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Apparent Cytotoxicity and Intrinsic Cytotoxicity of Lipid Nanomaterials Contained in a COVID-19 mRNA Vaccine

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Abstract

The medicinal preparation called *Comirnaty* by Pfizer-BioNTech is an aqueous dispersion of lipid nanomaterials, intended to constitute, after thawing and dilution, the finished product for intramuscular injection. In the present study, we examine some evident chemical-physical criticalities of the preparation, particularly regarding the apparent and the intrinsic pKa (*acid dissociation constant*) of its main excipient, the ionizable cationic lipid ALC-0315. The very high value of its intrinsic pKa causes, after internalization and endosomal escape of LNPs, a sudden increase of its cationic charge concentration and consequently the formation of pro-inflammatory cytokines and ROS (reactive oxygen species), that can disrupt the mitochondrial membrane and release its content, cause RNA mistranslation, polymerization of proteins and DNA, DNA mutations, destruction of the nuclear membrane and consequent release of its content. Additionally, the apparently low pKa value (6.09) of ALC-0315 associated with other lipids in the LNP, is not suitable for intramuscular application. Its value is too low to enable a proper transfection of host cells, despite what is stated by EMA (European Medicines Agency) in its Assessment report dated 19 February 2021, in flagrant contradiction with the same bibliographic source therein cited. Furthermore, the exceptional penetrability, mobility, chemical reactivity and systemic accumulation of uncontrollable cationic lipid nanoparticles, with high cytotoxicity levels, shed in unpredictable biological locations, even far from the site of inoculation, are all factors that can lead to an unprecedented medical disaster. Meanwhile, further immediate studies and verifications are recommended, taking into consideration, in accordance with the precautionary principle, the immediate suspension of vaccinations with the COVID-19 mRNA- LNP-based vaccines.

Keywords: *mRNA vaccine, LNP, lipid nanoparticles, ROS, reactive oxygen species, pKa, apparent pKa, intrinsic pKa*

INTRODUCTION

Lipid nanoparticles (LNPs) in the two COVID-19 mRNA-LNP-based vaccines (Comirnaty by Pfizer/BioNTech and Spikevax by Moderna Therapeutics) are formed by four different types of lipids: an ionizable cationic lipid whose positive charge binds to the negatively charged backbone of the mRNA, a polyethylene glycol (PEG)-linked lipid that helps prolonging the half-life of the composition, a phospholipid to facilitate the formation of a

two-layer structure, and cholesterol having a function of membrane fluidity modulator/stabilizer (Figure 1).

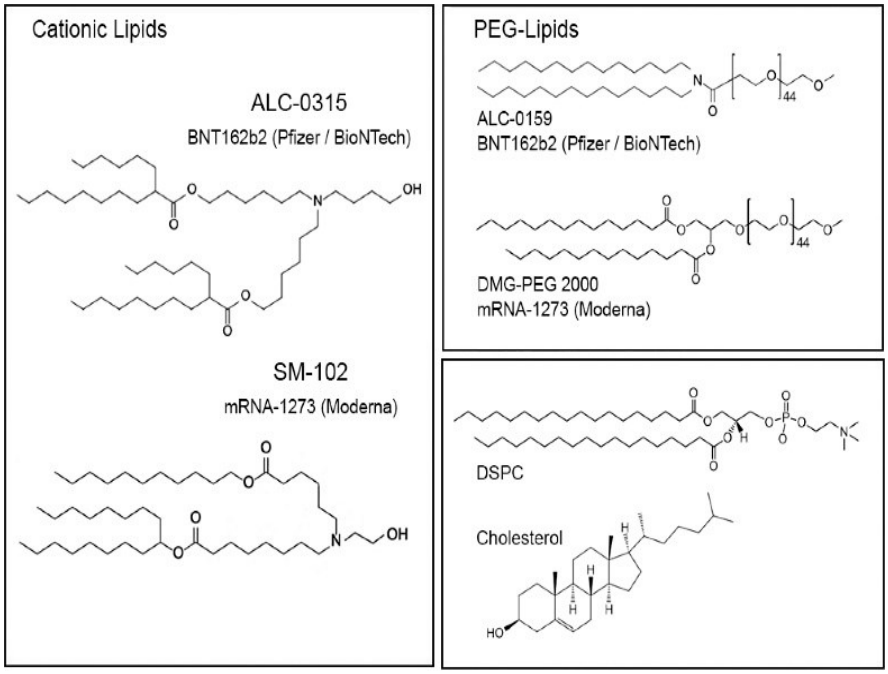


Figure 1. "Structures of the lipid constituents of the LNPs of the COVID-19 mRNA vaccines" reprinted from Figure 8, page 16989 from the article by Tenchov, R., Bird, R., Curtze, A. E., & Zhou, Q., entitled "Lipid nanoparticles ─ from liposomes to mRNA vaccine delivery, a landscape of research diversity and advancement" published in *ACS Nano* 2021, 15, 11, 16982–17015, 15(11), 16982–17015, https://pubs.acs.org/doi/full/10.1021/acsnano.1c04996. Copyright © by the authors 2021 and licensed under CC-BY 4.0.

These nanoparticles have the primary purpose of encapsulating the mRNA, protecting it from enzymatic degradation and assisting its penetration into the cells of the host organism, after intramuscular injection (Nance & Meier, 2021).

The messenger RNA (mRNA BNT162b2) of the medicinal product Comirnaty by Pfizer/BioNTech, which is expected to encode the viral Spike protein inside the host cell, is encapsulated in lipid nanoparticles formed by the two functional lipids ALC-0315 ((4-hydroxybutyl (azanediyl) bis (hexane-6,1-diyl) bis (2-hexyldecanoate)) and ALC-0159 (2 ([polyethylene glycol]-2000)-N,N-ditetradecylacetamide), and the two structural lipids DSPC (1,2-Distearoyl-sn-glycero-3-phosphocholine) and cholesterol.

