a hydrophobic segment or multiple such segments termed as fusion peptides (FPs) [31-33]. The FPs are buried within the core structures of fusion proteins in the prefusion state. The induction of fusion process leading to conformational change of the fusion protein exposes FPs. In particular, the multi-step cell fusion process initiates with the insertion of FPs to the host cell membrane. Interestingly, the isolated FPs demonstrate membrane fusion activity, and display specific interactions with lipid membranes. The fusogenic activity of FPs and their shorter sizes have drawn a vast interest to the investigations of virus entry to host cell. In-depth analysis of interactions of FPs with model membranes remains central of the examinations garnering important molecular insights of the virus host cell fusion process [34-38]. Notably, the use of full-length viral fusion proteins is quite challenging for high-resolution studies in complex with membranes. Biophysical methods, fluorescence resonance energy transfer (FRET) and optical spectroscopy (CD, FTIR) techniques are extensively used examining binding and perturbation of membranes of several important viral FPs [39-43]. Solution and solid-state NMR methods are playing vital roles in gaining atomistic information of FPs in complex with model membrane systems [44-54]. The primary structures of FPs contain a high proportion of hydrophobic amino acids and FPs of type I fusion proteins e.g. influenza virus HA peptide, HIV-1 gp41, are rich in alanine and glycine. FPs are sparingly soluble in aqueous solutions and often form heterogenous aggregated species. As a result of high interfacial hydrophobicity, FPs are assumed to be spontaneously partitioned to lipid membranes from aqueous solutions. A free energy value of -7.6 kcal/mol was estimated for the transfer of 20 residue influenza virus HA peptide into SUV at pH 5.0 [38]. In addition, it had been proposed that binding of 8-18 FPs of HA would yield required free energy change, 60-120 kcal/mol, for the stabilization of curved membrane lipid membrane which is initially essential for fusion process [38]. Insertion of FPs into lipid bilayer is known to support stable structures with propensity for stable oligomerizations [41]. The FPs of influenza virus HA peptide, HIV-1 gp41 belonging to the type I fusion protein have been serving as an archetypal example for mechanistic studies with model membranes.



**Figure 4:** Ribbon representation of micelle bound structures of fusion peptides (A) HIV-1 gp41 (pdb 2ARI), (B) influenza HA (pdb 1IBN) and (C) full length influenza HA (pdb 2KXA). The N-terminal helical segment of HIV-gp41 inserted into micelle has been colored in purple.

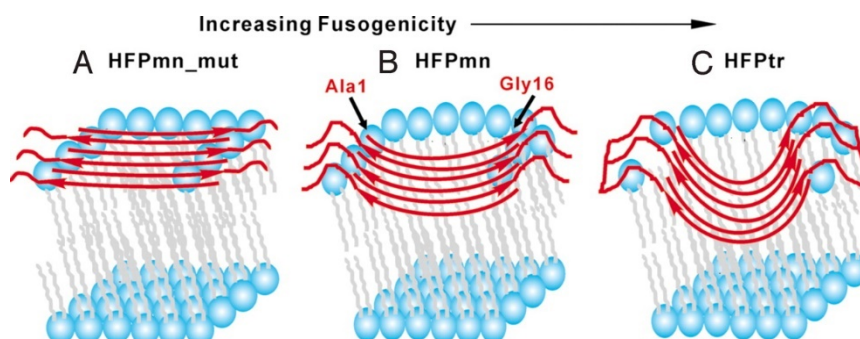
Both HA and HIV-1 gp41 FPs assume monomeric  $\alpha$ -helical structure, determined by solution NMR methods, in detergent micelle (**Figure 4**) [44,45,55]. A large part (residues Ile4-Ala15) of helical structure of the FP of HIV-1 gp41 in SDS micelle was deduced to be deeply inserted into the micelle core akin to the orientation of transmembrane helices [55]. Initial solution NMR and EPR studies determined an inverted “V” shaped or boomerang like helical structure of the 20-residue long HA FP in DPC micelle [44]. The non-polar face of the amphipathic helical structure probably be embedded into the monolayer of the lipid bilayer. Interestingly, a tightly packed helical hairpin structure of HA FP was determined in DPC micelle by solution NMR. This 23-residue construct of HA FP contains additional three conserved residues Trp<sup>21</sup>-Tyr<sup>22</sup>-Gly<sup>23</sup> at the C-terminus [45]. The helical hairpin structure of HA FP might undergo a structural change to a continuous long helix during fusion pore formation. It is conceivable that virus fusion would require higher order oligomerization or association of the fusion proteins. These oligomeric states are likely to confine fusion-induced perturbations to localized areas of the interacting bilayers. Several studies demonstrated that

FPs including HA and HIV-1 GP41 self-associate in model membranes containing cholesterol [37, 56, 57].



