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Bioprocessing Technology Trends of RNA-Based Therapeutics and Vaccines

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ABSTRACT

In 2014, the monoclonal antibodies market had the highest growth rate (19%) for the number of new molecules in the pipeline. DNA and RNA therapeutics were not far behind, achieving 12% year-over-year growth. Industry analytics data suggest that the RNA-based therapeutics market will reach $1.2 billion by 2020.

This article reviews the current dynamics in the RNA therapeutics/vaccines market as well as differences between small-interfering RNA (siRNA), RNA interference (RNAi), microRNA (miRNA), and messenger RNA (mRNA). In addition, the authors outline the general production processes for these platforms, the challenges encountered during process development and production, and the strategies to overcome them.

MARKET OVERVIEW

RNA-based therapeutics target the treatment of diseases such as diabetes, cancer, tuberculosis, and some cardiovascular conditions. There is currently a great deal of money being put into this relatively new class of therapeutics and vaccines, which is projected to grow 12% in 2016 and reach $1.2 billion by 2020 (1). The 2015 research and development (R&D) biotech pipeline is shown in **Figure 1**. There are more than 700 nucleic acid-based therapeutics (DNA and RNA) in the pipeline and more than 60% of the nucleic acid-based therapeutic pipeline is in preclinical development. It is interesting to note that 35% of such pipeline is focused on oncology (2, 3).

Several companies (approximately 160) and many academic institutes (approximately 65) are developing RNA-based therapeutics. **Table I** provides a non-comprehensive list of a few (4). Two companies have marketed RNA-based therapies: NeXstar and Ionis Pharmaceuticals. There are 12 mRNA vaccines in development, seven of which are being developed by Curevac (Germany). Based on current outlook, the RNA therapeutics market seems more promising than the market for DNA therapeutics.

From a partnership perspective, Ionis Pharmaceuticals has entered into a global collaboration with Janssen Biotech, Inc. to discover and develop antisense drugs to treat autoimmune disorders of the gastrointestinal tract (5), and Merck & Co. (MSD) has bet $100 million on Moderna's mRNA technology (6). Moderna also has previously announced collaborations

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Figure 1: R&D biotech pipeline expansion.

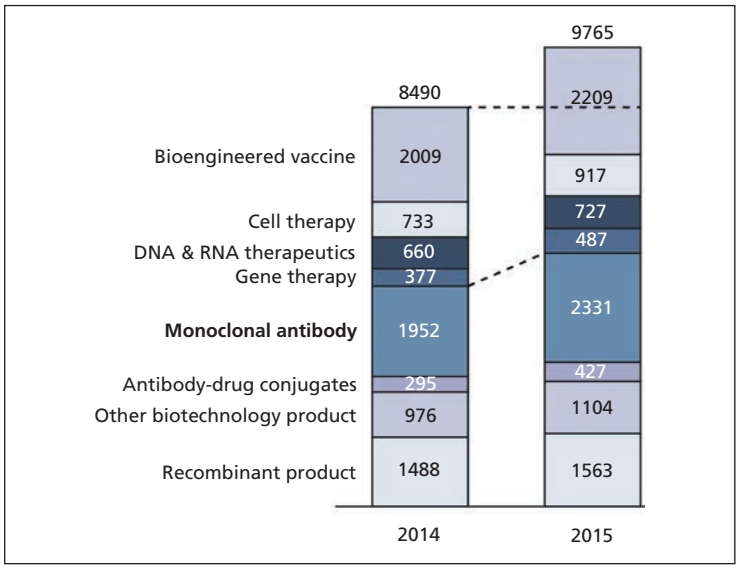
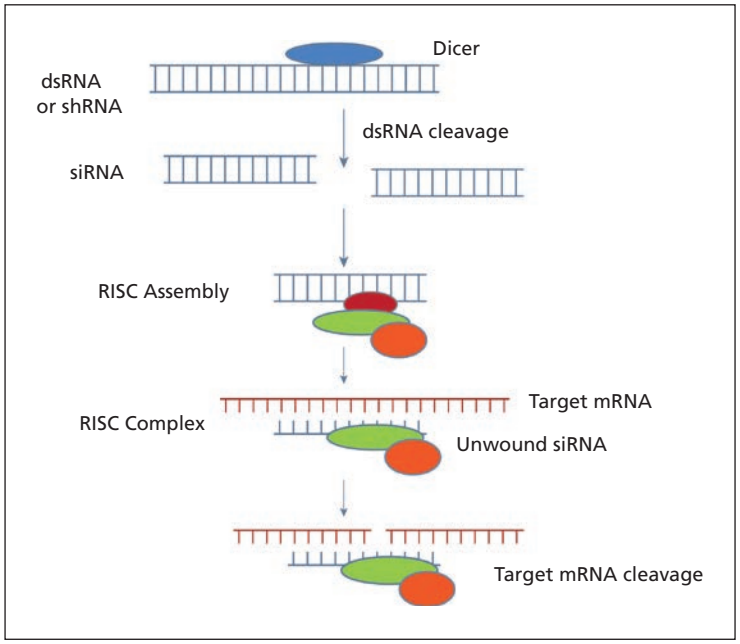


