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Differences in Vaccine and SARS-CoV-2 Replication Derived mRNA: Implications for Cell Biology and Future Disease

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Abstract
Codon optimization describes the process used to increase protein production by use of alternative but synonymous codon changes. In SARS-CoV-2 mRNA vaccines codon optimizations can result in differential secondary conformations that inevitably affect a protein's function with significant consequences to the cell. Importantly, when codon optimization increases the GC content of synthetic mRNAs, there can be an inevitable enrichment of G-quartets which potentially form G-quadruplex structures. The emerging G-quadruplexes are favorable binding sites of RNA binding proteins like helicases that inevitably affect epigenetic reprogramming of the cell by altering transcription, translation and replication. In this study, we performed a RNAfold analysis to investigate alterations in secondary structures of mRNAs in SARS-CoV-2 vaccines due to codon optimization. We show a significant increase in the GC content of mRNAs in vaccines as compared to native SARS-CoV-2 RNA sequences encoding the spike protein. As the GC enrichment leads to more G-quadruplex structure formations, these may contribute to potential pathological processes initiated by SARS-CoV-2 molecular vaccination.

Introduction
The simplification of scientific jargon in the realm of public health can lead to the construction of a false consensus. One such over-simplification exists in our discussions surrounding the expression of SARs-CoV-2 spike protein in mRNA vaccines. This spike protein is often referred to as being bio-equivalent to the naturally expressed spike protein in SARs-CoV-2. Accordingly, it is suggested that this "may" constitute a safer immunological exposure as the rest of the genes responsible for replication of the virus are omitted. This often leads one to assume that the pathologies that arise from vaccine expressed spike protein should be a subset of those you might experience with the full-length live virus. The mRNA vaccines have the benefit of being a non-replication competent immune exposure, but are the spike proteins truly equivalent?

In this line of questioning, one must ask what is the purpose for codon optimizing a viral mRNA that is already adapted to its host? This does not come risk free. The potential dangers of codon optimization have been raised for *in vivo* applications [1]. Even synonymous codon changes incorporated into mRNA vaccines can alter the expected encoded protein conformation as the translation speed and efficiency can result in different protein folding. Despite identical amino acids, the altered conformation can function differently as compared to synonymous codon replacements of native mRNAs that have been put in place under the selective pressure of evolution of parasite-host adaptation. Codon optimization strategies for the development of mRNA vaccines can result in immune de-regularities, affect epi-transcriptomic regulation, and can lead to disease progression [1 2].

Methods

We used open source, publicly available software for every step of the analysis.

Sequences utilized

Vaccine derived mRNAs were downloaded from Dae Eun Jeong *et al.*

https://virological.org/t/assemblies-of-putative-sars-cov2-spike-encoding-mrna-sequences-for-vaccines-bnt-162b2-and-mrna-1273/663

Using BLAST of the Wuhan Hu-1 reference sequence against the vaccine derived RNAs, we extracted the Wuhan Hu-1 spike sequence. Query13637:21560-25382 NC_045512.2 Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome

GC content

GC content of each sequence was calculated[3]. Biologicscorp.https://www.biologicscorp.com/tools/GCContent/

RNAfold analysis

Using default conditions from a tool known as RNAfold, secondary structures were predicted[4]. http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi

G4 Identification

We used QGRSMapper to calculate G4 motifs in the three mMRNA sequences.https://bioinformatics.ramapo.edu/QGRS/index.php[5]

Frameshift evaluation

We used IGV2.4.16 to visualize all 6 reading frames of the 5' UTR[6-8].

Results and Discussion

To address the implied equivalency of virus derived spike protein vs mRNA derived spike protein, we explore the impact of codon optimization on the secondary structure of the natural RNA encoded spike protein and compare this to the secondary structure of the mRNA vaccines. The most obvious alteration from codon optimization is the increase in the GC content of the mRNAs (Figure 1.)