**Figure 5:** (left panel) 2-D  $^{13}\text{C}$ - $^{13}\text{C}$  PDSO (Proton-Driven Spin Diffusion) spectrum of membrane associated 23-residue long HIV fusion peptide in solid state. (right panel) Secondary chemical shift of  $^{13}\text{C}\alpha$ ,  $^{13}\text{C}\beta$  and  $^{13}\text{C}=\text{O}$  of residues of HIV fusion peptide. The figure was adapted with permission from reference 58. Copyright (2008) American Chemical Society.

Solid state NMR methods showed transition of  $\alpha$ -helical to  $\beta$ -sheet conformations of FPs of HIV-1 gp41 and HA in membrane bilayers [50, 58]. **Figure 5** shows a representative example of solid state NMR studies of GP41 FP in lipid bilayer [58]. The secondary chemical shifts suggested  $\beta$ -sheet conformations of the HIV FP in cholesterol containing membrane. The oligomeric  $\beta$ -sheet structures of HIV-1 gp41 FP were correlated with the fusogenic activity (**Figure 6**) [54].



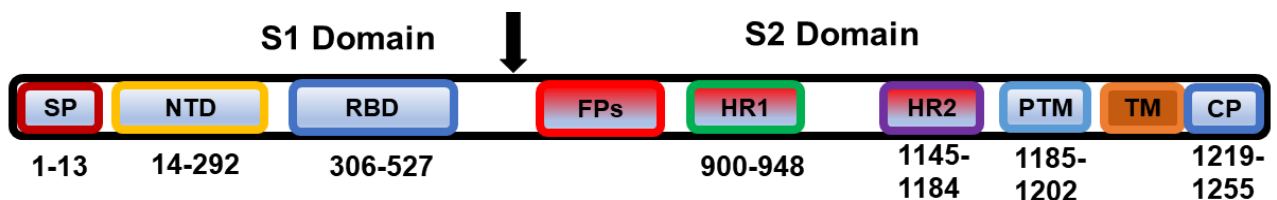
**Figure 6:** Schematic models of  $\beta$ -sheet assembly and localization of HIV-1 gp41 FP in lipid bilayer. HFP<sub>mn-mut</sub>, HFP<sub>mn</sub>, HFP<sub>tr</sub> represent mutated inactive FP, WT FP and cross-linked FP, respectively. The figure has been reproduced from ref 54 with permission.

Structures of FPs could significantly be influenced depending on membrane mimetic environments. As stated above, the FP of HIV-1 gp41 assumed helical structure whereas aggregated  $\beta$ -sheet conformations were deduced in cholesterol containing lipid bilayers. These observations appear to indicate that multiple conformations of FPs may be playing important roles in membrane fusion. Although, determination of atomic resolution structures is challenging in lipid bilayers, therefore, structural works utilizing systems of lipid nano-disks or bicelles could be employed for better understating on the effect of membranes [59-62]. It is worthy to mentioned that host protein is known to be important to aid in membrane fusion of HIV. Peptide fragments from abundant human semen protein prostatic acidic-phosphatase (PAP) or semen-derived enhancer of virus infection (SEVI) demonstrated enhanced infectivity, by a factor of 400,000-fold, of HIV [63, 64]. Ramamoorthy and coworkers established a novel mechanism of PAP peptide fragment mediated enhancement of HIV infection [65-67]. The 39-residue long PAP<sub>248-286</sub> fragment forms amyloids like fibril structures which are efficient in membrane fusion and increased infectivity of HIV. It has been proposed that SEVI amyloids bind to both the membranes of virion and host cells acting as a bridge towards efficient membrane fusion [65-67].