In this narrative review, the purpose is to provide a detailed and documented account of the currently existing scientific evidences proving the toxicity and hazardousness of cationic lipid nanomaterials contained in mRNA vaccines, with particular attention to Comirnaty by Pfizer/BioNTech, and the serious proven contradictions, omissions and non-compliances by both the manufacturers and the regulatory bodies responsible for the scientific evaluation, supervision and safety monitoring of medicinal products.

REGULATORY NON-COMPLIANCES AND ABSENCE OF TOXICOLOGICAL STUDIES

ALC-0315 and ALC-0159 are classified by EMA as *novel excipients*, *never previously used in a medicinal product in Europe* and *not registered in the EU Pharmacopoeia* (EMA/707383, p. 23).

Of the two, the most important functional lipid is ALC-0315, instrumental to the formation of spheroidal lipid nanoparticles. ALC-0315 is an ionizable cationic amino-lipid consisting of a tertiary amine with a hydroxy-butyl and two exilic groups esterified with 2-hexyldecanoic acid (Segalla, 2023).

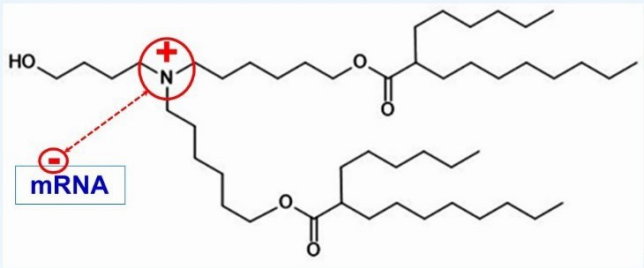


Figure 2. Molecular structure of cationic lipid ALC-0315.

Thanks to its particular tertiary aminic structure, ALC-0315 tends to be protonated in a neutral or moderately low pH environment, thus giving rise to the formation of *cationic nanoparticles*, i.e. having a predominant positive surface charge. This positive charge is critical as it is what allows the formation of nano-complexes with negatively charged genetic materials such as mRNA (Figure 2).

Experimental data, however, have shown that cytotoxic and genotoxic effects are enhanced if nanoparticles have a positive charge (Kanasty et al., 2012; Fröhlich, 2012; Barone et al., 2017). As admitted even by BioNTech (co-owner, together with Pfizer, of the Comirnaty vaccine) in its patent *RNA Formulation for Immunotherapy* dated November 26, 2019, the *elevated toxicity* attributed to *positively charged liposomes and lipoplexes* makes them problematic and unsuitable for use in pharmaceuticals. The reference is to formulations of RNA encapsulated in cationic lipid nanoparticles — i.e. very similar to those used in Comirnaty — and called, in this context, "lipoplexes":

> Unfortunately, for positively charged liposomes and lipoplexes elevated toxicity has been reported, which can be a problem for the application of such preparations as pharmaceutical products (patent US 10,485,884 B2)

Nevertheless, EMA, in its Assessment report dated 19 February 2021, surprisingly asserts:

> No genotoxicity nor carcinogenicity studies have been provided. The components of the vaccine formulation are lipids and RNA that are not expected to have genotoxic potential. (EMA/707383, 2021, p. 55)
>
> As per guidance, no genotoxicity nor carcinogenicity studies were performed. The components of the vaccine (lipids and mRNA) are not expected to have genotoxic potential. This is acceptable to the CHMP. [1] (EMA/707383, 2021, p. 56).

REACTIVE OXYGEN SPECIES (ROS) FORMATION AND LIPID NANOPARTICLE TOXICITY

In stark contrast to what EMA asserts, nanoparticles consisting of monovalent cationic lipids have been shown to be significantly efficient in inducing cell death through the production of *reactive oxygen species* (ROS) (Yun et al., 2016). There is overwhelming evidence that overproduction of ROS is the main cause of nanoparticle biotoxicity. By concentrating mainly in lysosomes, mitochondria, and the nucleus of the cell, and generating ROS at those sites, positively charged nanoparticles can cause devastating consequences. Numerous studies irrefutably confirm that nucleotides components

[1] CHMP: European Committee for Medicinal Products for Human Use.

of cellular DNA and RNA constitute a significantly vulnerable target to the aggression of ROS generated by nanomaterials (Imlay et al., 1988; Maki et al., 1992; Demple et al., 1994). The major challenges for the excessive use of cationic nanomaterials are their dose-dependent toxicity, hepatotoxicity, and pulmonary inflammation by promoting the release of reactive oxygen species and increasing intracellular calcium levels (Ozpolat et al., 2014; Lee et al., 2013; Zhang et al., 2014). Moreover, cationic liposomes can interact with negatively charged cellular constituents, such as opsonins and serum proteins, resulting in hemolysis, i.e. the rupture or destruction of red blood cells (Buyens et al., 2012). In addition, LNPs can induce activation of the immune system resulting in complement activation-related pseudoallergy (CARPA), an acute immunological response that can lead to anaphylactic-like shock (Szebeni et al., 2014).

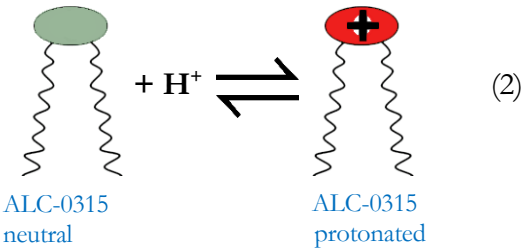
INTRINSIC pKa OF THE AMINO-LIPID ALC-0315

The amount of an ionizable compound being protonated (i.e. positively charged) in an aqueous solution, at a certain pH, is defined by the value of its *acid dissociation constant* (pKa)[2].

The pKa value of a ionizable compound defines the pH at which its functional ionizable groups are 50% in ionized and 50% in a de-ionized form. Since ALC-0315 contains a protonatable tertiary amine head group with an *intrinsic* pKa of 9.6 (Zhang et al., 2022), this implies that, at a pH value of 9.6, there would be 50% of the molecules in the protonated form (R_3NH^+) and 50% in the neutral form (R_3N), according to the equation:

$$R_3N + H^+ \rightleftharpoons R_3NH^+ \quad (1)$$

which, for ALC-0315, can be simplified and schematized as follows:



(2)

The neutral, non-protonated form of ALC-0315 (represented in green) will express the least toxicity, while its fully protonated form (represented in red) will express the maximum toxicity due to the disrupting interactions of its cationic charges with anionic parts of endosomal, lysosomal and mitochondrial membranes (Figure 3).

