The endosomal decomplexation rate between mRNA and the excipient cationic lipid may influence the translation extent and efficacy of mRNA vaccines.

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Editorial

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...Let my people go...

Exodus 5:1

Overcoming immune evasion by substituting the nucelobase pseudouridine (Ψ) for uridine (U) in the mRNA to be used as vaccines, has been a major contributor to clinical adoption. This substitution minimizes the indiscriminate recognition of exogenous mRNA by pathogen-associated molecular pattern (PAMP) receptors, such as the toll-like receptors and the retinoic acid–inducible gene 1(RIG-1) and removes a major hurdle to cellular delivery. Ψ substitution has also been shown to increase the thermodynamic stability of the mRNA structure by favorable base stacking and hydrogen-bonding with water in the major groove. Other efficacy enhancing effects include increased functional half life of the Ψ substituted mRNA due to the stabilization of secondary structure that is independent of codon optimality. This in turn leads to increased ribosome engagement, increasing ribosome density, decreased ribosome pausing and decreased hydrolysis by ribonuclease.

A factor in the chain of events leading to the delivery of the mRNA to the cytosol, that has thus far been neglected, is the escape of the mRNA from the endosome into the cytosol. This may be because this step is so cargo-transport-inefficient to begin with, (only 1-2% of endocytosed cargo finds its way into the cytosol) that no significance is thought attributable to the discrimination of mRNA endosomal release from different LNP formulations, so long as adequate antibody titers are generated by the vaccine. Such a presumption may prove fallacious, especially if it turns out that there is an absence of significant magnitude between unsubstituted and Ψ substituted mRNA cellular delivery at comparable doses. Such may be the case for mRNA vaccine efficacy differences between CureVac's unsubstituted mRNA vaccine and the Moderna and Pfizer-BioNTech m1Ψ substituted vaccines.

For the mRNA cargo to escape the endosome, the intermolecular ionic forces of attraction between the cationic lipid and the predominantly anionic mRNA must be overcome so that the cationic lipid can form what is generally termed as an 'inverted hexagonal phase' within the slightly acidic pH environment in the endosome. This packing architecture is generally considered to be a prelude to endosome membrane disruption and eventual cargo escape. This implies

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that the attractive intermolecular forces between the mRNA and the cationic lipid be relatively weak to begin with – but not so weak as to prevent cell ingress. The substitution of 1N-methyl pseudouridine (m1Ψ) for uridine (U) in the mRNA has been shown to decrease mRNA-to-RNA binding protein interactions, presumably because m1Ψ increases the thermodynamic stability and the melting temperature, Tm , of the RNA by facilitating base stacking. (This may also be a reason why less m1Ψ substituted mRNA– when compared with its unsubstituted counterpart – is detected in stress granules post endsosomal escape, and may also constitute a factor in the higher mRNA translation of the former). A mechanism similar to this may exist between m1Ψ substituted mRNA and the cationic lipid. The relatively weak(er) intermolecular forces of attraction between them may allow easier disentangling or dismantling of the m1Ψ mRNA-cationic lipid complex, thereby ultimately leading to a faster and more effective means of escape of the mRNA from the endosome into the cytosol. It has been reported that m1Ψ modification to mRNA – regardless of length - does not affect its encapsulation or loading efficiency into lipid nano particles (LNP), nor does it affect the particle size or the morphology of the formed LNPs'. It seems plausible therefore, that the relative decrease in the strength of non-covalent forces of complexation between the m1Ψ modified mRNA and the cationic lipid; compared to its non-substituted counterpart; must be responsible in part for enhanced protein expression of the m1Ψ modified mRNA.

Decomplexation of the mRNA cargo from the endocytosed LNP is an important determinant of endosome escape. Regardless of whether the LNP cationic lipid component induced ingress of anions into the endosome, the so called proton-sponge effect, or the cationic lipid induced destabilization of the endosomal bilayer lipid membrane is responsible for mRNA delivery to the cytosol; decomplexation of the cationic lipid from the mRNA must occur as a pre-requisite for these events. The pH and composition of the endosomal mileau, and/or other lipid components of the formulation; specifically their chemical structure and properties; must be capable of, or conducive to overcoming the strength of the intermolecular forces between the mRNA and the cationic lipid component of the LNP. The stoichiometric ratio of the cationic lipid to the mRNA is important when egressed as exosomes. Decomplexation enhancers influence transfection efficiency as do self-degrading cationic lipid components; some of which increased protein translation greater then that obtained with MC3, the currently used cationic lipid.

Curevac's formulation consists of lipid components essentially similar to those constituting the Pfizer-BioNTech and Moderna formulations. The U in the mRNA of the Curevac's formulation is not modified, whereas all the Us' in the mRNA of the latter two formulations have been substituted with m1Ψ. This suggests that the change in free energy, ΔG, of binding between mRNA and the cationic lipid of the Curevac's formulation may be (more negative; greater thermodynamic stability) than that between the mRNA and the cationic lipid of the Pfizer-BioNTech and the Moderna formulations. The ease of decomplexation between the two in the Curevac's formulation as well as the subsequent formation of an inverted hexagonal phase formed by the cationic lipid may be lesser when compared to the formulations where the U is substituted with m1Ψ. Consequently, the amount of mRNA that escapes the endosome may be lesser for the Curevac's formulation. It is noteworthy that a greater dose of the Curevac's formulation is required to generate similar antibody titers as those observed with m1Ψ substituted formulations. Furthermore, a greater amount of non-substituted mRNA in the Curevac's formulation post-endosome escape may end up in stress granules when compared with its modified counterpart. The end result may be that a lesser amount of the SARS-CoV2 receptor binding domain (RBD) domain is presented to T-cells in the Curevac's formulation.

The hypothesis presented in this editorial is difficult to test. This is because *in vitro* binding studies between mRNA and cationic lipids have not been found to correlate with endosome escape, probably because these experiments are not entirely representative of the sequence of events and the specificity of the reactions occurring inside the endosome. The change in the forces

of non-covalent interaction between the mRNA and the cationic lipid resulting from changing the cationic lipid may change the rate and extent of endosomal escape but cannot be unequivocally confirmed to be the cause of increased translation, because other influencing factors such as LNP biodistribution, cellular uptake, exosome egress, stress granule uptake, immune evasion, mRNA stability and/or degradation kinetics in the LNP and so on may confound the results. Analogous to the biblical call to action, the quantitative and efficient delivery of the mRNA to the promised land of the cytosol will ultimately be viewed as the sole arbiter of efficacy, in which the intermolecular attractive forces between the cationic lipid excipient and the (substituted or unsubstituted) mRNA play a part of an as-yet undetermined magnitude.