### **Structural Aspects of SARS-CoV-1 Fusion Protein**

Coronaviruses (CoVs) are zoonotic pathogens subdivided, based on genomes and serology, into four different categories alpha, beta, gamma and delta [68, 69]. CoVs are enveloped viruses with a positive sense single stranded RNA as genetic element [68]. CoVs can infect both animals and humans causing variety of diseases including respiratory, enteric, hepatic and neurological of various level of severity. Strains of alpha CoVs, HCoV-NL63, HCoV-229E, and beta CoVs, HCoV-HKU1, HCoV-OC43, are known to be involved in respiratory tract infection, often mild, to humans [69]. The coronavirus came into spotlight during 2002-2003 during SARS (Severe Acute Respiratory Syndrome) outbreak occurred initially in mainland China followed by other parts of the world [70]. The SARS epidemic had caused over 8000 infections and close to 800 deaths globally. In 2012, Middle East Respiratory Syndrome coronavirus (MERS-CoV) has emerged in Saudi Arabia leading to a total of 2494

infections and 858 associated deaths [71]. The recent pandemic of SARS-CoV-2 is overwhelming and has been discussed later. Belonging to the beta coronavirus family SARS-CoV, now known as SARS-CoV-1, and MERS-CoV both are transmitted from animals, presumably certain species of bats passing to intermediate hosts, to human [72-74]. The trimeric S or spike glycoprotein at the envelop of the CoVs appears to be determining in host specificity [20, 75]. The S protein of CoVs including SARS-CoV-1 and MERS helps the virus to bind to the receptor of the host cells and host-virus fusion. The S protein can be divided into two domains S1 and S2 which can be proteolytically cleaved in some CoVs [76, 77]. The S1 domain of S is largely involve in receptor binding whereas the S2 domain is responsible for host-virus membrane fusion [78-82]. The structural features of the S2 domain of CoVs demonstrated a type I fusion protein machinery like well investigated fusion proteins of influenza and HIV viruses. However, the S proteins of CoVs are much longer and appeared to be more complex.



**Figure 7.** Functional domains of SARS-CoV-1. SP: Signal Peptide, NTD: N-terminal Domain, RBD: Receptor Binding Domain, FPs: Fusion Peptides, HR1: Heptad Repeat1, HR2: Heptad Repeat2, PTM: Pre-transmembrane, TM: Transmembrane, CP: Cytosolic Part. The arrow indicates potential furin cleave site of S protein.

The full-length S of SARS-CoV-1 is 1255 amino acid long with several functional domains and potential multiple proteolytic cleavage sites at the boundary of S1 and S2, and also S2' site (**Figure 7**). Cryo-EM structures of S proteins from mouse coronavirus (MHV), human coronavirus KHU1, SARS-CoV-1 and MERS-CoV are reported in the prefusion state [83-85]. These structures revealed trimeric architecture of the S protein with extensive packing between S1 and S2 domains. Further, the fusogenic components of the S2 domain including HR1, fusion peptides are found to be buried within the core of the trimeric structure, whereas, the receptor

binding domain of S1 can be amenable for interactions with the host cell. The S protein is expected to undergo a large conformational change from the latent prefusion state to fusion active state upon receptor binding and priming by host proteases. The x-ray structures of the S2 domain of S protein largely on HR1, HR2 and HR1/HR2 complex revealed canonical trimeric coiled-coil helical structures akin to type I fusion system [79, 80]. The HR1/HR2 complex forms a bundle of trimeric six helix whereby three short helices of HR2 can be found to be tightly packed along the long three helices of HR1. The quaternary association of HR1 and HR2 would bring the fusion peptides and viral TM helix at proximity, presumably facilitate membrane fusion process (**Figure 3**).

### **Multiple Fusion Peptides (FPs) of SARS-CoV-1**

Although membrane fusion of SARS-CoV-1 belongs to the type I fusion system, the large S protein of SARS-CoV-1 encodes number of regions in the S2 domain with membrane binding and/or membrane fusion activity [86-95]. The existence of multiple potential FPs in the S2 domain of S protein in SARS-CoV-1 is unique in comparison to type I fusion proteins of HIV and influenza viruses. Notably, the single FP, located at the N-terminus, has been known to be responsible for membrane fusion of HIV-1 gp41 and HA influenza viruses [24-26]. Intuitively, membrane fusion mechanism of SRAS-CoV-1 and other CoVs may be complex involving several distinct stages. The upstream part of the HR1 (residues 892-972) of S2 domain of S protein of SARS-CoV-1 appears to be involved in membrane fusion (**Figure 7**). Several research groups have identified membrane binding and fusogenic peptides or potential FPs from the upstream region (residues 758-890) of HR1 (**Table 1**).

