Commentary

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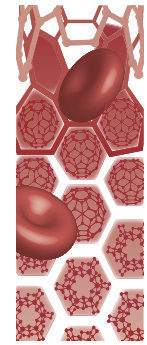

2021: an immunotherapy odyssey and the rise of nucleic acid nanotechnology

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"Started in late 2019, an unprecedented global pandemic infection by the SARS-CoV-2 virus created biomedical pressure that very quickly resulted in the development of vaccines based on mRNA (Pfizer/BioNTech and Moderna)."

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Therapeutic nucleic acids

Nucleic acids are core biochemical constituents characteristic to all known forms of life. Both types of nucleic acids, DNA and RNA, can pass genetic information to subsequent generations or transfer genes horizontally between different organisms. DNA and RNA regulate translation of the genetic blueprint and all physiologically relevant cellular processes through various means of gene expression modulation or direct interactions with proteins. Despite their crucial biological roles, the importance of both nucleic acid polymers has not yet translated to serving as a major specific target or therapeutic by themselves. However, the onset of nucleic acid therapeutics is on the horizon, symbolized by recently approved medications including the small interfering RNAs (siRNAs) Onpattro® and Givlaari®, as well as antisense oligonucleotides, aptamers and several other therapeutic nucleic acids (TNAs) that are currently undergoing various phases of clinical trials [1,2].

Started in late 2019, an unprecedented global pandemic infection by the SARS-CoV-2 virus created biomedical pressure that very quickly resulted in the development of vaccines based on mRNA (Pfizer/BioNTech and Moderna). The functional principle of mRNA vaccine is very elegant and straightforward. The mRNA coding for a specific antigen, in this case the SARS-CoV-2 spike (S) protein, encompassed by a lipid vesicle, is released into the cytoplasm where it is further recognized and translated by the host's cellular machinery. The viral S glycoprotein is then either displayed on the membrane of transfected cells or dispersed from the dead cells. Following the successful expression of S protein, its presence recruits the adaptive immune system to produce antibodies which bind S glycoprotein and neutralize the virus [3–5]. A similar approach is also applied in mRNA-based vaccines designed against other infectious diseases or used for cancer immunotherapy and protein replacements, just to name a few [6,7].

While the idea of mRNA therapeutics is not novel and dates back several decades, it had to overcome several major drawbacks that hindered clinical applications of mRNAs or any other TNAs as an active compound in medicine. However, now, the main obstacles, such as chemical instability, uncontrolled immunostimulation, poor translation and inefficient intracellular delivery are being increasingly solved by the incorporation of modified nucleotides, purification steps, rational design and development of advanced lipid formulations, respectively [8]. The programmability of nucleic acid sequences allows for an immediate response for the synthesis of mRNA of the most current (mutated) viral antigens.

Current mRNA vaccines only deliver information for the translation of antigens, but the 5′ and 3′ untranslated regions of mRNA can potentially encode regulatory sequences to enhance the therapeutic outcome of a given vaccine. In this synergistic approach, one could join 5′ and/or 3′ ends of coding mRNA sequences with sequences adopting functional secondary structures that would, for example, decrease the rate of degradation, bind to specific

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proteins or regulate translation. Depending on the therapeutic strategy of mRNA vaccines, one could employ and combine several groups of functional or structural motifs (e.g., virus derived motifs that may modulate cell host defense system). On the other hand, implementation of protein specific aptamers, could exert agonistic or antagonizing function to the specific targets [9]. Also, introduction of RNA switches selected to respond to the presence of specific proteins or small molecules, could conditionally regulate the translation of downstream mRNA sequences [10]. Together, the combination of these structural elements fused into one strand offers the engineering of next generation vaccines to combat infections, cancer and other diseases. Therefore, the modular nature of nucleic acids offers additive potential for combination therapies.