Figure 2: An insight into the RNAi pathway. Small hairpin RNA (shRNA) is a class of double-stranded RNA (dsRNA). The dsRNA is cleaved or degraded by a "dicer" enzyme into oligonucleotide segments called small interfering RNA (siRNA), which then enter a cell to form the RNA-inducing silencing complex (RISC). The siRNA strands then separate or unwind to form the activated RISC complex, which can then target messenger RNA (mRNA), bind to it, and cleave it.



with Alexion, AstraZeneca, and the Defense Advanced Research Projects Agency (DARPA) totalling $450 million. Moderna has raised $625 million in equity funding (7, 8).

RNA interference (RNAi) and RNA antisense technologies appear to be dominating the market. RNAi is a gene-silencing technology in which RNA molecules inhibit gene expression by targeting and destroying specific mRNA molecules. RNA antisense technology involves synthesizing an RNA strand that binds to a specific mRNA or to a splicing site on a pre-mRNA molecule to prevent translation. The major challenges associated with the commercialization of these RNA-based therapies are toxicity and drug delivery.

RNA-BASED THERAPEUTICS

With the advent of RNA-based therapeutics and their potential in treating a variety of chronic diseases, it is important to note the number of enabled technologies used to exploit the RNA mechanism/pathway, some of which are discussed in the following.

RNAi

RNAi technologies work by "silencing" or turning off a gene through the use of its own DNA sequence (**Figure 2**). The process is initiated by double-stranded RNA (dsRNA) that expresses either as a small or short hairpin RNA (shRNA) or as a microRNA (miRNA) transcript. Using this silencing mechanism, RNAi is commonly used to gain a better understanding of gene function, which can then be used to generate additional targeted therapeutics (9). Small interfering RNA (siRNA) and miRNA are the core elements of RNAi technology based therapeutics.

siRNA

RNAi utilizes a "dicer" enzyme to cut dsRNA into 21 oligonucleotide segments, called siRNA. These siRNAs can then bind to a specific family of proteins called Argonaute proteins, of which there are two classes: Ago and Piwi. Ago proteins bind to siRNAs or miRNAs, while Piwi proteins bind to Piwi-interacting RNA (piRNA) and are used to silence mobile genetic elements. The siRNA, miRNA, or piRNA complex bound to the Argonaute protein is called the RNA-induced silencing complex (RISC). Once bound to the Argonaute protein, one strand of the dsRNA is removed and the remaining strand binds to and directs the degradation of the complementary RNA target sequence, which then leads to the loss of protein expression (10).