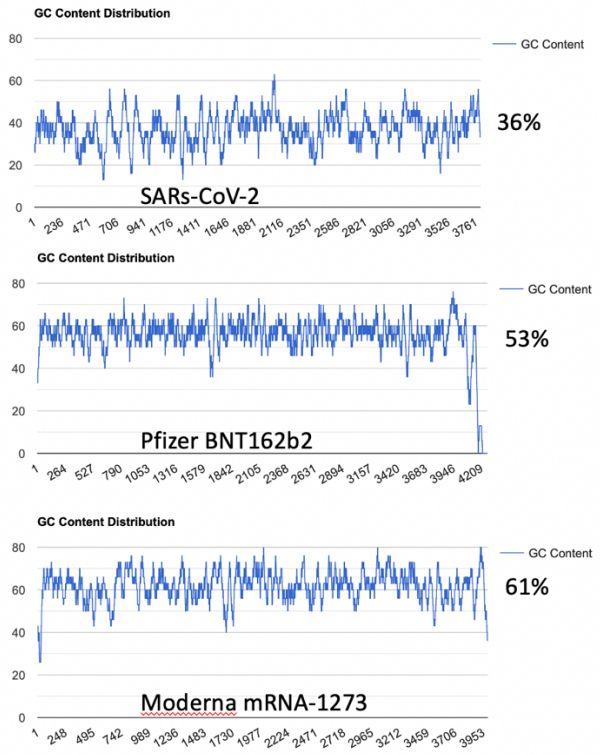


Figure 1. Top: GC content of SARs-CoV-2 Spike Protein. Middle: GC content of codon optimized Pfizer BNT162b2 vaccine. Bottom: GC content of codon optimized Moderna mRNA-1273. https://www.biologicscorp.com/tools/GCContent/

This increased GC content significantly alters mRNA secondary structure. Using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) we see the changes to secondary structure in the vaccine derived mRNAs compared to the native virus (Figure 2)[4]. This is a result of codon optimization that was likely performed without the consideration of secondary structures like quadruplex G formation.

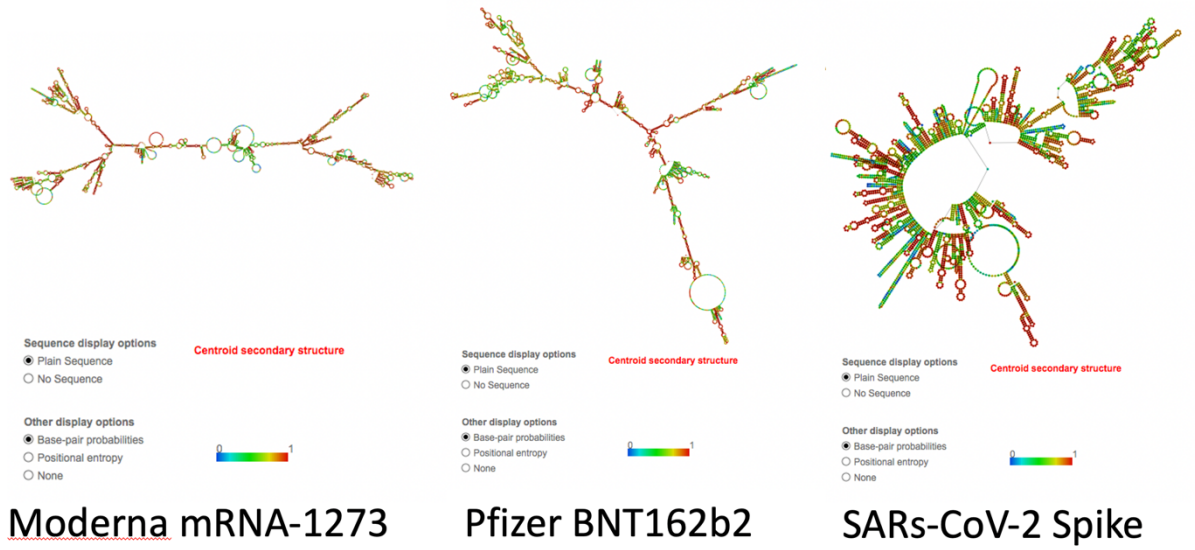


Figure 2. The three different Spike Protein Sequences (Moderna (left), Pfizer (middle), SARs-CoV-2(right)) analyzed with RNAfold.

Not only are the secondary structures of the mRNA noticeably different, of note, is the increased number of quadruplex G formations in the codon optimized mRNA vaccines (Figure 3). Quadruplex G formations (G4s) in SARs-CoV-2 are highly conserved across over 16,466 SARs-CoV-2 genome sequences [9]. They are believed to play a critical role in transcription and translation of SARs-CoV-2 peptides [10-12]. G4 formations in the RNA sequence for nucleocapsid protein have been proposed as attractive drug targets to eliminate nucleocapsid expression. This is achieved by using compounds that stabilize quadruplex G formations [13].