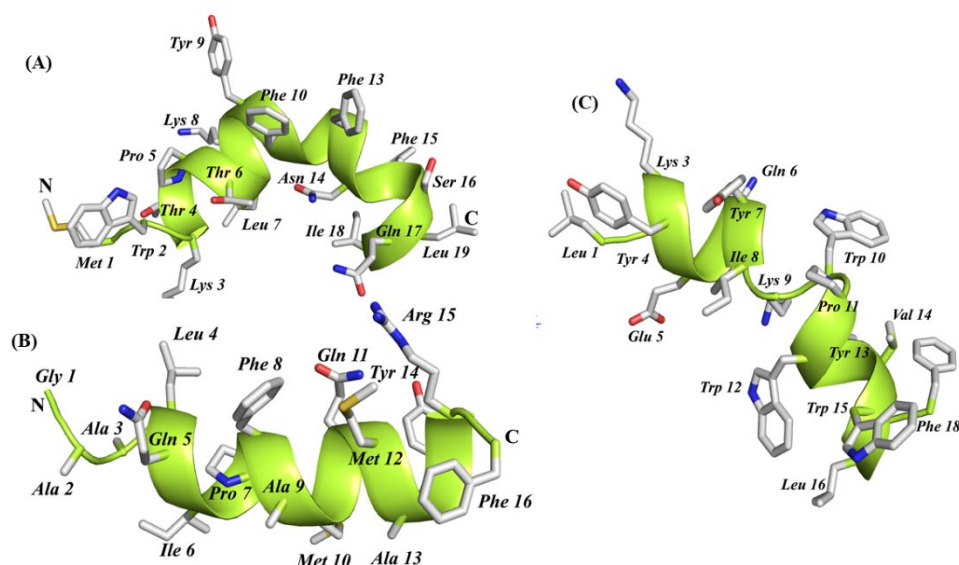
**Table 1:** A list of potential FPs of SARS-CoV-1 upstream of coiled-coil HR1

Sequence of FPs	Reference
<sup>770</sup> MYKTPTLKYFGGFNFSQIL <sup>788</sup> (FP)	86
<sup>798</sup> SFIEDLLFNKVTLADAGF <sup>815</sup> (IFP1)	93
<sup>873</sup> GAALQIPFAMQMAYRF <sup>888</sup> (IFP2)	88
<sup>858</sup> ALISGTATAGWTFGAGAALQIPFAMQMAYR <sup>886</sup>	79

Based on interfacial hydrophobicity, Wimley and co-workers identified two regions, residues 770-788 and residues 864-886, in the S2 domain as potential FPs [86]. It was noted that the potential FP (residues 770-788) is located 9 amino acid downstream of a predicted furin cleavage site ( $^{758}\text{R-N-T-R}^{761}$ ) between S1 and S2 domains of the S protein. Also, the location of the potential FP (residues 770-788) at the extreme end of S2 domain is very similar to FPs of HIV-1, influenza and paramyxoviruses. Further functional and biophysical analyses confirmed fusogenic activity of the synthetic peptide encompassing residues 770-788 [86,81,92]. Studies demonstrated high extent of phospholipid membrane partitioning, membrane fusion and leakage of the FP. On the other hand, Villalain and co-workers have characterized a fusogenic sequence of residues 873-888 (Table 1), also termed as internal fusion peptide or IFP2, demonstrating high binding affinity and leakage from model membranes of various compositions [88]. The IFP2 appeared to be binding with negatively charged phospholipids with higher affinity compared to zwitterionic lipids [88]. It may be noteworthy that the IFP1 is located at the C-terminus of a proteolytic cleavage at residues 797/798 at S2' site. This would suggest that after proteolytic priming of S protein at S1/S2 and S2' sites, the IFP1 would remain a part of the fusion protein whereas FP, residues 770-788, will not be covalently bonded to the rest of the S2 domain (**Figure 7**). In a later study, Whittaker and co-workers has defined a yet another segment of residues 798-815 in proximity to the S2'cleavage site as a fusion peptide or IFP1 [93]. Mutagenesis studies on the full-length S protein identified residues L803, L804 and F805 that are critically involved in fusion. Biochemical analyses with synthetic peptide of IFP1 demonstrated ability of this peptide in membrane fusion. In addition to these fusogenic peptides, a C-terminal segment adjacent to TM or PTM (residues 1185-1202) is known to confer strong membrane partitioning (**Figure 7**). Further, ~170 amino acids long polypeptide between HR1 and HR2 also appeared to be containing peptide motifs with membrane interacting properties and virus-cell fusion inhibiting activity [94].

