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Non-Structural Proteins of SARS-CoV-2 as potential sources for vaccine synthesis

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ABSTRACT:

— ***Background:*** Coronavirus disease of 2019 (COVID-19) is a pandemic that the world is still not able to treat, vaccinate, or manage. In medical history, vaccines were the best option against viral infections. This article proposed the possibility of using the nonstructural proteins (NSPs) of Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as sources of vaccine material by extracting the NSPs peptides (particularly the small-sized NSP11 and NSP7), from a feasible and abundant protein: Albumin.

— ***Materials and Methods:*** The databases of SWISS-MODEL and Uniprot were used to compare NSPs sequences with sequenced proteins, including Albumin. Similarities in sequences between severe acute respiratory syndrome Coronavirus and severe acute respiratory syndrome coronavirus-2 were excluded; also, homologies of less than 20% were omitted.

— ***Results:*** Albumin has no homology to any of the SARS-CoV-2 NSPs. The highest value of similarity between SARA-CoV-2 NSPs and a non-coronavirus protein was the 49% similarity of NSP4 with RNA Polymerase of mouse hepatitis Virus. A tetramer (or a trimer) of NSP11 has 29% similarity with the *Human Astrovirus-2* capsid protein spike domain. NSP7 has no homology with any known sequence protein other than the coronaviruses proteins. Other resemblances of the SARS-CoV-2 NSPs to known sequenced proteins were 31% or less, and these were to proteins from human, bacterial, and viral sources.

— ***Conclusions:*** The extraction of SARS-CoV-2 NSPs from albumin is unlikely to occur because of absent sequence similarities. There are only partial homologies of nonstructural proteins to proteins from humans, bacteria, or other viruses.

— ***Keywords:*** SARS-CoV-2, COVID-19, Nonstructural proteins, Protein sequencing, Albumin.

INTRODUCTION

It is well known that in all Corona Viruses the 3' terminus contains Corona Virus canonical set of structural proteins, and these are four proteins[1,2]:

I. Nucleocapsid (N) protein, a basic RNA-binding protein.
II. Spike protein (S), a glycoprotein that contains oligosaccharides which bind with serine amino acid by o- glycoside bond.
III. Membrane protein (M) that spans the membrane.
IV. Envelope protein (E), a highly hydrophobic protein that covers the entire structure of the coronavirus, and for this reason the virus cannot move in blood.

On the other hand, two-thirds of the genome (in the 5' terminus) encodes a replicase polyprotein named polyprotein 1ab (pp1ab), which is comprised of two overlapping open reading frames (ORFs): ORF1a and ORF1b. These ORFs are then cleaved by viral proteases into 16 non-structural proteins (NSPs) that are involved in genome replication and transcription[3].

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The non-structural proteins are polypeptides without sub-protein ordinary structure (primary, secondary, or tertiary structures). The NSPs perform important and beneficial tasks for the viral pathogenicity:

- NSP1 inhibits protein synthesis in human cells and prevents the cell from performing antiviral functions[4].
- NSP2 had structural similarity a disease-inducing protein in the infectious bronchitis virus in birds[5].
- NSP3 is a big protein that can change the normal human protein shape[5].
- NSP4 protects new viruses that are recently been replicated in the host cell DNA[5].
- NSP5 possesses a cleavage protein capacity (helps the peptidase)[6,7].
- NSP9 can enter the nucleus carrying genetic materials of the virus to human cells and mediates virus proliferation[8].
- NSP12 seems to have an RNA polymerase activity[9].
- NSP13 seems to have a Helicase activity[9,10].
- NSP15 has an endoribonuclease activity; it is a good target for antiviral drugs[9].
- NSPs14 and 16 mediate the 5' methylation of Virus genome[9].

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) uses host protease enzymes and produces these 16 NSP types. The synthesis of the viral proteins occurs in the host cell Golgi apparatus[11].

