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Thomas Kramps · Knut Elbers *Editors*

RNA Vaccines

Methods and Protocols

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Preface

Vaccines are currently regaining attention from members of the medical and scientific communities but even the broader public, including heads of state. This level of public awareness of the fundamental relevance of vaccines for global human well-being has been rekindled by dramatic threats of rapidly emerging infectious diseases (predominantly caused by viruses) and increasingly widespread multidrug-resistant bacterial infections. Insect-borne Zika virus and Ebola fever are only the most recent examples demonstrating a persistent vulnerability of human society to such primordial threats. In another area, cancer immunotherapy, vaccines are a promising, innovative treatment modality, too. In future, integrated treatment regimens that include cancer vaccines may enable patients to better regain immunological control over the tumor, superseding or complementing today's immune checkpoint inhibitors.

RNA vaccines, the subject of this volume, span a spectrum from recombinant viruses to self-amplifying mRNA and nonreplicating mRNA vectors. Given this breadth, we firmly believe that RNA technology will eventually spawn vector platforms of enormous medical and commercial potential. All RNA vaccines share distinct features, which will likely contribute to their continuing relevance:

- Like viruses, they provide integrated stimuli to adaptive and innate immunity, i.e., antigen expression in situ and danger signaling, e.g., via toll-like receptor pathways.
- Like live vectors, they induce "balanced" immune responses that comprise humoral and cellular effectors as well as immunological memory.
- Synthetic RNA vaccines allow for a combination of different antigens without increasing the complexity of vaccine formulation, thus facilitating speedy and flexible production.
- Due to "vector neutrality" they generally allow for highly repetitive vaccination schedules with consistent boost potential and no or little immune response directed against the vector.
- Thermostable RNA vaccines could simplify transport and stockpiling even in the absence of a cold chain, a frequently underestimated hurdle for global disease control.

In any case, unlocking this potential will require continued optimization as well as informed choice of applications.

Thus, the aim of this volume is to facilitate both efforts by assembling an overview of the field and practical hints for vaccinologists in academia and industry. Different RNA vaccines exhibit diverse sets of trade-offs with respect to efficacy, reactogenicity, and handling that reflect the great versatility of this class of vaccines. To choose the best way ahead, a basic understanding of the regulatory framework, including aspects of nonclinical safety testing and good manufacturing practice, is essential. The scope of protocols included in this book is laid out and discussed in more detail (together with some scientific context and additional references) in the introductory, first chapter. The protocols include relevant pointers to current "best practice" with concrete tips and tricks in the notes section of each chapter.

Finally, we are well aware that the relevant body of knowledge is rapidly developing and cannot realistically be captured in a single volume. We, therefore, sincerely hope that this compendium may engender increased collaboration on RNA vaccines between basic and applied scientists in academia, government, and industry to develop future solutions for today's challenges. In any technological field, we need reliable maps that are drawn from facts and open discourse to safely navigate both hyperbole and pessimism. We hope that this book will offer helpful orientation.

Ingelheim am Rhein, Germany *Thomas Kramps*
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Chapter 1

Introduction to RNA Vaccines

Thomas Kramps and Knut Elbers

Abstract

RNA vaccines are attractive, because they exhibit characteristics of subunit vaccines and live-attenuated vectors, including flexible production and induction of both humoral and cellular immunity. While human proof-of-concept for RNA vaccines is still pending, the nascent field of RNA therapeutics has already attracted substantial industry and government funding as well as record investments of private venture capital. Most recently, the WHO acknowledged messenger RNA (mRNA) as a new therapeutic class. In this chapter, we briefly review key developments in RNA vaccines and outline the contents of this volume of Methods in Molecular Biology.