### Atomic-resolution Structure of FPs of SARS-CoV-1 in Membrane Mimetic Systems

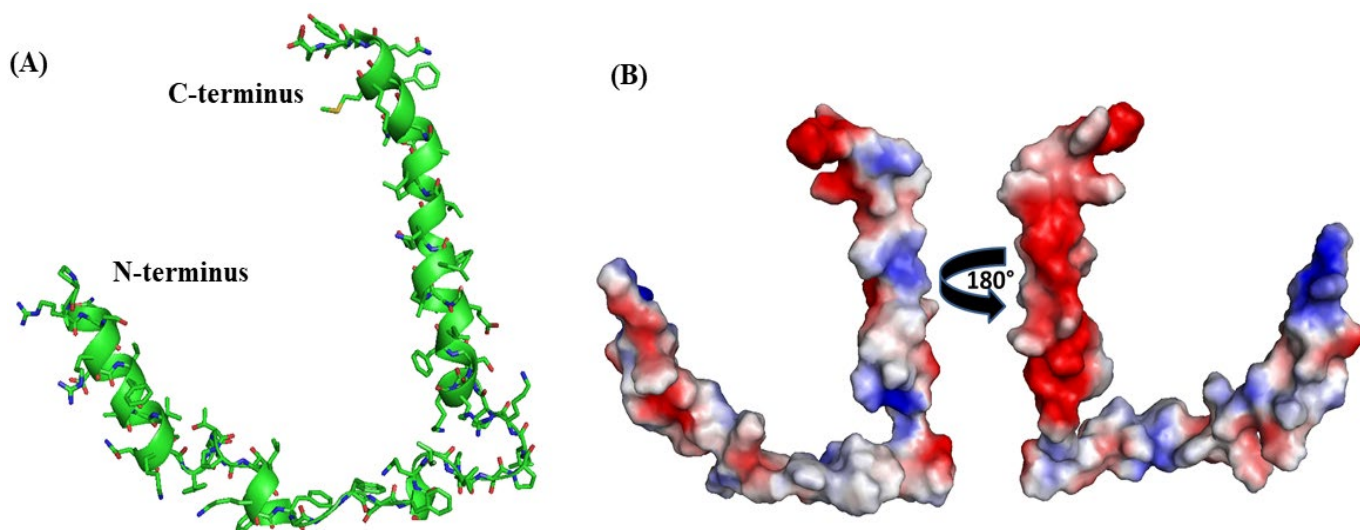
We have determined 3-D structures of FPs and PTM of SARS-CoV-1 in solution of DPC detergent micelle by NMR methods [96, 97]. The atomic resolution structure of the FP (<sup>770</sup>MYKTPTLKYFGGFNFSQIL<sup>788</sup>) revealed a bend helical structure presumably resulting from two Gly residues G780 and G781 at the center of the sequence (**Figure 8**). The outside periphery of the bend helical structure of FP is predominantly hydrophobic maintained by two patches that are consisted of sidechains of residues L776, Y778, F779 and F782 and the sidechains of residues F784, I787, and L788 (**Figure 8**). There are aromatic-aromatic stacking interactions involving residues Y778, F779 and F782. The inner face of the bent helix of FP appears to possess a polar face of residues K772, T775, N783, and Q786 (**Figure 8**). The DPC bound structure of the IFP2 (<sup>873</sup>GAALQIPFAMQMAYRF<sup>888</sup>) of S2 domain is defined by a rod-shaped helical structure encompassing residues L877-F888 (**Figure 8**). The IFP2 helix is mostly hydrophobic with a large non-polar face consisted of sidechains of residues I878, A881, M882, A885 and F888 whereas opposite face of the helix is amphipathic with a hydrophobic patch of residues L876, F880, M884 and potential polar interactions involving sidechains of residues Q883, Y886, R887 (**Figure 8**). NMR structure of the aromatic rich pre-transmembrane (PTM) region (residues <sup>1185</sup>LGKYEQYIKWPWYVWLGF<sup>1202</sup>) of S protein has also been determined in DPC micelle. The PTM is a conserved sequence among SARS-CoVs, and also observed in other viruses like HIV-1 and Eob. It has been postulated that the PTM segment could be involved in viral membrane fusion process [87]. Micelle-bound PTM assumes a fold of helix (residues K1187-Y1191)-loop (I1192-K1193)-helix (W1194-F1202) structure whereby the two helices were found to be independently oriented (**Figure 8**). The aromatic-aromatic packing interactions could be observed within the helix-loop-helix structure in distinct regions of PTM. Aromatic clusters are realized among residues W1194/Y1197/F1202, W1196/1199 and Y1188/Y1191 (**Figure 8**). NMR paramagnetic relaxation enhancement studies demonstrated that helical structures of FP and IFP2 are deeply buried within the hydrophobic core of the micelle whereas the helix-loop-helix structure of PTM is largely positioned at the micelle/water interface.