Viral infections could be prevented with an effective vaccine. Normal vaccines could be produced from a complete viral protein with a primary, secondary, tertiary, and sometimes quaternary structures[12,13]. The main sources of vaccine materials are vectors (such as Adenovirus coat), viral epitopes (the antibody attachment sites), viral DNA, viral surface proteins, or a complete protein synthesized by recombinant technology[7,13,14]. Apart from vaccination, a patient or an animal model whom been healed from coronavirus will gain an antibody mediated immunity against it[15].

The synthesis of a vaccine through DNA in viruses such as hepatitis B, or in bacteria like mycobacterium TB is relatively easier than in RNA viruses. SARS-CoV-2 is a single positive RNA virus, as well as SARS-CoV (Severe Acute Respiratory Syndrome Coronavirus). SARS Cov-2 and SARS CoV are closely related, as both viruses are beta Coronaviruses and share high similarities in structure and pathogenicity[16].The vaccine trials for the 2002 SARS CoV included inactivated coronavirus, adenovirus-vectored spike protein, and naked spike protein; however, all these were not tested in humans because of the SARS outbreak ceased[16]. No vaccine had yet been developed to combat SARS-CoV-2 and Coronavirus disease of 2019 (COVID-19)[16].

Through this article, the authors suggested the use of NSPs to synthesize a SARS-CoV-2 vaccine. If it is possible to trans-locate the NSPs to the blood, cellular and humoral immune response against SARS-CoV-2 could be stimulated. Albumin was elected by authors as a source of amino acids for the synthesis of NSPs. The choice of albumin came from its abundance, feasibility, and natural presence in plasma. It is more convenient to target smaller NSPs, as their obtainment from albumin is more likely. The sizes of NSPs per amino acid are[9]: NSP1: 180 amino acids, NSP2: 638 amino acids, NSP3: 1945 amino acids, NSP4: 500 amino acids, NSP5: 306 amino acids, NSP6: 290 amino acids, NSP7: 83 amino acids, NSP8: 198 amino acids, NSP9: 113 amino acids, NSP10: 139 amino acids, NSP11: 13 amino acids, NSP12: 932 amino acids, NSP13: 601 amino acids, NSP14: 527 amino acids, NSP15: 346 amino acids, NSP16: 298 amino acids. NSP11 and NSP7 were particularly chosen in this study because they are small and hence more likely to be extracted from other larger proteins.

MATERIALS AND METHODS

The research methodology was divided into two parts:

1. The first part was an experimental study aimed to produce SARS-CoV-2 NSPs from the albumin polypeptide chain. The steps suggested to synthesize NSPs from albumin were:
   I. Heating albumin in 100°C degree of temperature for 10 minutes.
   II. Waiting for 20 minutes to cool the albumin. Albumin became an inactive long polypeptide.
   III. SARS-CoV-2 depends on Golgi apparatus proteases enzymes to produce NSP 1-16; so, a protease enzyme such as papain enzyme (similar to angiotensin-converting enzyme type 11 that produces NSPs in COVID -19 disease) addition will produce the desired NSPs from the deactivated Albumin *in vitro.*
   IV. The NSPs are now available for intravenous (i.v) or subcutaneous (S/C) injection.
   V. NSPs injection should cause no allergy because the sequences of amino acids are from albumin structure, but at the same time internal cellular and humoral immunity will be activated when the NSPs reach the blood.
   VI. Now thin layer chromatography could be used to exclude running of individuals' amino acids. This biochemical technique had previously been used to remove NSPs and unwanted host cell proteins from the purified antigen vaccine[13]. Now this criterion could be used alternatively to extract NSPs as a vaccine material (Figure 1).