G4s are also implicated in recruiting viral SARs Unique Domain (SUD) of Nsp3 [14]. While the mRNA vaccines do not encode this non-structural protein, an increasing number of breakthrough infections must consider the biology of both the virus and the non-native vaccine derived mRNA. With increased vaccination rates and continued boosters, it will be increasingly important to understand the immune status of patients expressing both mRNA-based vaccine spike proteins concurrent with viral spike protein expression.

The changes to secondary structure can be observed in the G4 formations seen in each sequence using QGRSMapper (Figure 3) [5]. Similar trends were observed using G4-iM Grinder (Personal communication Belmonte)[12].

Figure 3. Three sequences analyzed with QGRSMapper. Only four G4 motifs (yellow) are identified in the Spike coding region of the virus mRNA (left). 19 G4 motifs are identified in Moderna (middle) and 9 G4 motifs are identified in Pfizer (Right).

Does the increase of 4 to 19 G4 motifs alter the translation efficiency of these proteins? Do they create truncated spike proteins not seen with the natural virus?

Fidelity of Spike protein expression

To address these questions, there is another unique feature of the mRNA vaccines that must be considered. The uracil's in the vaccines have been replaced with N1-methylpseudouridine. The use of N1-methylpseudouridine in these mRNAs will further complicate the folding predictions as N1-methylpseudouridine (m1Ψ) has promiscuous base pairing with G and A and is known to create errors in translation [15-17]. These m1Ψ replacements are excellent for evading host RNAses but they are also implicated in Toll Like Receptor biology and this is something unique to mRNA vaccines. While m1Ψ enables slower degradation of mRNAs, it comes at the cost of camouflaging the mRNA from the immune system that targets viral RNA secondary structures [18]. Immune receptor TLR3,7,8 and RIG-1 are intimately involved in targeting such secondary structures and their response is RNA modification dependent [19]. This has been described as "reprogramming the innate immune and adaptive immune response" [20]. This may explain the increased rate of Herpes Zoster, HHV6/HHV7 (Pityriasis rosea) and Epstein-Barr reactivation post mRNA vaccination [21-26]. These reactivations are also seen with SARs-CoV-2 infection. Further investigation is required to understand if the mRNA vaccines exacerbate or curtail these reactivations.

Codon optimizations and pseudouridine replacements alter the secondary structure significantly and likely change the Toll Like Receptor activity one might find with the native virus [27]. How these receptors behave in the presence of both vaccine-derived spike mRNA and viral derived spike mRNAs is a nascent field. However, this field is of interest to many physicians concerned about chronic diseases including cancer [28]. Jiang *et al*. note that the spike protein localizes to the nucleus and significantly alters DNA damage and repair pathways via modified VDJ recombination required for adaptive immunity [29]. Many of these vaccine trials demonstrated

lymophocytopenia and neutropenia 2 weeks after injection [30 31]. Down regulation of the innate immune system concurrent with reduced DNA repair may lead to carcinogenesis[32].

Notably, promiscuous bases like inosine and pseudouridine are known to stabilize quadruplex Gs [33 34] further exacerbating the impact of G4 quartet formation with codon optimization.

How much m1Ψ is present in these mRNAs? The only independent DNA sequence released for these mRNA vaccines were sequenced on Illumina platforms that are blind to pseudouridine [35]. The exact m1Ψ density (while reported by the manufacturers to be 100%) has not been independently sequence verified. The most parsimonious synthesis approach described by Nance *et al.* replaces all uracil's with m1Ψ via polymerase incorporation [18]. Such an approach would also leave the stop codons prone to pseudouracil stop codon read through described by Fernandez *et al.* [36]. Pseudouridine is also known to create ribosomal frameshifts. Penultimate to the ΨGA ΨGA stop codon in the BNT162b2 vaccine is an out of frame human amino acid sequence of unknown function (AAG23172.1) (Figure 4). Xia *et al.* also makes note that UGA stop codons are more prone to read through and +1 frameshifts suggesting these mRNA derived stop codons may not be as effective as viral derived stop codons [15].