**Figure 8.** Ribbon representation of the structures of the fusion peptides, determined in DPC micelle, of SARS-CoV-1 (A) FP (B) IFP2 (B) and (C) PTM. Figure has been prepared using PyMOL using pdb coordinates of FPs [96].

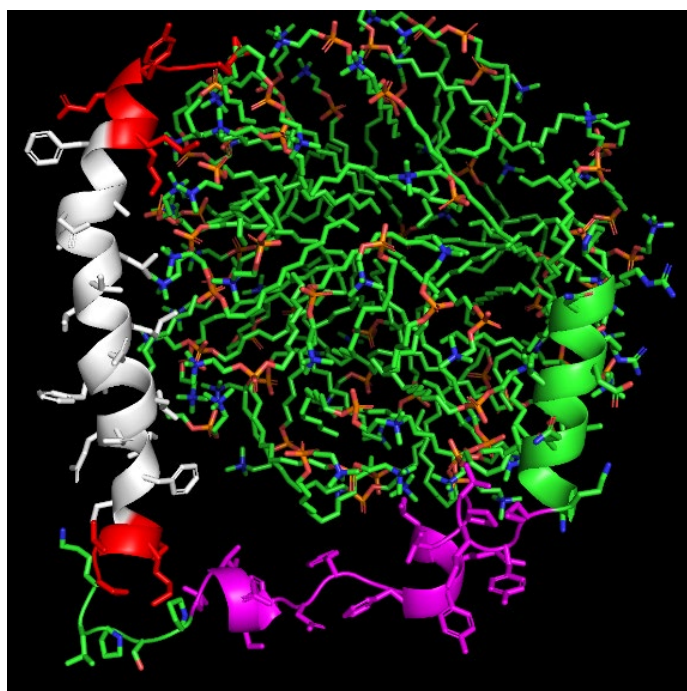
Observations of multiple adjacent fusogenic peptides in the S2 domain prompted us to examine structure and membrane localization of a 64-residue long, residues R758-E821, or LFP (long fusion peptide) in detergent micelle solution [97]. The primary structure of LFP contains FP (residues 770-788) and IFP1 (residues 798-815) with additional residues at the N and C-termini. LFP was over-expressed in *E. coli* as a fusion protein containing an 81-residue long prodomain of human furin followed by an Asp-Pro sequence for formic acid digestion [89,90]. The construct also contains an additional six residue His-tag at the N-terminus for affinity purification resulting a fusion protein His<sub>6</sub>-Prodomain-D-P-LFP. Atomic resolution structure, <sup>15</sup>N relaxation and micelle localization were investigated by heteronuclear NMR methods in DPC micelle solution (**Figures 9, 10**). 3-D structure of LFP demonstrated existence of discretely folded helices connected by several loops (**Figure 9A**). The C-terminal region of LFP defines a long  $\alpha$ -helix including residues T795-Y819 with a kink at residue D812. It may be noted that IFP1, residues 798-815, is included within the C-terminal helix. The FP segment (residues 770-788) in LFP assumed a helix-loop-helix structure, although, the isolated FP delineated a bend helical structure (**Figure 8**). Interestingly, the N-terminal residues R761-

Q769 of LFP assumed an amphipathic helical conformation (**Figure 9A**). The helical structures of LFP were found to be motionally rigid experiencing fast motion in ns-ps time scale.



**Figure 9.** (A) Ribbon representation of 3-D structure of LFP of SARS-CoV-1 showing discretely folded helices. (B) Electrostatic potential surface of LFP, positively charged, negatively charged and uncharged amino acids are marked as blue, red and white color.

Electrostatic potential surface shows that the long C-terminal helix of LFP has a negatively charged surface at one face whereas non-polar and positively charged residues occupy the opposite face (**Figure 9B**).



**Figure 10.** Topology of LFP docked onto DPC micelle. The FP and IFP1 are colored as purple and white ribbon, respectively.

Paramagnetic relaxation NMR indicated that the middle portion of LFP is deeply buried in zwitterionic DPC micelle. Docking of LFP/DPC delineated potential localization of the LFP in micelle surface (**Figure 10**). The structure of LFP of SARS-CoV-1 suggested that multiple membrane active regions of the S protein might be involved in membrane fusion in a concerted or synergistic fashion.