   To optimize the search of a suitable protein for slicing and manufacturing NSPs, a sequence homology scan could be performed. Several databases are available online which offer sequence similarity scanning for proteins, RNA, and DNA.
2. The second part was an analytical, database dependent study performed within the period from the 1st to 7th June 2020. All of this part of the methodology was performed online. The databases of SWISS-MODEL (The Center of Molecular Life Sciences, University of Basel, Basel, Switzerland), and Uniprot (A collaboration between The European Bioinformatics Institute (EMBL-EBI), the SIB Swiss Institute of Bioinformatics and the Protein Information Resource (PIR)) were used to compare NSPs sequences with sequenced proteins in the databases. Similarities in sequences with

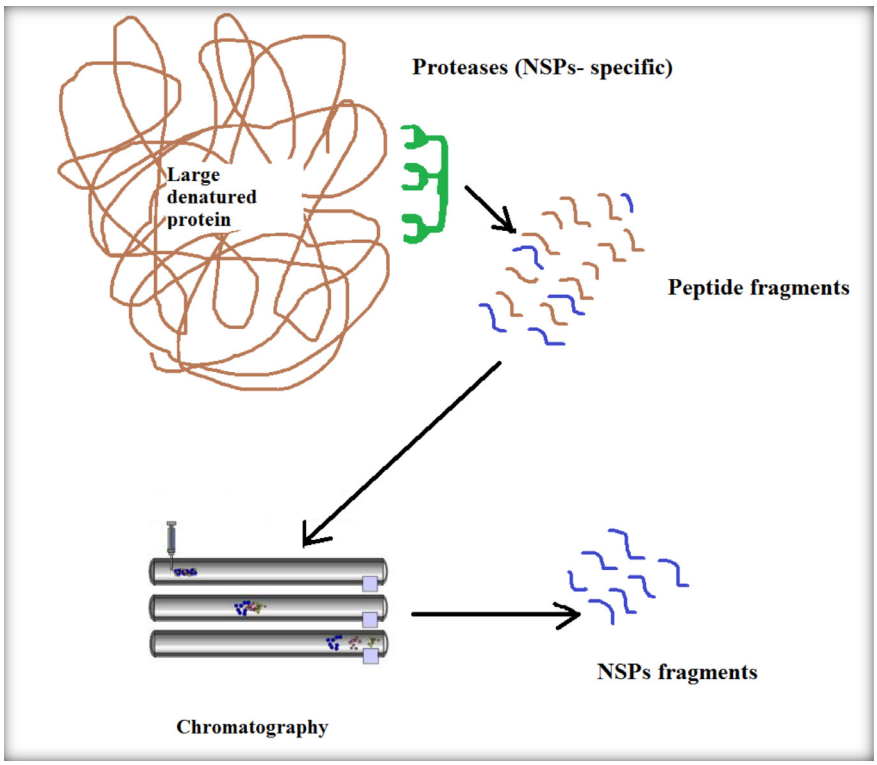

Figure 1. The suggested steps of the synthesis of NSPS from proteins (in this case, albumin); subjecting heat-denatured protein to the proteases of Golgi apparatus cleaves albumin into small fragments including the NSPs, these, in turn, will be run in Thin-layer chromatography (or any suitable chromatographic technique) where NSPs could be isolated.

severe acute respiratory syndrome Coronavirus and severe acute respiratory syndrome coronavirus-2 were omitted; also, protein-protein complexes and similarities less than 20% in the structure were not mentioned. Due to the short sequence of NSP11, which is not comparable in the database, a trimer and tetramer of the NSP11 were used in the comparison.

RESULTS

It had been found that albumin, a protein containing 585 amino acids[17,18], has no homology with neither NSP7, NSP11 (Figure 2), or any other Covid-19 NSP, therefore the experimental part of the study was not performed.

Other proteins sequences similarity detection showed that a trimer (or tetramer) of NSP11 has 29% similarity with *Human Astrovirus*-2 capsid protein spike domain, a 242 amino acids tertiary protein[19,20]. NSP1 has a 29% similarity with *Haemophilus Influenzae* Hypothetical Protein HI-1011. NSP4 has 49% similarity in structure with the RNA directed RNA Polymerase of mouse hepatitis Virus protein, which considered the highest value of similarity between SARA-CoV-2 nonstructural protein and a non-coronavirus protein.