Therapeutic NANPs

In the last two decades, self-assembly of different TNAs into discrete nanostructures has given rise to a variety of bottom-up designed multifunctional nucleic acid nanoparticles (NANPs) with various architectures and potential in biotechnology and medicine. The functional versatility and combination of multiple noncoding nucleic acids (antisense oligonucleotides, siRNAs, miRNAs, CpG motifs, aptamers, ribozymes, etc.) within individual NANPs has allowed simultaneous regulation of a plethora of physiological processes [11–13]. In addition, the intrinsic physicochemical properties defined by the design of individual NANPs dictate their immunorecognition and allow for synthesis of NANPs with regulated immunostimulatory properties [14,15], thus offering an innovative tool that can expand the immunotherapeutic potential when used in combination with mRNA vaccines or alone.

For decades, the efforts of the nanotechnology drug delivery community were focused on masking nanoparticles from immune recognition [16]. However, it recently became evident that the intrinsic property of the immune system to clear particulate materials that researchers tried to avoid creates limitless opportunities for harnessing this property. These features then can be directed against diseases by modulating the immune response to synergize with the drug's mechanism of action delivered by nanocarriers. Delivery of NANPs using different types of carriers and the generation of immune responses that vary both quantitatively and qualitatively provide a good example of this phenomenon and will be reviewed in more detail below.

Immunorecognition of nucleic acids

The activation of cell defense systems is based on the recognition of self from nonself nucleic acids, which is an essential quality to combat against invading pathogens, mostly of viral origin. The innate immune response is the front line against viral infections. In this battle, the pattern recognition receptors (PRRs) are the first to encounter the invading agent or materials derived from it, that are recognized by so-called pathogen-associated molecular patterns (PAMPs). The foremost PAMPs sensors reside on the cell surface or in endosomal compartments, where Toll-like receptors (TLRs), expressed predominantly in immune cells, are the most prominent. The intracellular surveillance is comprised of receptors, such as RNA sensing RIG-I-like receptors, DNA-sensing cyclic GMP-AMP synthase and IFI16 that are also expressed in nonimmune cells [10]. Triggering of elaborate cellular immune signaling cascades results in the production of cell defense proteins such as chemokines, proinflammatory cytokines and more importantly, interferons (IFNs) that affect self or surrounding cells in an autocrine or paracrine way to activate further expression of so-called interferon-stimulated genes to cease viral replication and further spread. In the best-case scenario, at the end of chain reactions triggered by innate immune cells and recruitment of the adaptive immune system, the antiviral state in the tissues is established. The insight into cellular innate immune pathways has allowed us to determine critical parameters such as chemical composition, sequence, intracellular localization and relative quantities of nucleic acids, as the most relevant to their immunorecognition [17].

Interestingly, almost the same is true for NANPs. While the knowledge of immunostimulatory properties of viral nucleic acids is inspirational for NANP design, their predefined topology and mode of delivery does not allow simple assumptions about NANP immunorecognition, despite the ability of human cells to assess and process NANPs by employing the same defense strategies under viral infection. Accordingly, it is hard to predict the immunostimulatory potential of different NANPs based solely on the knowledge about immunostimulatory characteristics of individual NANP constituents. The assembly of components into NANPs obtains new qualities including overall NANPs immune behavior. Since immunorecognition of NANPs is not just a sum of their building parts, the thorough systematic experimental evaluation of NANPs immune properties is required. From a clinical perspective, this situation is reflected by the US FDA through a separation of TNAs and NANPs into different classes.

Regulation of NANPs immunorecognition

Similar to other types of nanomaterials, the effects of NANPs on cells are determined by their physicochemical properties [14,18]. However, NANPs have several unique attributes not present in other nanomaterials – their macromolecular nature and polyanionic structure that prevent immediate interaction between NANPs and cells. The assembled NANPs can be delivered to target tissues by two main strategies that can have a potent impact on consecutive signaling pathways [9]. Thanks to the receptor-targeting aptamers, NANPs can potentially be transported to specific cells as naked structures while NANPs surrounded by lipid formulations can enter any cells nonspecifically. Upon aptamer mediated delivery, NANPs must be released into the cytoplasm to promote their intended functions. However, despite many experimental observations, there is still a lack of reliable pathways for NANPs delivery to cytoplasm using aptamers [9]. On the other side, aptamers offer great potential in immunotherapy where they could be applied as extracellular stimulators of anticancer immunity by releasing immune checkpoints [9].