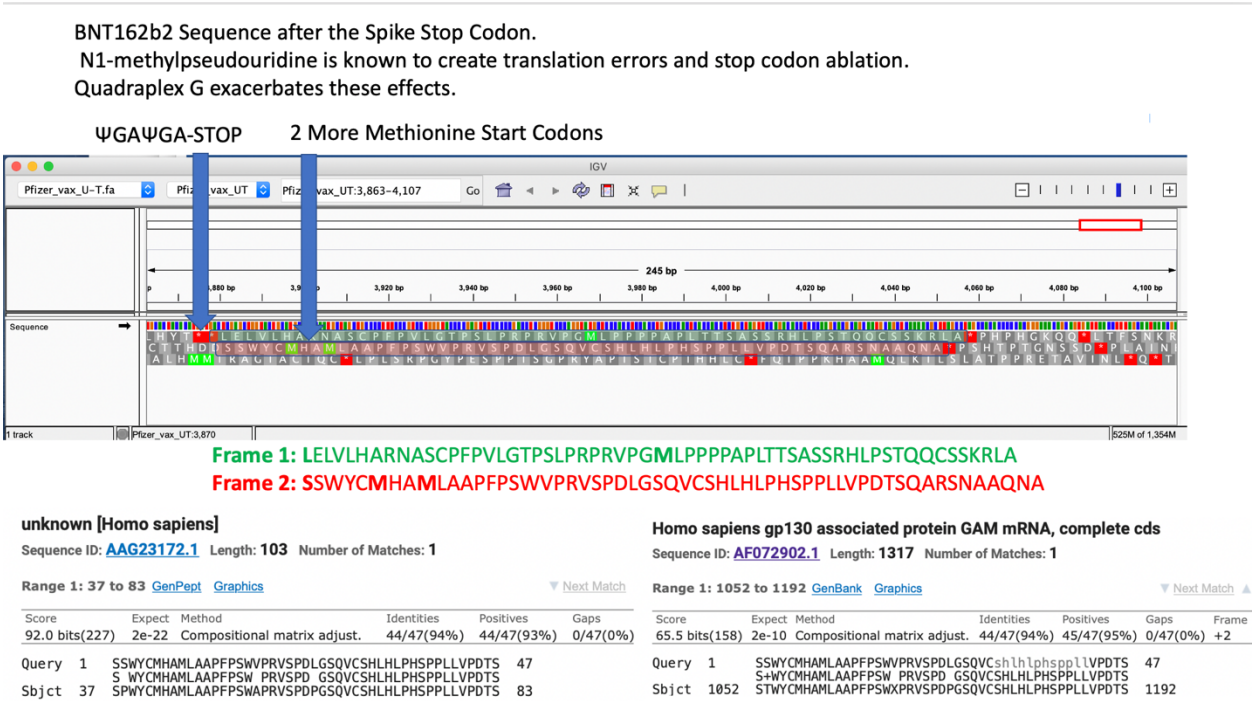


Figure 4. Top: IGV view of the BNT162b2 mRNA sequence as published by Dae-Eun Jeong *et al.* focused on the Amino acid sequence 3' to the stop codon. N1-methyl Pseudouridine based Read through of this codon could lead to chimeric Spike-Human peptides (Red text). Bottom track:Read through amino acid sequence BLASTp alignment delivers a Human hit. tBLASTn of amino acid sequence had homology to human gp130.

Expression of chimeric spike-human peptides may be unique to pseudouracil based mRNA vaccination and raises concerns over immune de-regulation including autoimmunity that may develop with such chimeric inoculations.

Hypothetical viral: mRNA vaccine interactions

With any virus or vaccine that enables latent virus reactivation we must consider the case of viral recombination. Chimeric RNAs are more likely to form with mRNAs that have degenerate bases. In this hypothetical case, a non-replicative pseudouridylated mRNA may hitchhike into a replication form via recombination with a live virus.

Relevantly, *in vitro* and *in vivo* experiments using human and mouse RNAse L chimeras, and chimeric Mouse Hepatitis Virus (coronavirus) expressing recombinant L* from Theiler's murine encephalomyelitis virus (picornavirus) showed that chimeric MHV viruses are functional. These efficiently express RNAse L inhibitors and hence interfered with prompt interferon responses[37]. Linking to SARs-CoV-2, the subunit 1 (S1) persists for up to 15 months post infection and is related to post-acute inflammatory sequalae presenting with neurological complications[38]. 75% of Patients with persistent RNAemia also present long COVID[39].