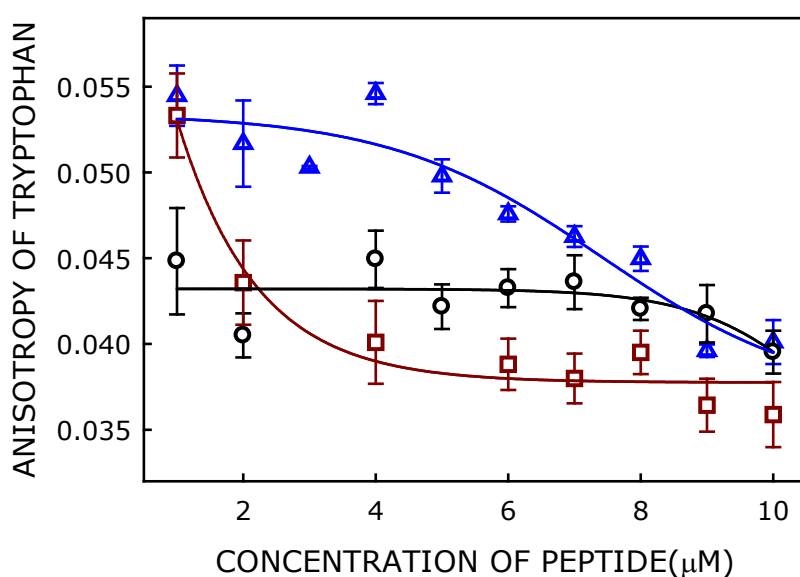
### **Effect of membrane composition on the structure of SARS-CoV-1 fusion peptide**

The fusogenic property of the membrane generally depends on its composition and curvature [100]. The lipid composition has its direct effect on the membrane physical properties; however, lipid composition further influences the structures of fusion proteins and peptides. The lipid composition provides lateral and transversal heterogeneity to the membrane, where the proteins and peptides behave differentially pertaining to the solubility, structure and oligomeric status. Therefore, lipid composition plays an important role in the secondary structure of fusion proteins and peptides, as well as membrane fusion.

The structural flexibility is the hallmark of fusion peptides [100]. The binding, conformation and function of fusion peptides are sensitive to the lipid composition of the membrane. SARS-CoV-1 fusion peptide shows preferential binding to the membrane containing negatively charged lipids [101]. In addition, the depth of insertion of SARS-CoV-1 fusion peptide is more while membrane contains negatively charged lipids, and induces higher content leakage compared to the zwitterionic membranes. The differential scanning calorimetry measurements demonstrated that the peptide induces elevated membrane perturbation while membrane contains negatively charged lipids [102].

Recently we have shown using circular dichroism that the peptide adopts  $\beta$ -sheet conformation in the membrane comprising phosphatidylcholine and phosphatidylcholine/cholesterol [103]. Our results demonstrated that the binding affinity of the peptide enhances with increasing concentration of membrane cholesterol. Moreover, the

peptide undergoes concentration-dependent oligomerization in cholesterol containing membrane. We utilized fluorescence anisotropy to determine the oligomerization of the SARS-CoV-1 fusion peptide. Homo-fluorescence resonance energy transfer (Homo-FRET) is an energy transfer process between two or more same fluorophores, when it shows smaller Stokes shift [104]. The energy transfer from one molecular dipole to another molecular dipole in different orientation results in depolarization of the emitted light, thereby decreasing fluorescence anisotropy [105]. Therefore, the concentration dependent drop of fluorescence anisotropy is correlated to the oligomerization. Interestingly, tryptophan demonstrates Homo-FRET [106], and the tryptophan in the peptide had been utilized to determine peptide oligomerization. **Figure 11** demonstrated the anisotropy of tryptophan as a function of peptide concentration. The fluorescence anisotropy of tryptophan does not show any change with concentration in the membrane without cholesterol, i.e. phosphatidylcholine/phosphatidylglycerol (POPC/POPG) membranes. The fluorescence anisotropy dropped with the peptide concentration in the membrane containing 10 and 20 mol% of cholesterol, nonetheless the anisotropy the decrease was initiated in much lower peptide concentration while membrane contains 20 mol% of cholesterol. In addition, the change in fluorescence anisotropy was much steeper in the presence of 20 mol % cholesterol compared to that in the presence of 10 mol % cholesterol [103].