Comparative research between the delivery of pure NANPs or NANPs in a complex with carriers suggests that delivery of naked NANPs does not trigger the TLR mediated cellular defense system [14]. For induction of an IFN response, NANPs have to be complexed with a suitable carrier that promotes their uptake by a specific route while keeping the NANPs' structure intact. The subsequent stimulation of the immune system depends on the combinations of various factors. The size, dimensions, composition and connectivity are the main architectural parameters that influence the immunorecognition of NANPs. In experimental models of human peripheral blood mononuclear cells (PBMCs) freshly collected from healthy donors, RNA globular NANPs (cubes) associated with different carriers induced higher responses in cytokine production than their DNA analogs or any other planar or fibrous RNA NANPs [14]. The stimulation of cellular immunity by RNA cubes was also qualitatively different in comparison with DNA cubes. While RNA cubes triggered expression of all members of type I IFNs (α, β and ω), signaling molecules important for antiviral and antitumor immune responses and IFN III (λ), DNA cubes induced only IFN α and ω. As for the rest of the surveyed NANPs, the planar RNA and DNA structures were more immunostimulatory than fibrous NANPs. Interestingly, considering the RNA structures, the size is more important for IFN induction than mass; however, no similar correlation was observed in DNA NANPs.

Our team has demonstrated that PBMCs internalize NANP/lipofectamine complexes through scavenger receptors via the endosomal pathway, with some RNA NANPs mostly recognized by TLR7 [14,19]. PBMCs are a heterogeneous group of blood cells, but it appears that due to their phagocytic function, monocytes and to a lesser extent, lymphocytes are responsible for NANP uptake. Plasmacytoid dendritic cells (pDCs) respond to viral infection by synthesizing large quantities of type I IFNs, exceeding levels of any other immune cells [14]. In addition, pDCs constitute a bridge between innate and adaptive immune systems. As mentioned above, sensing NANPs involves signaling cascades that are characteristic for antiviral defense systems; thus, pDCs are the primary source of IFNs in response to NANPs. Interestingly, in pDCs, all NANPs stimulate strong IFN responses, regardless of their structures and composition. It seems that variability in IFN activation for various NANPs observed in PBMCs results from the intercellular interactions within PBMC subpopulations [14,19].

Carriers in complexes with NANPs are another crucial factor that determines the uptake and subsequent spectrum of cytokines produced by PBMCs. The influence of a carrier's chemical composition on cytokine expression is illustrated by the contrast of different NANP-carrier complexes. For example, lipofectamine 2000 (L2K) is less efficient in NANPs' intracellular delivery when compared with the PAMAM dendrimer G5-NH_2 [20]. Thus, NANP-L2K complexes produce IFN I and III expression while NANP-PAMAM complexes activate so called 'danger signals' (IL-1α)-cytokines associated with stress, trauma and cytokine storm (IL-1β, IL-6 or TNFα). Different methods of cell entry offer an explanation for the distinct affects, such that cationic PAMAM dendrimers perforate cell membranes while the lipid-like L2K is endocytosed [20].

The effect of NANPs' chemical composition on the activation of immune responses was also confirmed in cell models of human microglia in a comparative study with NANPs composed of RNA, DNA and 2′F-modified oligonucleotides with the same sequences, connectivity, shape, size and charge, differing only in chemical composition, displayed distinct behavior in subcellular accumulation and immunogenicity [21]. Variability can be further increased by the application of different carriers; herein, L2K or DOTAP transported various NANPs to cells with diverse efficiencies. This observation is informative for the engineering of NANPs used for delivery to specific subcellular compartments. Interestingly, 2′F modifications of pyrimidines substantially altered the immunoquiescence of DNA NANPs, as they activated NF-κB and IFN regulatory factors, leading to pro-inflammatory IL-6 and

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IFN-β production. This induction was not triggered by TLR recognition but rather by RIG-I detection through employment of RNA polymerase III [21].