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Table I: Biopharmaceutical companies developing RNA-based therapeutics and vaccines. siRNA=small interfering RNA, miRNA=microRNA, and mRNA=messenger RNA.

siRNA	miRNA	mRNA
Kyowa Hakko Kirin	Andes Biotechnologies	CureVac
Silence Therapeutics	Mirna Therapeutics	Biontech RNA Pharmaceuticals
Debiopharm	miRagen Therapeutics	Boehringer Ingelheim
Marina Biotech	Marina Biotech	Johnson & Johnson
Ipsen	Moderna Therapeutics	Ludwig Institute for Cancer Research
Alnylam Pharmaceuticals	Alnylam Pharmaceuticals	BioNTech
Sanofi Pasteur	Sanofi Pasteur	Sanofi Pasteur
Tekmira Pharmaceuticals	Tekmira Pharmaceuticals	
NanoCarrier	Regulus Therapeutics	
Dicerna Pharmaceuticals	Biogen Idec	
BioCancell Therapeutics	GlaxoSmithKline	
Samyang Group	AstraZeneca	
Silenseed	Ionis Pharmaceuticals	
siRNAsense	Les Laboratoires Servier	
Reference Biolabs	Celsion	
Avena Therapeutics	Rosetta Genomics	
Lipella Pharmaceuticals	Santaris Pharma	
Arrowhead Research	Shire	
	InteRNA Technologies	
	Alexion Pharmaceuticals	
	t2cure	
	Rigontec	
	Microlin Bio	

It has been reported that synthetic siRNA is able to knock down targets in various diseases *in vivo*, including hepatitis B, human papilloma virus, ovarian cancer, bone cancer, hypercholesterolemia, and liver cirrhosis. Only a few molecules of siRNA per cell are required to produce effective gene silencing (11). siRNAs are most commonly delivered into cells using microinjection or a transfection agent. Many companies now offer siRNA-delivering reagents to simplify this process (12).

miRNA

miRNA do not code for proteins, as they belong to specific class of non-coding RNAs. miRNA are 19–25 nucleotides in length and are encoded within introns (i.e., the portions of the gene sequence that are not expressed in the protein) (13). miRNA acts as a guide strand for the RISC complex to its mRNA target in vertebrates. Approximately 30% of genes in the human genome are regulated by miRNA (14).

Though siRNA silencing requires exact match between target and small interfering RNA, miRNA are non-specific and can exert action through imperfect base pairing. In addition, miRNA triggers translation inhibition (i.e., prevents the RNA from synthesizing protein from amino acids), while siRNA triggers mRNA degradation.

mRNA

mRNA, which codes for protein, is an essential component of the central dogma of life (DNA→mRNA→protein). mRNA is transcribed from a DNA template. mRNA takes the genetic code from DNA to the ribosome where the mRNA is translated to protein. There has been a significant increase in mRNA-based therapies in large part due to the many advantages that mRNA has over DNA in relation to gene expression and transfer. While RNAi and antisense RNA technologies are used primarily for gene

silencing, mRNA technologies are often used in vaccines or gene therapy (15). In both cases, after injection into the human body, mRNA is translated to protein, which can ultimately replace a missing protein (therapeutic) or induce an immune response (preventive approach). The production of synthetic mRNA for therapeutic use is relatively straightforward, and the challenges associated with its stability and delivery have been tackled through scientific advances in recent years (16).

RNA-BASED VACCINES

Conceptually, mRNA-based vaccines are a simple approach to inducing an immunological response by delivering the coding genetic element as a translation-ready molecule. Upon direct vaccination with mRNA molecules, dendritic cells (antigen-presenting cells) take-up, process, and encode the target antigen, which in turn induces an immune response. Typically, mRNA vaccines are produced by *in-vitro* synthesis through an enzymatic process. Such a synthetic process can be tightly controlled, resulting in a quality and predictable product profile. mRNA can be easily tailored to offer a specific immunogenic profile and pharmacokinetics (17). mRNA's stability and antigenic properties can be easily manipulated by changing codon or modifying base pairs.