**Figure 11.** Change in average fluorescence anisotropy of tryptophan with varying concentration of POPC/POPG (80/20 mol%) (black, o), POPC/POPG/Chol (70/20/10 mol%) (blue, Δ) and POPC/POPG/Chol (60/20/20 mol%) (red, □) membranes. All experiments were carried out in a 10 mM phosphate buffer of pH 7.4 at 25 °C. The concentration of lipid was kept constant at 200 μM and the emission was monitored at 350 nm by exciting at 295 nm. See the Materials and Methods section for more details. The figure has been adapted from [103] with appropriate permission.

The oligomerization of fusion protein is an important event for the successful fusion between the virus and host cell. Cryo-electron microscope results of SARS-CoV-1 showed that it is trimeric in pre-fusion stage, hence prompting our expectation of it to form a six-helix bundle in the post-fusion conformation [107]. In that context lipid composition-dependent oligomerization of SARS-CoV-1 fusion peptide is extremely significant in understanding the entry of the SARS-CoV-1.

The inconsistency in secondary structure of SARS-CoV-1 fusion peptide obtained from different methodologies could be due to the difference in the membrane environment for these measurements. In addition, the oligomeric status of the peptide in membrane milieu depends on the lipid composition of the membrane. Yet, the structure of the peptide in physiologically relevant condition is still imperceptible. Keeping in mind the higher binding affinity of the peptide in presence of negatively charged lipid, a high-resolution structure of the peptide in presence of negatively charged lipid would be helpful in elucidating the physiologically active conformation of the fusion peptide. Apart from oligomerization of fusion peptides, it has been shown that the C-terminal derived peptide of SARS-CoV-1 E protein undergoes oligomerization and may be important for infection process [108,109].

### **Membrane Fusion of SARS-CoV-2 (COVID-19)**

Pandemic due to coronavirus was predicted after SARS and MERS outbreaks. However, no vaccines or effective drugs were developed for the mitigation of the threats. A new strain of coronavirus called SARS-CoV-2 or COVID-19 is demonstrating a rapid spread all over the world which was initially found in Wuhan, China. SARS-CoV-2 pandemic, so far, has caused nearly 500,000 deaths globally with an infection of over 11 million people. The

high level of infectivity of SARS-CoV-2 compared to SARS-CoV-1 could be related to an efficient cell entry of the virus. Although, the molecular mechanism of the cell entry process remains unclear, binding of the virus to the host cell receptor is an important step in successful infection. Studies have shown that the isolated RBD of SARS-CoV-2 binds to ACE2 with tighter affinity compared to SARS-CoV-1, indicating potential for higher infectivity [107, 110-113]. However, paradoxically, experiments with the full-length S protein have evidenced either similar binding affinity or even lower affinity of RBD to human ACE2 in comparison to SARS-CoV-1 [111-113]. The cryo-EM structure of SARS-CoV-2 showed that the RBD domain could be hidden within the S protein structure for immune evasion [111]. Therefore, other factors might be responsible for high infectivity of SARS-CoV-2. Protease cleavage of S protein has been postulated to be one of the factors responsible for high infectivity of SARS-CoV-2 [112-114]. An efficient membrane fusion mechanism between SARS-CoV-2 and host cell could also be responsible for the high level of infection. Sequence comparison of S proteins domain between SARS-CoV-1 and SARS-CoV-2 indicated high level of conservation both for the S1 and S2 domains [115]. Nevertheless, variations can be observed for the fusogenic regions of the S2 domain between two viruses. Although, effect of these variations for an efficient membrane fusion remains to be examined.

### **Concluding remark**

Emerging and re-emerging viral infections are continuous threats to human kind. The worldwide pandemic caused by SARS-CoV-2 has already claimed almost half a million lives, and there is no sign of any improvement yet. Hence, there is an obvious need to understand the infection process, the first step of which is the fusion between host cell and the virus. The spike (fusion) protein present on the viral envelope of coronaviruses mediates the fusion between host cell and viral membranes. An atomistic understanding of the structure of fusion protein would, therefore, be important to understand the fusion process. Structure of the fusion peptide would be particularly vital in this regard, since it plays a crucial role in host invasion. Interaction of the fusion peptide with host membrane is also a critical factor, since it is a function of membrane composition. Taken together, a comprehensive understanding of the

atomistic structure of fusion protein and peptide, and their interaction with membranes could lead us to a better understanding of the host-virus fusion, hence suggesting an appropriate intervention at an early stage of infection.

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