[2] Acid dissociation constant (pKa): the pH at which molecules are half dissociated. pKa is of utmost importance for understanding drug absorption and biodistribution in the systemic circulation. pKa measurements enable the proportion of the molecules in the ionized (charged) or deionized state to be determined (P. Patel et al., 2021).

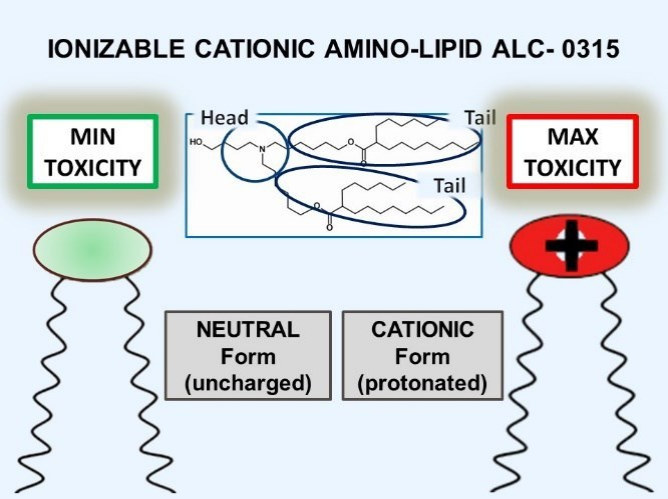


Figure 3. Representation of charged and uncharged forms of ALC-0315.

The value of the pKa of an ionizable compound is of utmost importance for understanding its absorption and biodistribution, and, as we will see shortly, also for consistently estimating its cytotoxicity. Measurements of the pKa make it possible to calculate the exact proportion of ionized (positively charged) and deionized (neutral) molecules of ALC-0315, at a certain pH.

The relationship between the ratio of the protonated form to the non-protonated form and the pKa of the ionizable molecule is regulated by the *Henderson-Hasselbach equation*, which, in the case of a tertiary amine, can be written as follows:

$$\text{Log } [R_3N]/[R_3NH^+] = \text{pH - pKa} \qquad (3)$$

or

$$[R_3N]/[R_3NH^+] = 10^{(\text{pH} - \text{pKa})} \qquad (4)$$

where R_3N is the deprotonated form (neutral) and R_3NH^+ is the protonated form (cationic) of ALC-0315.

The Henderson-Hasselbach equation clearly shows that there is a strict correlation between the pH of the aqueous medium, the pKa of the ionizable substance and the relative concentrations of its cationic and neutral forms. This provides as well a good assessment of its theoretical cytotoxicity (according to the principle that, within the same number of moles, the more protonated a species is, the greater the resulting cytotoxicity). The resulting pKa determines also the ionization behavior and surface charge of the ionized nanoparticles, which substantially influence their stability, potency [3], and toxicity (Alabi et al., 2013).

At pH values below pKa, the predominant chemical species is the protonated form (cationic, more cytotoxic) of the amino-lipid, while at pH values above pKa, the predominant chemical species is the basic de-protonated (neutral, less cytotoxic) form of the amino-lipid.

[3] Potency is an expression of the activity of a drug, in terms of the concentration or amount needed to produce a defined effect (Neubig et al., 2003)

Applying equation (4) above, at a physiological pH of 7.4, we will have:

$$[R_3N]/[R_3NH^+] = 10^{(7.4-9.6)} = 0.006309573$$

which simply indicates that, in a physiological environment, 99.37% of the molecules of ALC-0315 are protonated, thus expressing their maximum cytotoxicity.

APPARENT pKa OF LIPID NANOPARTICLES

To reduce cytotoxicity of cationic liposomes and nanomaterials in general, a relatively effective solution has been the modulation of their acid dissociation constant (pKa), whereby values of 7 or lower were demonstrated to be of importance in RNA encapsulation and in vivo activity. When the amino-lipid is inserted, together with other structural lipids, within the structure of a lipid nanoparticle, its pKa can undergo a lowering of 2-3 units, due to the forces of interaction with anionic species or with polar portions of the other contiguous lipids.

The new lower value achieved for the particle is called *apparent* pKa (or *surface* pKa), to distinguish it from the original *intrinsic* pKa, i.e. the pKa value of the amino-lipid measured *before* the formation of the lipid nanoparticle.[4]

The apparent pKa of a lipo-nanoparticle is then defined as the pH at which 50% of the ionizable lipids, associated to that LNP, is protonated.

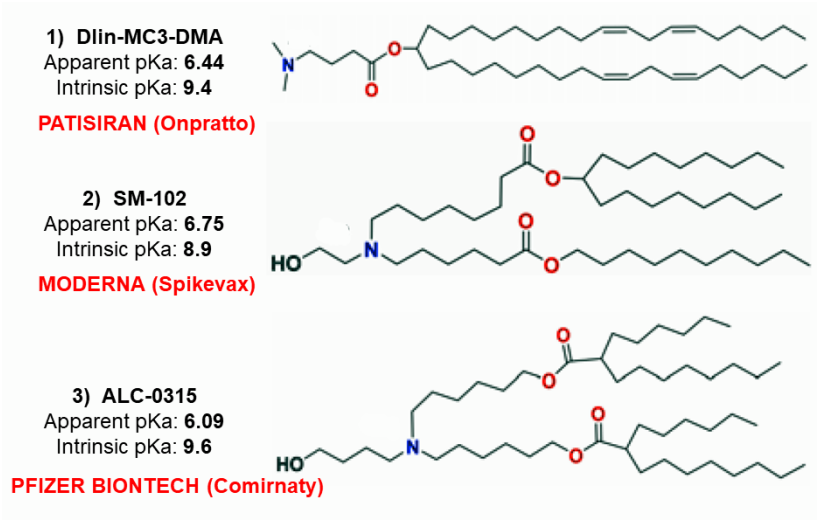


Figure 4. Structure and pKa values of amino-lipids: 1) DLin-MC3-DMA (*Onpratto/ Patisiran* by Alnylam): apparent pKa 6.44, intrinsic pKa 9.4 2), SM-102 (*Spikevax* by Moderna): apparent pKa 6.75, intrinsic pKa 8.9 3) Alc-0315 (*Comirnaty* by Pfizer BioNTech): apparent pKa 6.09, intrinsic pKa 9.6.