Key words RNA vaccine, Messenger RNA, Self-amplifying RNA, Replicon, RNA virus vector

1 Introduction

Vaccination remains a key medical innovation. In essence, vaccines stimulate the immune system to form a prophylactic or curative response against a given disease and could offer a powerful treatment modality for a wide range of conditions with unmet medical needs [1]. However, realizing this conceptual potential faces considerable challenges [2]. In many instances, insufficient understanding of immune correlates and mechanisms of protection are major impediments [3]. Also, induction of potent effectors and long-lasting memory can be difficult, e.g., against pathogens localized at mucosal or immune privileged sites. The induction of effective T cell responses or of broadly neutralizing antibodies in particular remains a key challenge in addressing mutable microbial pathogens [4, 5]. Finally, an additional layer of complexity exists in individualized approaches, for example in the tailored immunotherapy of cancer [6].

However, the recent integration of vaccinology and "omics" technology offers exciting prospects of addressing such challenges [3]. For example, they may allow researchers to systematically unravel correlates of protection [7] or better understand dynamic

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host–pathogen interactions [8, 9]. On the other hand, we still lack validated vaccine platforms that complement such analytic capabilities and facilitate effective vaccine development [10]. Suitable vaccine technologies would enable high-throughput screening for protective antigens as well as rapid synthesis and testing of selected lead compounds [11]. Due to their simplicity and versatility, synthetic RNA vectors offer particular promise as tools for rapid screening and development of vaccine products than traditional approaches (including lower cost) [12–17].

2 Messenger RNA and Self-Amplifying RNA (Replicon) Vaccines

2.1 Historical Background

The concept of synthetic RNA vaccines is not new, but ingenious: In a seminal paper published a quarter century ago, Wolff et al. first showed that injection of uncomplexed messenger RNA (mRNA) led to protein expression in mice [18]. Instead of applying the protein antigen, RNA vaccines carry genetic information for endogenous protein expression in the vaccinee, similar to infection with a virus. In short order of this initial discovery, the immunogenicity of the format was shown in different test systems (reviewed in ref. 12), but overall the impression prevailed that producing and handling synthetic RNA vectors were prohibitive in terms of cost and complexity. By and large, attention focused on plasmid DNA technology or recombinant viral vectors instead [19].

Initial efforts by groups that pioneered mRNA vaccines mostly addressed cancer immunotherapy with no validated benchmarks to compare and optimize the format [20–23]. While some researchers favored direct injection of naked mRNA [20, 24], others used in vitro transfection of dendritic cells (DC) with mRNA to boost immunogenicity [25, 26]. For both approaches, academic and start-up initiatives established clinical grade (GMP)-conform production and provided important basic data on the safety and immunogenicity in humans [13]. The first successful preclinical proof-of-concept studies of prophylactic RNA vaccines in small and large animals, which also included head-to-head comparison with licensed comparators, have been reported only relatively recently [27–29]. These studies indicated principal feasibility and encouraged extended testing of an mRNA-based prophylactic vaccine in a first human clinical trial (NCT02241135). These activities involved increasing industry and government funding and led to record investments of venture capital [30]. Most recently, the WHO acknowledged mRNA as a new therapeutic class with its own international nonproprietary nomenclature (the suffix "-meran" as first used for "nadorameran," a rabies-specific vaccine) [31, 32].

2.2 Vector Design

RNAs are composed of strings of alternating nucleotides (generally uridylate, adenylate, guanylate, and cytidylate) which can also be subject to chemical modification [33]. Synthetic RNA vaccine vectors contain an open reading frame that encodes the antigen of interest and optimized, *cis*-acting flanking structures: the 5′ and 3′ untranslated regions (UTRs) flanking the open reading-frame (ORF), terminal 5′ 7-methyl guanosine cap structure (cap), and 3′ polyadenylated tail (polyA). Ultimately, all these elements serve to increase antigen yield by maximizing the rate of translation and/or vector persistence within transformed cells through interactions with regulatory proteins, other RNAs, and metabolites. As such, the 5′ cap, 5′ UTR, ORF, 3′ UTR, and polyA offer relevant targets for optimization of mRNA vectors [22, 34]. In the sequence of events leading to protein synthesis, translational initiation is rate-limiting and tightly regulated by the orchestrated recruitment of *trans*-acting factors to specific RNA sequences. Thus, improving translational initiation by sequence optimization is also important for the design of better mRNA vectors. We believe that continuing optimization will result in greater carrying capacity, further increasing potency, reducing cost, and facilitating the formulation of multivalent products.