NSP6 has 24% similarity to the Ca^{2+} binding protein calbindin D9k of humans, also it has 29% similarity to human Mitochondrial NADH dehydrogenasel beta subunit 5, and 27% similarity in structure to the B5 subunit of ovine NADH: ubiquinone oxidoreductase. NSP9 presented a 28% similarity in structure to human Urokinase plasminogen activator. NSP12 showed 27% similarity with the RNA-dependent RNA polymerase of Foot and mouth disease virus, 29% similarity to the RNA-dependent RNA polymerase of *Coxackie B3 virus*, 28% resemblance of Poliovirus RNA Polymerase, and 27% resemblance to the RNA dependent RNA polymerase of Norwalk virus.

NSP13 has a 31% similarity to human Upf1 helicase core protein and 27% similarity to the RecBCD enzyme subunit of *E-coli*. NSP14 showed 24% similarity to the protein 3' repair exonuclease 2 of humans. NSP16 has 27% homology in structure with the *Zika virus* NS5 methyltransferase. NSP7, the second choice of the authors, has no homology with any known sequence protein other than the coronaviruses proteins.

Other resemblances of SARS-CoV-2 NSPs to known sequenced proteins, if present, were less than 20%. NSP2, NSP3, NSP%, NSP7, NSP8, NSP10, and NSP15 have no significant homology with a known protein sequence. Table I summarizes the most important detected homologies of SARS-CoV-2 NSPs to SWISS-MODEL and Uniprot sequenced proteins.

DISCUSSION

The main goal of this research was to detect the possibility of using Albumin as a source of peptides forSARS-CoV-2 NSPs, particularly NSP11 and NSP7. The ability to extract the NSP peptide from the abundant Albumin could be of benefit in synthesizing an active vaccine for COVID-19 disease. It will be better to extract SARS-CoV-2 NSPs form a pre-existing protein to spare the time, money, and effort needed for recombinant protein vaccine synthesis.

Figure 2. Comparison of the sequence of amino acids in Albumin (above) and NSP7 and NSP11 (below); no similarities found even for the short NSP11.

The proposed steps to synthesize NSPs from Albumin could be summarized as follows: denaturing albumin to an inactive polypeptide; breaking down albumin with Golgi's apparatus NSPs-specific proteases; running the peptide fragments obtained from Albumin in Chromatography to isolate and purify NSPs from other fragments, which would be available for administration through intravenous, subcutaneous, or intramuscular routes.

Early in the research process, it had been found that Albumin has no structural similarities to NSP11, NSP7, or any NSP of COVID-19. The similarities found to sequenced proteins were within a range of 48% at best and decreasing to less than 5%. These similarities were to human, viral, and bacterial proteins. Coronaviruses proteins were not mentioned, and they were of 73% homology or more[9]. Most of the results of the similarity assessment gave references to intracellular proteins; the only exception was the 29% homology of NSP11 trimer or tetramer to the *Human Astrovirus* 2 Capsid protein spike[17,18,19]. These results are disappointing when related to the study prospect, partially because the incomplete homology is not enough to extract an NSP from the protein, and partially due to the type of proteins with partial homology; none of these proteins is as available or affordable as Albumin.

According to Buchholz et al[42], the only protein capable to induce antibodies formation in Coronaviruses is the spike protein. This 2004 study on SARS CoV tested different combinations of SARS viral proteins: Spike protein (S), Membrane protein (M), Envelope protein (E), and Nucleoprotein (N). Any combination of proteins that spared the (S) protein failed to elicit neutralizing antibody response[40].The spike protein is the best option of a protein to induce antibody formation but is also an important factor in the virulence of SARS-CoV and SARS-CoV-2. This 1273 amino acid containing protein has no homology with any protein other than those of coronaviruses[19]. For the 2002 SARS-CoV, the inactivated virus vaccine proved to be superior to the adenovirus coated spike protein and nucleoprotein vaccines[43].

The vaccine trials for SARS-CoV-2 are still ongoing, and plasma transfer from the recovered subjects would probably and hopefully confer immunity to the host against COVID-19, as it was against SARS[44]. New studies declared that the peaks of Immunoglobulins G (IgG) and M (IgM) titers could occur earlier in SARS CoV-2 than in SARS CoV[15].