Retroviral and non-retroviral RNA sequences are abundantly integrated into mammalian DNA[40]. These inserted viral sequences exist in the form of long interspersed nuclear elements (LINEs). These LINE based retrotransposons mobilize and also transcribe human DNA not associated with the LINE sequences forming pseudogenes [41]. These can be active in disease onset including tumorigenesis [42]. As an example, the polio RNA sequences are identified with a 100 % sequence homology to human chromosomes and are associated with cancer progression[43]. These require attention for possible production of chimeric mRNAs with SARS-CoV-2 native or vaccine derived mRNAs, especially during meiotic division[44], stem cell differentiation and cancer[45].

Furthermore, human endogenous retroviruses (HERVs) constitute the 8% of human DNA. These are mobile genetic elements related to etiopathogenesis of inflammation and neurogenerative disease[40]. Due to viral transactivation of HERVs the herpesviridae reactivation is implicated in the onset of multiple sclerosis [46]. Another example of reactivation disease is Ebstein Barr virus associated head and neck cancer. Notably, HERVs induce neurological autoimmunity by interfering with innate immunity in mice [47].

Moreover, the presence of chimeric SARS-CoV-2 mRNAs with other viral sequences has recently been reported [48 49]. Other studies describe a decrease in the interferon response[50 51] and emergence of Herpes Zoster Virus infection post SARS-CoV-2 molecular vaccination[23 21]

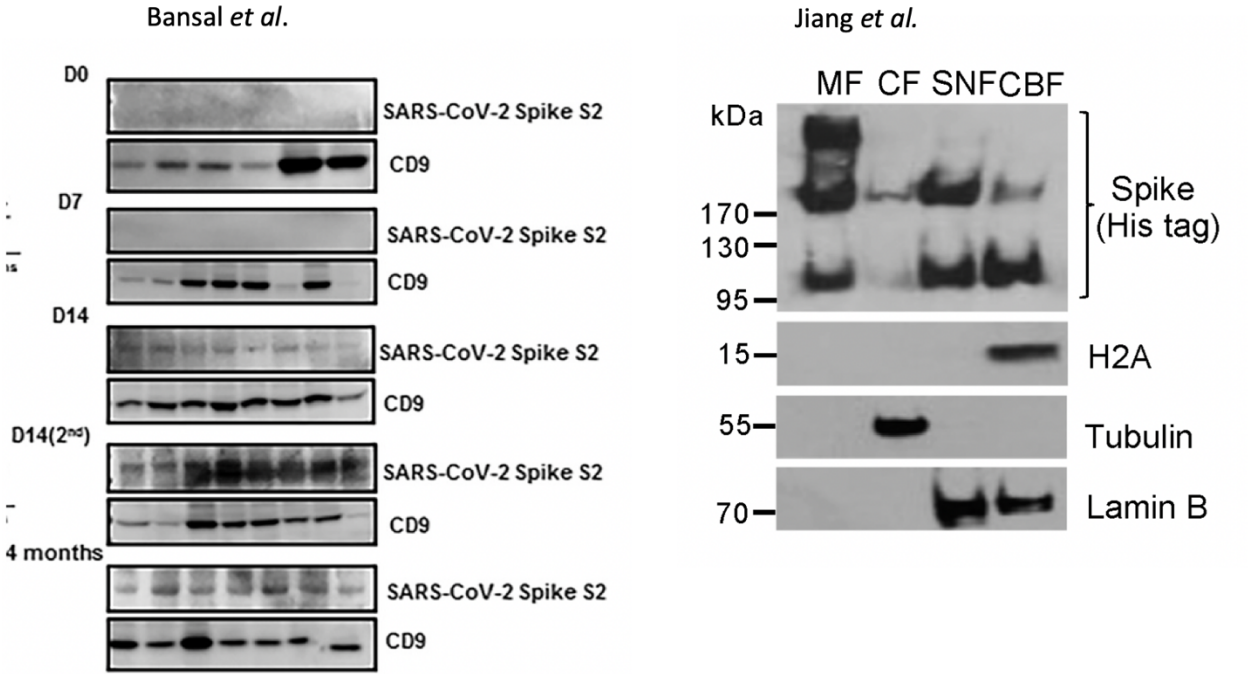
It is important to understand these are not documented cases of SARs-CoV-2 viral recombination but such hypothetical cases resonate with recently published clinical data[52]. Likewise, virus to mRNA recombinations increase in likelihood with mRNAs that use degenerate bases like m1Ψ[53]. Notably, the natural Ψ:U ratio of mRNAs under normal conditions is markedly low (0.2-0.6 %) compared to the 100% Ψ substitutions in synthetic SARS-CoV-2 mRNAs[54]. RNA based Pseudouridine substitutions determine the cell's fate and differentiation[55]. Specifically, pseudouridylations of mRNAs are subject to activity of PUS7 writer protein which is a key