For example, the lipid nanoparticle used to encapsulate Onpattro (a drug for the treatment of hereditary amyloidosis and administered via intravenous infusion) contains the FDA-approved amino-lipid DLin-MC3-DMA (intrinsic pKa 9.4), whose LNP apparent pKa gets thus lowered to 6.44. The apparent pKa of LNPs formed by amino-lipid SM-102 and used to encapsulate the Moderna vaccine Spikevax, from the intrinsic value 8.9, gets down to 6.75. The apparent pKa of ALC-0315-based LNPs is reduced from 9.6 to 6.09 (P. Patel et al., 2021; Zhang et al., 2022; and see Figure 4).

[4] The apparent pKa of LNPs is dependent not only on the pKa of individual lipid but also on the molar ratio of all the lipids. Each lipid has a distinct pKa which can be changed by modifying its headgroup and the hydrophobic tail. Therefore, one strategy to adjust the apparent pKa of LNPs is to chemically modify the lipid. Another strategy is to use a mixture of two or more lipids with different pKa values and adjust their ratio to achieve the desirable apparent pKa (P. Patel et al., 2021).

The apparent pKa of nanoparticles can be measured by different techniques. TNS[5] fluorescence titration is considered the most accurate method: amino lipid pKa values are determined for each LNP by measuring the fluorescence of TNS during titration at different pH values (Jayaraman et al., 2012; P. Patel et al., 2021). An apparent pKa between 6 and 7 has been generally estimated as the optimum range for the development of efficient nanoparticles for RNA delivery. Nanoparticles with lower pKa values have insufficient ionic charges and polarity at neutral pH, thereby leading to aggregation of the nanoparticles and consequent instability of the whole composition. On the other hand, nanoparticles with a higher pKa exibit more positive charges at physiological pH, which results in higher stability but, unfortunately, also in higher toxicity (Figure 5).

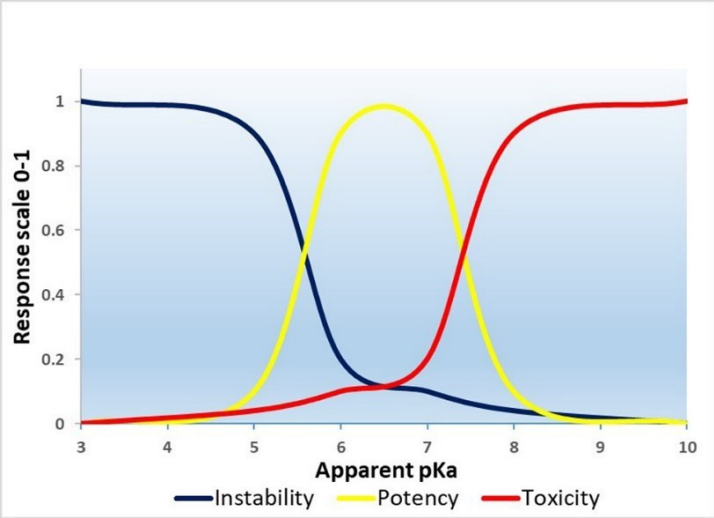


Figure 5. The Effects of pKa on the Instability, Potency, and Toxicity of Nanoparticles.
Reprinted from Trends in Pharmacological Sciences, Vol. 42(6), 448–460, Patel et al., 2021, *The Importance of Apparent pKa in the Development of Nanoparticles Encapsulating siRNA and mRNA*, Page No. 458, Copyright © 2021, with permission from Elsevier Ltd.

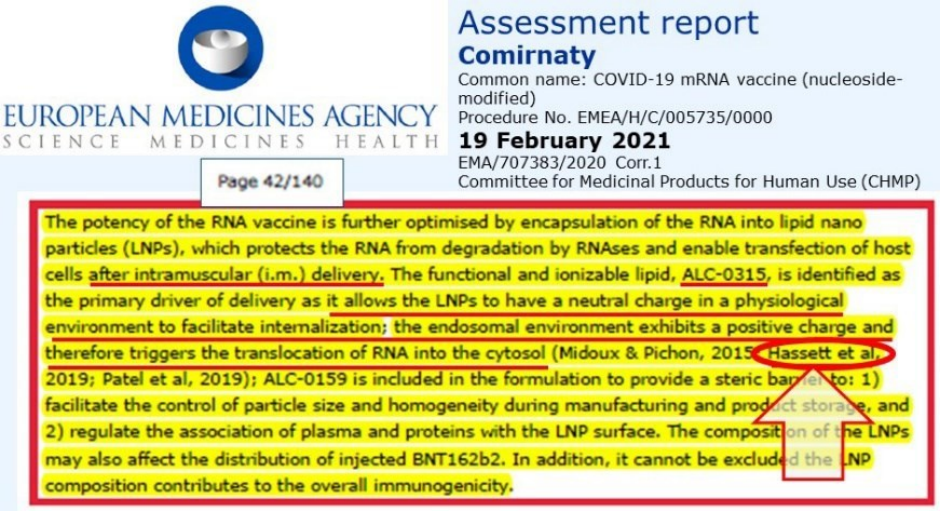
When the pH of the preparation medium is below the apparent pKa of the lipid nanoparticles, the amino groups are protonated and bear a positive charge that interacts with negatively charged RNA to form stable cationic nanoparticles.