This study was performed with a minimum budget, in a short time, and with high accuracy (the databases are of high precision and under continuous assessment)[45]. As a disadvantage, this study considered analyzing similarities of NSPs to known protein structures while the surface proteins of the virus are best to be considered in vaccine synthesis.

The authors are planning to explore the structural proteins homology of SARS-CoV-2 Nucleocapsid protein, Spike protein, Membrane protein, and Envelope protein. Currently the authors are working in summarizing the vaccine literature of *Haemophilus Influenzae*, *Pyrococcus furiosus*, *Bacillus subtilis*, *Mouse hepatitis Virus*, *Hu-*

Table 1. Homologies of SARS-CoV-2 NSPs to some proteins other than Coronaviruses. Table excluded homologies of 20% or less, protein-protein complexes, or protein-DNA complexes*.

SARS-CoV-2 NSP	Homology to Albumin	Detected homologies-other than to Albumin or Coronaviruses	% Similarity	References
NSP1	No	1. *Haemophilus Influenzae* Hypothetical Protein.	29%	17,21, 22
		2. V-type ATP synthase subunit F from *Pyrococcus furiosus*	30%	23
		3. Pyrimidine-nucleoside phosphorylase from *Bacillus subtilis*	31%	24
NSP2	No	None		17
NSP3	No	None		17
NSP4	No	RNA directed RNA Polymerase (Mouse hepatitis Virus protein).	49%	17,19,25
NSP5	No	None		17
NSP6	No	1. EF-hand Ca2+binding protein calbindin D9k (Human).	24%	17,19,26
		2. Mitochondrial NADH dehydrogenase1 beta subcomplex subunit 5 (Human)	29%	17,27
		3. NADH: ubiquinone oxidoreductase subunit B5 (Ovine)	27%	17,28
NSP7	No	None		17
NSP8	No	None		17
NSP9	No	Urokinase plasminogen activator (Human)	28%	17,19,29,30
NSP10	No	None		17
NSP11	No	Capsid protein spike (Human Astrovirus 2)	Trimer: 29% Tetramer:29%	17-20
NSP12	No	1. RNA-dependent RNA polymerase (Foot and mouth disease virus)	27%	17,19,31
		2. RNA-dependent RNA polymerase (Coxackie B3 virus)	29%	17,19,32
		3. Poliovirus RNA Polymerase	28%	17,19,33
		4. RNA dependent RNA polymerase (Norwalk virus)	27%	17,19,34
NSP13		1. Upf1 helicase core (Human)	31%	17,19,35,36,37
		2. RecBCDenzymesubunit+-(E-coli)	27%	17,19,38
NSP14	No	3' repair exonuclease 2 (Human)	24%	17,29,39
NSP15	No	None		17
NSP16	No	NS5 methyltransferase (Zica Virus)	27%	17,19,40,41

*The full list of homologies is not mentioned here, but representative examples are given.

man Astrovirus 2, *Foot and mouth disease virus*, *Coxackie B3 virus*, *Poliovirus*, *Norwalk virus*, and *Zica Virus*, as all the above mentioned pathogens showed different degrees of protein homologies to the NSPs of SARS-CoV-2. *Human Astrovirus* 2 Capsid protein spike, in particular, is a good target for further study.

CONCLUSIONS

This research was performed to investigate the possibility of vaccine formation for the emerging COVID-19 disease from the SARS-CoV-2 nonstructural proteins. The aim of the first part of the study was to try synthesizing nonstructural proteins from other larger proteins *in vitro* by degradation with proteases, separation of fragments by Chromatography, and extraction of the NSPs, which will be injected to the patient to reach the blood and induce an immune response. The authors chose Albumin as a target for degradation, but there was no homology between the Albumin peptide sequence and any of the NSPs. The second part of the study was to detect homologies of NSPs to known protein sequences in databases. Similarities of NSPs to known sequenced proteins were, at best, 49% similarity of NSP4 with the RNA directed RNA Polymerase of mouse hepatitis Virus protein. The authors are welcoming suggestions concerning the choice of the homologous Protein and the suitable non-structural protein(s).

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Conflict of Interest:
The authors declare that they have no conflicts of interest.

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