regulator of protein translation and determinant of stem cell growth and differentiation[55]. Dysregulation of PUS7 activity correlates with agitated protein synthesis in stem cells which leads to hematological disorders and aggressive stem cell acute myeloid leukemia[56]. Also pertinent to the detection of the SARs-CoV-2 recombination hypothesis is that many SARs-CoV-2 sequencing methods (ARTIC) rely on SARs-CoV-2 specific PCR primers that are often blind to recombination events [57]. Given the paucity of sequence information on the vaccines and the peptides expressed in-vivo with such mRNAs, further work is required to confirm pseudouridine induced promiscuous translation or viral recombination with mRNA vaccines.

Limited evidence of pure vaccine derived spike protein expression in humans

There is limited literature describing fidelity of mRNA vaccine derived spike protein expression. The best *in-vivo* evidence of heterogeneous translation of vaccine derived spike protein is from Figure 2C in Bansal *et al.* where the SDS-PAGE of vaccinated patients' exosome-derived spike proteins exhibit broad banding patterns (Figure 5). To rule out electrophoretic artifacts from membrane derived proteins, LS/MS-MS should be performed to confirm or negate the spike translation heterogeneity. Additionally, spike proteins expressed from a DNA based plasmid in HEK293T cells described by Jiang *et al*. demonstrate 3 different bands on SDS-PAGE. These 3 bands represent the glycosylated, full length and fragmented spike proteins and their expression appears cell fraction dependent. DNA based expression does not contain error prone pseudouridine and one would expect more translational error from the m1Ψ mRNA-based vaccine expression systems.

Figure 5. Reproduced from Bansal *et al*. and Jiang *et al*. Vaccine induced, exosome-derived Spike proteins exhibit broad banding patterns (left). DNA plasmid-based expression of Spike in HEK293T cells demonstrates translational heterogeneity in a cell fraction dependent manner.



While these truncated or elongated promiscuous translation products may be rare events, this does bring to focus the question of dosage and spike protein duration with mRNA vaccines that ablate many components of the innate immune system [20].

Quantity of Spike Protein

How much spike is too much? Bansal *et al* note that Spike protein can be detected circulating on exosomes membranes 4 months post vaccination [58]. Virus derived Spike protein has been detected 15 months later in post-acute sequelae patients (PASC or long COVID)[38]. The vaccine programs have not existed long enough to form a valid comparison in the same tissues. Patterson has recently presented data that both the S1 and S2 segments of spike protein are recoverable from vaccinated persons many months after immunization (personal communication).

mRNA based vaccination has also been shown to provide log scale higher anti-spike antibody production compared to natural infections [59]. While some imply this equates to better immunity, it likely comes at the cost of higher spike protein titers and spike protein is believed to be toxic and a potential inhibitor of DNA damage and repair [29].

Estimates of mRNA transfection efficiency have been described by Pardi *et al*. using a luciferase mRNA [60] transfected into mice. While this does suggest more than 1 protein is synthesized from each mRNA transfected, it is not clear this result can be superimposed onto mRNA derived spike proteins which localize to the nucleus. Quantitative measurement of spike protein expression levels of vaccinated individuals is lacking in the peer reviewed literature.

Toxicity of spike protein

There are multiple modes of spike protein toxicity. Some is attributed to spike protein induced coagulopathy and mitochondrial damage [61]. Other toxicity may be a result of Staphylococcus Enterotoxin B (SEB) sequences in the spike protein [62-65].

These SEB motifs share sequence homology to neurotoxic peptides from Cobra's and are classified as bioweapons [66 67]. It should be emphasized that short peptides like the SEB motif may have different properties when cloned into spike proteins and the bioweapon classification has not been applied to the SARs-CoV-2 SEB motif. This motif is unique to SARs-CoV-2 amongst coronaviruses.