OPTIMAL AND NON-OPTIMAL pKa RANGE OF LNPs FOR INTRAMUSCOLAR ADMINISTRATION OF mRNA VACCINES

According to the EMA Assessment Report, dated 19 February 2021 (EMA, 2021, page 42), in the physiological environment, where the pH is 7.4, these lipid nanoparticles, thanks to the contribution of the primary driver ALC-0315, have a neutral charge:

> The potency of the RNA vaccine is further optimized by encapsulation of the RNA into lipid nanoparticles (LNPs), which protects the RNA from degradation by RNAses and enable transfection of host cells after intramuscular (i.m.) delivery. The functional and ionizable lipid, ALC-0315, is identified as the primary driver of delivery as it allows the LNPs to have a neutral charge in a physiological environment to facilitate internalization; the endosomal environment exhibits a positive charge and therefore triggers the translocation of RNA into the cytosol (Midoux & Pichon, 2015; Hassett et al, 2019; S. Patel et al, 2019; and see Figure 6).

[5] TNS: 2-(p-toluidino)-6-napthalene sulfonic acid.


EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

Assessment report
Comirnaty
Common name: COVID-19 mRNA vaccine (nucleoside-modified)
Procedure No. EMEA/H/C/005735/0000
19 February 2021
EMA/707383/2020 Corr.1
Committee for Medicinal Products for Human Use (CHMP)

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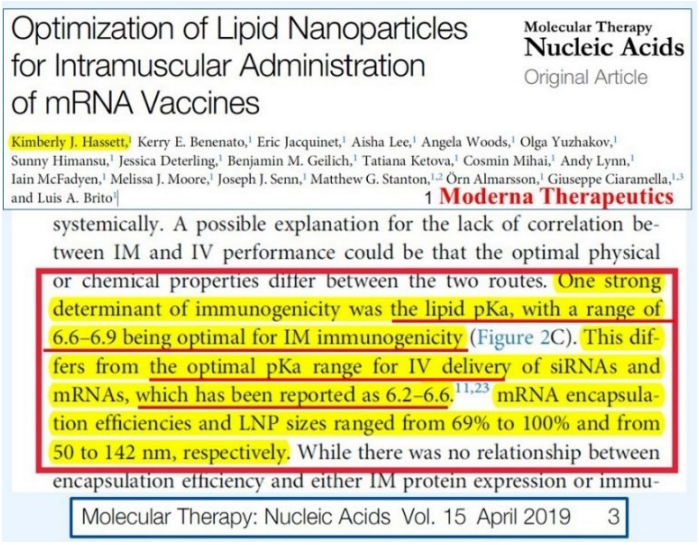
The potency of the RNA vaccine is further optimised by encapsulation of the RNA into lipid nano particles (LNPs), which protects the RNA from degradation by RNAses and enable transfection of host cells after intramuscular (i.m.) delivery. The functional and ionizable lipid, ALC-0315, is identified as the primary driver of delivery as it allows the LNPs to have a neutral charge in a physiological environment to facilitate internalization; the endosomal environment exhibits a positive charge and therefore triggers the translocation of RNA into the cytosol (Midoux & Pichon, 2015; Hassett et al, 2019; Patel et al, 2019); ALC-0159 is included in the formulation to provide a steric barrier to: 1) facilitate the control of particle size and homogeneity during manufacturing and product storage, and 2) regulate the association of plasma and proteins with the LNP surface. The composition of the LNPs may also affect the distribution of injected BNT162b2. In addition, it cannot be excluded the LNP composition contributes to the overall immunogenicity.

Figure 6. EMA Assessment Report on Comirnaty by Pfizer/ BioNTech, dated 19 February 2021, page 42.

From such statement, it is clear that EMA, by even calling it *the primary driver,* has undoubtedly understood that ALC-0315 is an instrumental and determinant factor in facilitating and *optimizing* the internalization of LNPs into the endosomal environment and the consequent translocation of mRNA into the cytosol, *after intramuscular (i.m.) delivery*.

However, it seems that the author of the EMA assessment report has omitted to assess scrupulously, if not having willingly ignored, what is reported in one of the scientific references cited in support of such a claim, namely, the 2019 article by Hassett et al., with the eloquent title: *Optimization of Lipid Nanoparticles for Intramuscular Administration of mRNA Vaccines*.

Unexpectedly, all 19 authors of this paper are either current or previous employees of Moderna Therapeutics and own stock options and/or shares in the company, the main Pfizer's competitor for what regards mRNA COVID-19 vaccines.


Optimization of Lipid Nanoparticles for Intramuscular Administration of mRNA Vaccines

Molecular Therapy
Nucleic Acids
Original Article

Kimberly J. Hassett,[1] Kerry E. Benenato,[1] Eric Jacquinet,[1] Aisha Lee,[1] Angela Woods,[1] Olga Yuzhakov,[1] Sunny Himansu,[1] Jessica Deterling,[1] Benjamin M. Geilich,[1] Tatiana Ketova,[1] Cosmin Mihai,[1] Andy Lynn,[1] Iain McFadyen,[1] Melissa J. Moore,[1] Joseph J. Senn,[1] Matthew G. Stanton,[1,2] Örn Almarsson,[1] Giuseppe Ciaramella,[1,3] and Luis A. Brito[1]

1 **Moderna Therapeutics**

systemically. A possible explanation for the lack of correlation between IM and IV performance could be that the optimal physical or chemical properties differ between the two routes. One strong determinant of immunogenicity was the lipid pKa, with a range of 6.6–6.9 being optimal for IM immunogenicity (Figure 2C). This differs from the optimal pKa range for IV delivery of siRNAs and mRNAs, which has been reported as 6.2–6.6.[11,23] mRNA encapsulation efficiencies and LNP sizes ranged from 69% to 100% and from 50 to 142 nm, respectively. While there was no relationship between encapsulation efficiency and either IM protein expression or immu-

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Figure 7. Extract of scientific article by Moderna Therapeutics (2021) on optimization of LNPs for intramuscular and intravenous administration (Hassett et al, 2019).

But what is even more unexpected is found on page 3 of this paper, with regard to the *optimal* value of pKa (rightly defined as a *strong determinant*) that lipids for intramuscular administration should have:

> […] One strong determinant of immunogenicity was the lipid pKa, with a range of **6.6–6.9** being optimal for IM [intramuscular] immunogenicity […]. This differs from the optimal pKa range for IV [intravenous] delivery of siRNAs and mRNAs, which has been reported as **6.2–6.6**. (Figure 7).