Ongoing clinical trials show that mRNA can be delivered as naked mRNA; immobilized on particles or in liposome nanoparticle; or transfected in dendritic cells *in vitro* resulting in a discernible immune response and protective efficacy. mRNA can also act as an adjuvant and mRNA also has been explored to stimulate the innate immune system through toll-like receptors (18). RNA-based vaccines are comparatively simple to produce and can be developed, manufactured, and administered in a short time period, therefore, they are suitable for pandemic situations. Thermostability of mRNA vaccines can also significantly contribute to their low cost, as they do not require cold-chain distribution.

MANUFACTURING RNA-BASED BIOPHARMACEUTICALS

As more experimental RNA drugs move through the clinic and into large-scale trials, the demand for efficient and cost-effective manufacturing strategies will grow (19). RNA-based biopharmaceuticals are inherently susceptible to endonucleases, so special handling is required for production and purification. Degradation of product during manufacturing adds heterogeneity and chemical instability to the product. Therefore, the manufacturing and purification methods used in RNA-based therapeutics differ from that of DNA and other proteins (20).

mRNA purification (post-chemical synthesis) includes concentration precipitation, extraction, and chromatographic methods (including high-performance liquid chromatography) (19). The purpose of the upstream concentration and diafiltration step is to concentrate (if lower titer) and change the buffer to the necessary pH and conductivity for the first chromatography step. The objective of the final concentration and diafiltration step is to de-salt and achieve the necessary final concentration prior to sterile filtration. A 5-kD membrane cut-off is generally used for concentration and diafiltration in mRNA processes. Because siRNA are smaller than mRNA, a 1-kD membrane cut-off is used for adequate retention of the siRNA product (21).

CHROMATOGRAPHIC PURIFICATION STEPS

Since the breakthrough discoveries of catalytic RNAs in the early 1980s and RNA interference in the late 1990s, more than 50 RNA or RNA-derived therapeutics have reached clinical testing. In RNA purification, despite the different techniques such as arginine-affinity, ion-pairing reversed-phase, or pellicular anion exchange, the traditional ion-exchange (IEX) media—especially anion exchange (AEX)—remains the most popular technique used in both pure RNA and RNA packaged for delivery (22, 23, 24, 25).

Sm and Sm-like proteins, which can form heteromeric complexes or bind to various RNAs, were proven to contain ancient RNA-binding motifs (Sm domain) with oligo(U) specificity (26). Fractogel TMAE (MilliporeSigma), a strong anion-exchange resin, was used for the purification of small nuclear ribonucleoproteins (snRNP). The snRNP molecule was eluted with Tris/HCl

and 300 mM NaCl. Ribonucleoprotein and uncoupled RNA were separated from free protein, and the sample was immediately used for negatively stained electron microscopy.

Both AEX and reversed-phase (RP) technologies are widely used in the RNA purification process. Quarternary amine (Q) and Dimethylaminoethyl (DMAE) chemistry are among the choices for AEX (27, 28, 29, 30). One study proved that a few AEX resins can be used for RNA purification with optimized experimental conditions to achieve high dynamic binding capacity. In this study, among the 18 AEX medias that were screened, only four resins—Q Sepharose FF (GE Healthcare), POROS 50HQ (Applied Biosystems), Q Ceramic HyperD F (Pall), and Fractogel DEAE (MilliporeSigma)—showed baseline separations of RNA and plasmid DNA (31). After optimized loading and eluting conditions, Fractogel DEAE had a wider range of operation, higher dynamic binding capacity, and complete separation of RNA in the breakthrough from plasmid in the elute. The high recovery, robustness, and reproducibility also met the requirement for large-scale manufacturing. These binding and elution conditions can be utilized as a starting point for optimal experimental conditions in RNA purification.