RNA replicon vaccines present a complementary approach and very interesting alternative to non-replicating mRNA vectors [35]. This alternative setup makes use of accessory viral elements that lead to self-amplification of the messenger RNA [36]. A major strength of this approach is that, due to self-amplification of the vector in vivo, high-level and long-lasting protein expression is readily feasible with available technology. A persistent challenge, however, remains in the lower yield and specificity of production of these much larger molecules and—arguably—interference by anti-vector immunity [35, 37].

2.3 Production

The typical product profile of synthetic RNA vaccines differs substantially from that of traditional protein- or pathogen-based vaccines:

- For synthesis of the RNA vector, only information about the nucleic acid sequence is required. Thereby, handling of infectious agents, environmental risks, or restrictions of global vaccine distribution can be eliminated [15].
- While it can take years and hundreds of millions of dollars for a new manufacturing facility for traditional vaccine products to become productive, RNA vaccines are produced by a highly standardized process with relatively minor adaptations to account for variations in sequence length or composition. This generally reduces lead-time and cost [15].

- RNA represents a relatively stable drug-substance, as long as exposure to RNase is prevented [38]. RNA can be lyophilized for prolonged storage at ambient temperature, greatly facilitating distribution and storage [27].

The manufacture of bulk RNA by enzymatic in vitro transcription is well established [38]. Alternative protocols to generate template DNA, e.g., by polymerase chain reaction, currently limit design, fidelity, and yield. They have been employed for antigen screening [11], but remain much less common and are not discussed in this book. In the context of cancer vaccines flexible antigen selection is key to match the most relevant antigens with a given cancer type or for the design of patient-specific vaccines [9]. Such personalized therapeutics recently received much stronger attention and several academic groups and biotech companies initiated efforts to validate RNA vaccines encoding patient specific neoantigens in the clinic [39].

2.4 Adjuvantation

RNA exerts direct immune-stimulating effects [33, 40]. This RNA-mediated adjuvanticity may be modulated by composition and formulation: In the case of synthetic RNA vaccines, factors such as stabilization against RNase-mediated degradation, particle size, and charge influence the localization of RNA in cells or lymphatic organs and their resulting adjuvant activity [41–43].

The signaling pathways involved in RNA-mediated stimulation of the immune system are understood in some detail [44–46]. Innate responses to RNA are induced by dedicated pattern recognition receptors (PRR) upon detection of aberrant localization or unusual structural features of the RNA adjuvant [47]. RNA-specific PRR include endosomal toll-like receptors (TLR) 3, 7, and 8, the cytoplasmic receptors retinoic acid inducible gene I (RIG-I), melanoma differentiation antigen 5 (MDA5), protein kinase R (PKR), and others. They are differentially expressed in various cells and tissues, ranging from narrow expression in specific immune cells like plasmacytoid dendritic cells (pDC) and B cells for TLR7, to virtually ubiquitous expression, e.g., for RIG-I and PKR [48]. The differential stimulation of these molecules and cell types will thus shape the immune response to a given RNA vaccine. In designing preclinical test strategies, it is important to keep in mind that expression patterns and specificities of RNA-specific PRR may vary between humans and an animal test species of choice [48].

Apart from deriving adjuvant effects from their chemical composition, protein-coding RNA vectors can serve as “genetic adjuvant”. Here, co-expression of antigen with immune modulatory factors, such as cytokines, would enhance interactions of antigen presenting cells with immune effectors [49]. Genetic adjuvants extend design space vastly, but also raise additional complexities regarding delivery and—possibly—safety.