The scientific article by Hassett et al., cited by EMA in support of the claimed ideal role played by ALC-0315 in the intramuscular administration of the Comirnaty vaccine, clearly and thoroughly explains that the optimal lipid pKa range for *intramuscular* applications should be between 6.6 and 6.9. Such range is considered optimal, as LNPs with those values of pKa produce an efficient immune response after intramuscular administration of mRNAs (P. Patel et al., 2021). It is therefore clear that ALC-0315 is NOT suitable for intramuscular delivery, since its apparent pKa (6.09) is much lower than the optimum (Figure 8), making the RNA vaccine composition too unstable and ineffective for intramuscular application. According to Hassett et al., it would not be suitable either for intravenous delivery, this later one requiring apparent pKa values between 6.2 and 6.6. The optimal range indicated by Hassett et al. is in sharp contradiction with the technical information that EMA highlights in its official assessment report on the medicinal product *Comirnaty,* with regard to its *primary driver* ALC-0315.

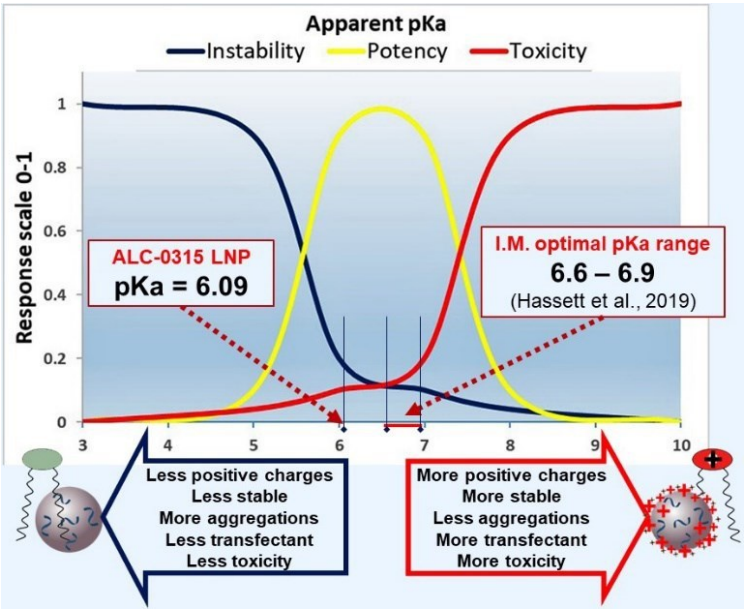

Figure 8. Lipid pKa of ALC-0315 with regards to instability, potency and toxicity. Modified from Trends in Pharmacological Sciences, Vol. 42(6), 448–460, Patel et al., 2021, *The Importance of Apparent pKa in the Development of Nanoparticles Encapsulating siRNA and mRNA*, page No. 458, copyright © 2021, with permission from Elsevier Ltd.

In the light of what has been so far exposed and what will be hereafter presented, asserting that Comirnaty LNPs enable transfection of host cells *after intramuscular (i.m.) delivery* and that ALC-0315 *allows the LNPs to have a neutral charge in a physiological environment to facilitate internalization,* is scientifically unacceptable and potentially misleading, as it promotes the idea that the composition of the LNP-based vaccine Comirnaty has been somehow "optimized" for *intramuscular inoculation*. Such statement is disavowed by the very reference which it is based on.

In the final analysis, it is evident (Figure 8), that the apparent pKa value of ALC-0315 is too low to be defined *optimal*, and that a so low value makes the entire structure of the LNP unstable, inducing the formation of aggregates and particulates, which may inhibit the transfection and inevitably influence, not only the efficacy of the product, but also both the biodistribution and the bioaccumulation of lipid nanoparticles in unexpected tissues and organs. Bioaccumulation can lead to blockage of small blood and lymphatic vessels, while an abnormal biodistribution means that cell

death and inflammation caused by the COVID-19 mRNA vaccine could occur in organs not foreseen by its biological destiny, such as the brain, placenta, and testes (Parry, P.I., et al., 2023; Zhou, Y., et al., 2018; Wick, P., et al., 2010).

Other significant evidences relating to the unsuitability of the medicinal product *Comirnaty* for intramuscular application, as well as its instability and inefficacy due to the formation of LNP aggregations and agglomerations caused by the addition of destabilizing ionic compounds (PBS pH buffer), have already been described in detail (Segalla, 2023).

PREDOMINANCE OF THE CYTOTOXIC PROTONATED LIPID FORM DURING THE ENDOCYTOTIC PROCESSES

In the acidic environment of endosomes, the amino groups are protonated and their positive charge promotes interaction with anionic endosome lipids, inducing destabilization of the endosome membrane and promoting the release of mRNA into the cytosol (Wan et al., 2014; Draz et al., 2014; Tam et al., 2013), as also briefly mentioned in the aforementioned EMA report:

> […] In general, following endocytosis of LNPs, the mRNA is released from the endosome into the host cell cytosol (Sahay et al, 2010; Maruggi et al, 2019).

The RNA-loaded nanoparticle, once penetrated by endocytosis into the cell, undergoes various passages through early endosomes, late endosomes and lysosomes, along a decreasing pH gradient (Figure 9), until its cationic charge causes the rupture of the endosomal membrane and the consequent release of RNA inside the cytosol (P. Patel et al., 2021).