Based on our experience, we conclude that manipulation of NANPs' physicochemical properties and their complexation with various types of carriers provides a predictable way to control the quality (classes of cytokines) and quantity (degree of the cytokine release) of the immune response to delivered NANPs. We observed reproducible indicators that RNA NANPs or NANPs with a higher proportion of RNA strands are more immunogenic than their DNA counterparts. Further, globular shapes elicited more potent IFN responses than planar structures, with fibrous particles being the weakest immunogens. The administration of naked NANPs does not lead to immune stimulation. Therefore, delivery of NANPs in complex with chemical carriers is necessary. The formulation of NANPs with carriers can have profound effects on NANP delivery and their subcellular localization. The various PRRs residing in individual cellular compartments differ in recognition of distinct PAMPs. Furthermore, distribution of certain PRRs is cell dependent. Accordingly, the nature of NANPs and their carrier affects particle routing and thus activation of corresponding PRRs. Downstream signaling usually merges to the expression of IFN types I and III but may differ in representation of individual IFN members and other cytokines. Generally, we can distinguish between two main types of the immune response – induction of antiviral and antitumor immunity or activation of danger signaling and induction of inflammasome. The uptake of NANPs and subsequent activation of immune defense systems differs between individual immune cell types and observed semifinal picture is; therefore, the result of intricate crosstalk between various cell subpopulations. Not surprisingly, the outcome of the induction of innate immune system is donor dependent.

Knowledge gaps in NANP technologies

Among the primary knowledge gaps impeding the field of therapeutic nucleic acid nanotechnology are our understanding of the absorption, distribution, metabolism, excretion, toxicological properties and pharmacokinetics. The overall dependence of these parameters on individual NANPs' physicochemical properties (e.g., size, dimensionality, composition), chosen therapeutic action (e.g., cell targeting, regulation of gene expression, immunostimulation) and complexation with other materials (e.g., lipids, polymers, inorganic materials) when delivery carriers are required [22,23] are another factors affecting employment of TNA in biomedicine. The last but not least determinant important for TNA application is the presence of NANPs at the site of injection and whether and how they are distributed to the systemic circulation upon local administration (e.g., subcutaneous, intraperitoneal, and intradermal routes). Extensive physicochemical characterization to identify attributes critical to both NANPs' safety and efficacy along with the development of formulation, delivery, packaging, and bioanalytical methodologies represent other areas of preclinical development experiencing substantial gaps. Since, all new products intended for use in humans have to undergo rigorous safety testing prior to their approval for clinical use, and immunotoxicity is one of the end points required for preclinical safety assessment, it is also important to improve current understanding of NANPs' recognition by the immune cells. Even after receiving the initial approval, drugs undergo postmarketing surveillance and can be removed from the market due to toxicity. Immunotoxicity is one of the common reasons for drug discontinuation and market withdrawal; particularly, hypersensitivity reactions and anaphylaxis [24,25], which further emphasizes the importance of improving our understanding of NANPs' immunological properties.

Among immunological safety studies, NANPs' interaction with blood coagulation (e.g., platelets, coagulation factors, procoagulant activity of cells) and the complement system, red and white blood cells are informative for estimating potential short-term toxicity outcomes. In terms of the long-term toxicity, it is important to understand whether or not NANPs, when used alone or in combination with other carriers, can break immunologic tolerance to self-nucleic acids and induce the formation of anti-dsDNA antibodies. The combined physicochemical properties of carriers and NANPs and their influence on NANPs' long-term toxicity is another important area of research that will inform safer design and use of NANPs as therapeutics. Government-sponsored resources available for the extramural research community to conduct such studies include the Nanotechnology Characterization Laboratory's standardized assay cascade [26].

Future areas of research important for the translation of NANPs to clinical applications include identification and development of alternative synthesis protocols that would allow inexpensive production of high-quality NANPs over a short period.

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