Even though the SEB motif is cloned into a different amino acid context, many of the symptoms observed in SEB ingestion are similar to SARs-CoV-2 symptoms as these super antigenic peptides are known for inducing cytokine storms. Cheng *et al.* suggest SARs-CoV-2 SEB may be responsible for multisystem inflammatory syndrome (MIS-C). Ahanotu *et al.* describe the symptoms of SEB intoxication as the following

"sudden onset of fever (40-41C), chills, headache, myalgia, non-productive cough. Some patients may develop shortness of breath and chest pain. Fever may last for 2-5 days and cough may continue for up to one month. Patients also present with nausea, vomiting, and diarrhea when the toxin is swallowed."

The conclusions of Ahanotu *et al*. are prescient and suggest the most likely method for delivering SEB as a bioweapon would be through the use of an aerosol.

"The use of SEB as a weapon of mass casualty is considered likely for several reasons, mainly high morbidity with ease of production and dispersion, the delayed onset of disease symptoms associated with high morbidity and low mortality and difficulty in diagnosis. Staphylococcal enterotoxin B is a superantigen capable of massive non-specific activation of the immune system. Because of the remarkable toxicity and stability, they would most likely be disseminated as an aerosol"

Finally, the raised GC content and the amplification of G4 quartets in vaccine spike protein mRNAs over the native SARS-CoV-2 spike protein mRNA, can amplify the already established interaction of SARS-CoV-2 RNA with RNA binding proteins[11 68]. This constitutes a potential cause of interference on epi-transcriptomic regulation of RNA G4 binding proteins. This may play a major role in the potential activation or deactivation of a pathological pathway [69]. In this respect, oncogenic RNA binding proteins like the mutant variants of p53 and mdm2 can readily form RNA-Protein binding complexes at polysomes with SARS-CoV-2 vaccine mRNA potential G-quadruplexes [70 71]. The vaccine mRNAs prolong their translation due to robust capping resisting natural mRNA decay pathways. This can trigger cancer initiation and progression[28].

Conclusions

The argument that the spike proteins synthesized by codon optimized mRNAs are identical to spike protein from the virus should be cautiously examined. There are several arguments that challenge this dogma. First, the biodistribution of non-specific LNP transfection of mRNAs does not discriminate towards ACE2 or CD147 expressing cell lines as seen with the virus. Second, the mRNA that encodes spike protein is known to be different in several regards. The mRNAs are known to have a 2 Proline substitution (K986P and V987P) (Department of Health and Human Services Patent US 10,960,070B2) altering the proteins conformation. The mRNAs are known to be codon optimized thus altering their secondary structure and their quadruplex G density in the spike protein mRNA. The mRNAs are known to have N1-methylpseudourine substitutions that alter translation fidelity and Toll Like Receptor recognition. Additionally, the expression levels and duration of these mRNAs may be longer and of higher copy number in many tissues that never experience natural virus infection. Finally, the pharmacokinetics of injection are different than infection. 60ug-200ug of Spike mRNA equates to 26 Trillion to 80 Trillion mRNA molecules injected in a few seconds. The pharmacokinetics of this bolus injection differs from that of viral replication that occurs over the course of a few days. If each of these mRNAs can produce 10-100 spike proteins and you have 30-40 Trillion cells, there may be a far greater systemic quantity and a much longer duration of spike protein exposure through the vaccination route than natural infection. Boosters given more frequently than a year will lead to total body accumulation of spike protein and further heighten the risk of disease in organs such as the brain, heart, bone marrow, and immune cells and tissues. This false equivalency may lead to an under appreciation of the symptomatology of vaccine based adverse events.

It should be emphasized that these results are an *in-silico* hypothesis supported by the peer-reviewed literature but further work is required to better characterize the homogeneity of spike

protein expression *in-vivo.* This work has not considered post translational modifications or the impact of the degenerate base pairing from N1-methylpseudouridine.

More than 20 months into this pandemic and we have millions of SARs-CoV-2 genomes sequenced. Lot to lot sequencing of the vaccines is non-existent. To this date, no raw reads for these vaccines exist in NCBI despite over a billion liability-free vaccinations. To fully understand RNA synthesis substitution errors, fragmentation errors or strandedness errors in the mRNA synthesis process, robust lot to lot sequencing should be performed and published. Given these mRNAs are prodrugs which code for a desired protein, where is the evidence that the conversion of this prodrug into a drug is of high fidelity? This seems to have been assumed as opposed to documented. This work suggests this assumption should be questioned. Public and transparent quality control of these often-mandated injections are required. This should include sequence verification and quality control of the various lots and evidence of the proteins these mRNA express in patients.

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