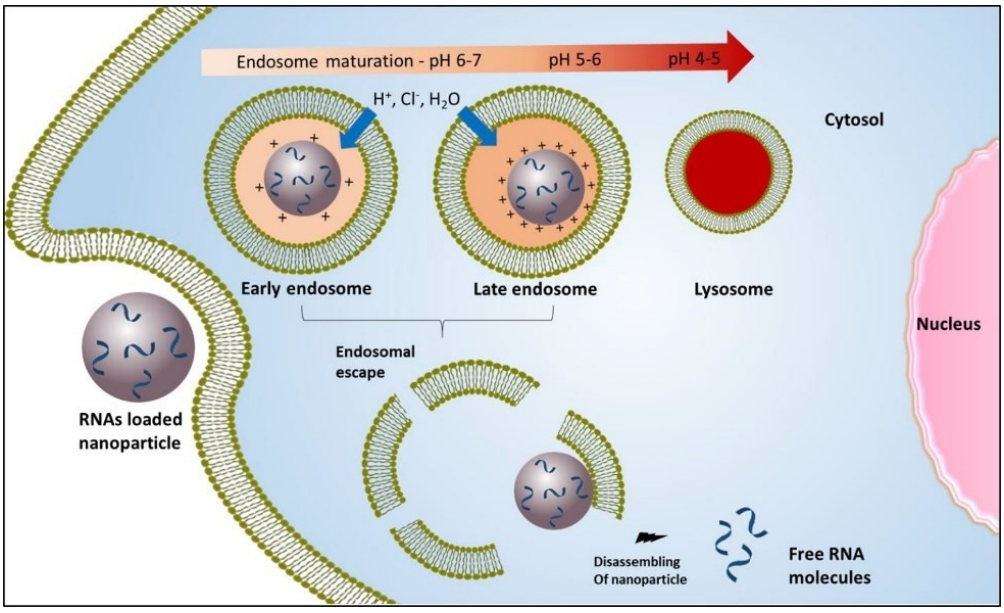

Figure 9. Delivery of RNAs into the cytoplasm through endosomal escape with ionizable nanoparticles. Once nanoparticles are taken up by the cells, the charges on the nanoparticle increase as the pH decreases below the pKa during endosomal maturation (pH 7–5.5). The charges on nanoparticles decrease in the cytosol and weaken the binding interaction with RNAs. Finally, the nanoparticles dissociate to release the RNAs and produce the desired activity. Reprinted from *Trends in Pharmacological Sciences, Vol. 42*(6), 448–460, Patel et al., 2021, *The Importance of Apparent pKa in the Development of Nanoparticles Encapsulating siRNA and mRNA*, Page No. 451, Copyright © 2021, with permission from Elsevier Ltd.

The pH thus decreases along the endosomal-lysosomal system: early endosomes have a pH between 6 and 7, late endosomes have a pH in the range of 5 and 6, and in lysosomes the pH can decrease to 4.5 (Hu et al., 2015).

Applying equation (4) to Comirnaty LNP composition, we can calculate the ratio $[R_3N]/[R_3NH^+]$ and, consequently, the percentage of the protonated species (i.e. the most cytotoxic), at various pKa and pH conditions, as follows:

- Before endocytosis: physiological pH 7.4, ALC-0315/LNP apparent pKa 6.09
- In early endosomes: pH 6.5, ALC-0315/LNP apparent pKa 6.09.
- In late endosomes: pH 5.5, ALC-0315/LNP apparent pKa 6.09.
- In lysosomes: pH 4.5, ALC-0315/LNP apparent pKa 6.09.
- In cytosol, after endosomal escape and LNP disassembling: pH 7.4, ALC-0315 intrinsic pKa 9.6.

The results are shown in Table 1 and Figure 10.

Table 1.

Henderson-Hasselbach equation applied to endocytosis of COMIRNATY - Pfizer/ BioNTech

PHASES OF ENDOCYTIC PATHWAY	pH	pKa	Ratio $[R_3N]$ / $[R_3NH^+]$	% Protonated form $[R_3NH^+]$	% Deprotonated form $[R_3N]$
ALC-0315/ LNP before endocytosis	7.4	6.09 (apparent)	20.42	4.67%	95.33%
ALC-0315/ LNP in early endosomes	6.5	6.09 (apparent)	2.57	28.01%	71.99%
ALC-0315/ LNP in late endosomes	5.5	6.09 (apparent)	0.26	79.55%	20.45%
ALC-0315/ LNP in lysosomes	4.5	6.09 (apparent)	0.03	97.49%	2.51%
ALC-0315 in cytosol after LNP disassembling	7.4	9.6 (intrinsic)	0.01	99.37%	0.63%

Another issue that cannot be overlooked is undoubtedly the enormous difference between the apparent pKa and intrinsic pKa values of the lipid ionizable species, particularly for the Pfizer preparation. Considering that these values are logarithmic values, a difference of 3.51 between the intrinsic pKa and the apparent pKa of ALC-0315 means that its *intrinsic* tendency to protonation (i.e. its *base strength*) is 3,236 times higher than the one expressed by its *apparent* LNP pKa. Similarly for SM-102, a difference between the two pKa values of 2.15 means an intrinsic protonating tendency 141 times higher than the apparent one. This elementary concept is the basis of the surprising and sudden leap in predominance of the protonated species (and therefore of its cytotoxicity) during the endocytotic pathway, as evidenced by the graph of Figure 10.

It should also be noted that the intrinsic pKa value (9.6) of ALC-0315 represents the absolute highest ever pKa value for a functional ionizable lipid used in cationic LNPs for immunotherapy. Being its pKa even higher than that of the ammonium ion (9.25), ALC-0315 expresses a base strenght about 2 times higher than that of ammonia itself, that is, it possesses ionizing powers twofold stronger than those of an equimolar aqueous solution of ammonia.