Overall, many biochromatography resins are suitable for RNA purification similar to use in other biomolecule separations. In many cases, the Fractogel resins showcased superior capacity and efficiency, largely due to the "tentacular" structure whereby functional groups are located at the end of long arms grafted to the bead surface, which circumvent the steric hindrance caused by large biomolecules (32).

FORMULATION AND DELIVERY

The most challenging aspect of RNA-based therapeutics is its delivery to target cells. Several methods have been explored and tested in clinical trials. Some of the most promising approaches are explained in the following passages.

Polymer conjugation/ chemical modification

Native RNA and RNA-based therapies are vulnerable to degradation from the many ribonucleases found within the cell. Chemical modification is one method for hardening the RNA against such enzymatic attacks. Modifications to the molecule can also increase its target affinity, decrease its undesired immunogenicity, and improve its overall efficacy. Hardening strategies include modifications to the backbone, sugar, or base of the RNA molecule.

Conjugation of the RNA therapeutic is a strategy that is increasingly being used for improved delivery and uptake. Alnylam Pharmaceuticals has adopted a method of conjugating an amino sugar derivative of galactose, *N*-Acetylgalactosamine (GalNac) to improve the delivery of siRNA therapies to the liver. The GalNac-conjugated siRNA is taken up by asialoglycoprotein receptors in the liver resulting in a fivefold increase in efficacy versus the parent molecule (33).

Arrowhead Research is developing a competing conjugation strategy. Arrowhead's delivery technology, termed Dynamic Polyconjugates (DPCs), is a siRNA bound to an endosomolytic polymer backbone via a disulfide bond. The endosomolytic polymer enables the quick and efficient release of the siRNA from the endosome. Arrowhead's most recent strategy includes attaching cholesterol to the siRNA and GalNac to the endosomolytic polymer, ensuring they are both delivered to the hepatocytes. The co-injection therapy was shown to increase the efficacy of siRNA-cholesterol 500-fold with a 90% knockdown (34).

Encapsulation

The dominant and most-studied strategy for the delivery of RNA-based therapeutics is lipid-based delivery systems. One successful platform is the use of stable nucleic acid lipid particles (SNALPs), which are lipid particles formed from a fusogenic lipid, cationic lipid, and PEG-lipid mixture. The SNALP delivery system has been developed and championed by Tekmira Pharma; the company now refers to it as LNP technology. According to Tekmira, the LNP "encapsulates siRNAs (also mRNA) with high efficiency in uniform lipid nanoparticles that are effective in delivering RNAi therapeutics to disease sites in numerous preclinical models" (35).

Another promising lipid delivery technology is the proprietary Smarticles delivery platform developed by Novosom and

now owned by Marina Biotech. Similar to SNALPs, the Smarticles technology can change their surface charge to facilitate both stability and endosomal release. Smarticles are capable of encapsulating both single- and double-stranded nucleic acid therapies. Smarticles are comprised of cationic, anionic, and neutral lipids. The negatively charged Smarticles avoid the often seen toxic effects of positively charged lipids at physiological pH but convert to a positive charge in the acidic environment of the endosome, facilitating its release. Other interesting encapsulation techniques involve PLGA nanoparticles (36, 37).

CONCLUSION

RNA-based therapeutics are a relatively new class of therapies that has bright prospects in the treatment and prevention of difficult-to-treat chronic and rare diseases. RNAi work by interfering with the transcription process, and thereby inhibit protein translation. Though such a therapeutic approach is highly selective and targeted, special care is required during the production of these therapies and vaccines because of their susceptibility to ubiquitous RNAse-induced degradation. Large-scale manufacturing of new class of therapeutics would require bioprocessing components, chemicals, and tools free from RNAse. Technology and tool providers need to consider making such products available to enable large-scale production of RNA-based therapeutics. The surmounting challenges related to potential toxicity and drug delivery need to be addressed before such products can be commercialized. However, new technologies are emerging to overcome some of these challenges, and the future of RNA-based therapeutics is very promising.

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