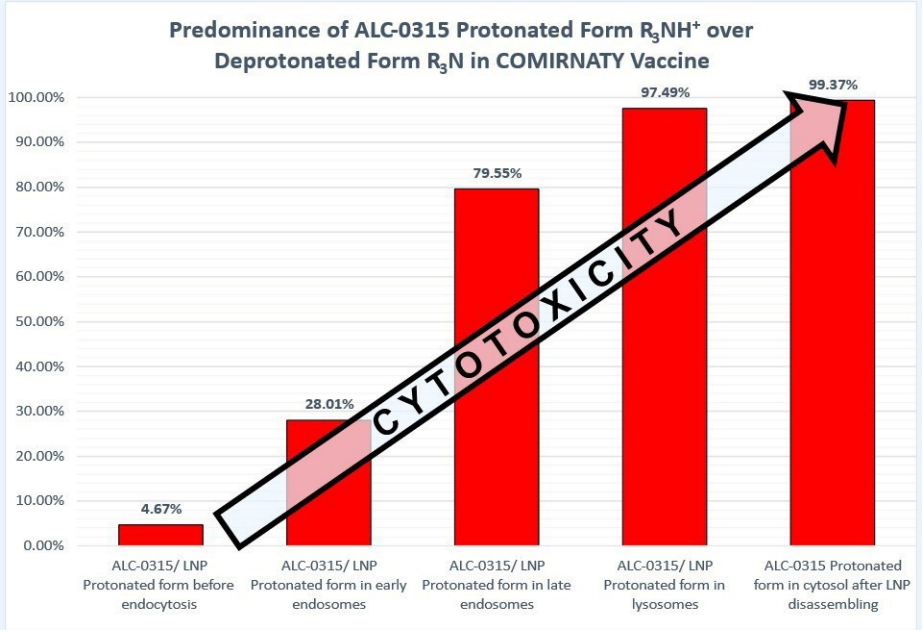

Figure 10. The main fundamental issue that emerges from these calculations is that, before the endocytotic process, the presence of the protonated form R_3NH^+ is relatively low and therefore less cytotoxic (4.67%), but then, as soon as the lipid nanoparticles pass through the phases of lysosomal digestion, disassembling and endosomal escape into the cytosol, the predominance of the cytotoxic protonated forms jumps up to nearly its maximum value (99.37%). The equilibrium of equation (1) shifts practically all to the right, that is, towards the production of the cationic and more cytotoxic species R_3NH^+.

Therefore, the molecules of ALC-0315, once penetrated and released into the cytosol, after the disassembly of the LNP envelope, reach maximum predominance of their cationic form, and thus express the maximum of their cytotoxicity, stimulating the secretion of pro-inflammatory cytokines and reactive oxygen species (Hou et al., 2021). These ROS, in turn, may have devastating toxicological consequences including genotoxicity (Yun et al., 2016; Yu et al., 2020), leading to very serious problems, in the medium and long term, particularly for parenteral applications, as previously known also to the manufacturer of the medicinal product Comirnaty (BioNTech patent US 10,485,884 B2, 2019). Furthermore, the exceptional penetrability, mobility, chemical reactivity and systemic accumulation of uncontrollable cationic nanoparticles, with high cytotoxicity levels, in unpredictable biological locations, even far from the site of inoculation, have predictably resulted in an unprecedented medical disaster. It should be noted that, with any agent that causes genetic damage, including cytotoxic anticancer drugs, there is a risk of cancer (including leukemia), and moreover there is a lifetime limit on the overall dose that can be tolerated. Thus, the prospect of frequently repeated COVID "booster shots," and also that of extending mRNA technology to vaccines against other pathogens or non-infectious diseases, conjures up a very grave public health risk (Palmer et al., 2022).

CONCLUSIONS AND OUTLOOK

The body of work presented in this review includes several physico-chemical, biochemical and toxicological evidences which clearly show and irrefutably demonstrate how the medicinal product named *Comirnaty COVID-19 mRNA BNT162b2* vaccine, is not only unsuitable for intramuscular inoculation, but also endowed with a high degree of toxicity whose devastating consequences can

manifest themselves both in the short term and in the medium and long term, due to the *shedding* effect of LNPs biodistribution and bioaccumulation.

The main reasons for such inadequacy and criticality are represented by the following factors:

- The apparent pKa value (6.09) of the ionizable lipid ALC-0315 is far from having been optimized for intramuscular delivery. Its value is actually too low to enable a proper transfection of host cells, despite what EMA incautiously states on page 42 of its Assessment report dated 19 February 2021, in flagrant contradiction with the same bibliographic source therein cited (Hassett et al, 2019).

- The intrinsic pKa value (9.6) of the ionizable lipid ALC-0315 is too high, which makes it a stronger base than ammonia itself (pKa 9.25) in aqueous solution, and makes it thus completely protonated once released into the cytosol of the host cell, at physiological pH.

- Such elevated cationic charge, acquired by ALC-0315 after its endosomal escape, stimulates the formation of pro-inflammatory cytokines and reactive oxygen species, that can disrupt the mitochondrial membrane and release its content, cause RNA mistranslation, polymerization of proteins and DNA, DNA mutations, destruction of the nuclear membrane and consequent release of its content (Yu et al., 2020).

- This kind of "troyan horse" mechanism determines the *apparently* low toxicity of the LNP before internalization, facilitating its penetration into the host cell. However, once penetrated and released into the cytosol, the LNP gets disassembled and its cationic lipid fragments are free to re-express their intrinsically elevated pKa and therefore their maximum intrinsic toxicity.

- An elevated chemical toxicity of the ionizable cationic LNPs, maximized in the Comirnaty vaccine by the extreme ionizing power of ALC-0315, nevertheless must be expected also in other and future vaccines that use the same delivery technology, namely the mRNA cationic LNP-based platform, regardless of whether they be directed against the spike protein, another SARS-CoV-2 antigen, or a different antigen or disease altogether.

- There is overwhelming scientific evidence that the lipid-nanoparticles used in the COVID-19 vaccines have been found to induce significant inflammatory cytokine secretion and macrophage inflammatory proteins with cell death. This pro-inflammatory effect of the cationic lipid-nanoparticles would increase the vaccine adjuvant immunogenicity of the COVID-19 mRNA vaccines and add to the adverse events caused by the toxicity of the spike protein itself (Ndeupen et al., 2021; Parry et al., 2023).

A Final Word

It is to be considered a matter of priority that thorough and long-term studies should be carried out in the appropriate institutional, clinical and forensic locations, especially in relation to any causal or con-causal links between what is presented here and the wide pathological heterogeneity of serious or lethal adverse events that have occurred, and are still occurring, after vaccinations, in order to adopt and expedite all appropriate corrective and preventive actions to protect public health, including discontinuing vaccinations with the COVID-19 mRNA-LNP-based vaccines in accordance with the precautionary principle, and in the light of the following:

Article 10 of the Nuremberg Code

> *During the course of the experiment the scientist in charge must be prepared to terminate the experiment at any stage, if he has probable cause to believe, in the exercise of the good faith, superior skill and careful judgment required of him, that a continuation of the experiment is likely to result in injury, disability, or death to the experimental subject.*

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