RNA technologies: mechanisms of action, applications and forms of delivery
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On behalf of the Federal Office for the Environment (FOEN) and the Swiss Expert Committee for Biosafety
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1 Introduction

1.1 Background

Ribonucleic acids (RNA) play an essential role in all cells of all organisms. Nevertheless, until recently, these molecules were overshadowed by the much better-known deoxyribonucleic acid (DNA). It was only the COVID-19 pandemic that brought RNAs in the form of mRNA vaccines into the spotlight: less than a year after the outbreak of the pandemic, mRNA vaccines against SARS-CoV-2 were available in most western countries. This was the result of decades of research into both the structure and function of RNAs and their use in therapy. In Switzerland, around 70% of the population was vaccinated against SARS-CoV-2 in April 2023, the vast majority with an mRNA vaccine.

RNAs occur naturally in all cells and fulfil a wide variety of functions. They play a prominent role in the production of proteins, for example by transferring genetic information from the DNA in the cell nucleus to the cytoplasm via messenger RNAs (mRNAs). However, RNAs also fulfil many regulatory functions in cells and can bind to a variety of target molecules. This makes them potentially suitable for a wide range of applications, particularly in medicine and agriculture. RNA technologies, especially for therapeutic applications, have been developed for over 30 years. The first RNA-based therapies were authorised at the turn of the century (see Section 2.2.2.1 ASOs). However, the technologies were not yet widely accepted at that time. Only in recent years have decisive technical advances been made, for example by stabilising RNA and introducing it efficiently into target cells. As a result, several new RNA therapies have been developed within a few years and public and industrial research into RNA technologies has intensified considerably (Mollocana-Lara et al., 2021). With the success of the mRNA vaccines against SARS-CoV-2, RNA technologies have now moved even more into focus. Various research groups and networks, e.g. the Swiss National Center of Competence in Research RNA & Disease (NCCR RNA & Disease), as well as private companies, are also working on the research and development of RNA technologies, particularly in the field of therapeutics.

1.2 Aim of the report

The aim of this report is to provide a technical and scientific overview of the various currently relevant RNA technologies. It provides information on the mechanisms of action of the various RNA-based approaches, their fields of application and products and their development status. The report is intended to help assess and discuss the benefits, risks and limitations of the various RNA technologies and their products. It is also intended to support the legal categorisation of RNA products.

1.3 Featured technologies

The report discusses various technologies based on RNA molecules. RNA stands for ribonucleic acid and consists of the sugar ribose and the four bases adenine, guanine, cytosine and uracil. Chemically, RNA differs from DNA (deoxyribonucleic acid) in that it contains the base uracil (U) instead of thymine (T) and that DNA lacks a hydroxyl group on the sugar-phosphate backbone compared to RNA. Unlike DNA, which is practically always double-stranded, RNA is often single-stranded. In contrast to DNA, RNA is also very susceptible to degradation by enzymes called ribonucleases, which are ubiquitous in organisms. In many RNA technologies, the RNA molecules are therefore chemically modified to increase their stability, among other things. These modified nucleic acids cannot be clearly classified as RNA or DNA if they do not have the hydroxyl group that distinguishes the two substances. There are also active substances that can consist of both RNA and DNA molecules, for example antisense oligonucleotides (ASOs) (see Section 2.1.1). RNA technologies are therefore chemically modified to increase their stability, among other things. These modified nucleic acids cannot be clearly classified as RNA or DNA if they do not have the hydroxyl group that distinguishes the two substances. There are also active substances that can consist of both RNA and DNA molecules, for example antisense oligonucleotides (ASOs) (see Section 2.1.1). RNA technologies are therefore not always clearly distinguishable from DNA technologies. Where appropriate, DNA-based methods are therefore occasionally mentioned in the report if their mechanism of action is the same as that of RNA technologies.

The report does not cover technologies that use RNA molecules to modify the genetic material of target cells. This includes in particular the CRISPR/Cas method for genome editing, which uses two RNA molecules in addition to the Cas enzyme. RNA viruses and virus-like vectors, such as replicons, are also not covered, as these differ significantly from the technologies presented here in various aspects, particularly with regard to biological safety.

The report focuses on RNA technologies that are being researched and developed with a view to applications in therapy and agriculture. It considers both technologies that are already established and have initial products on the market (such as mRNA vaccines) and approaches that are at an early stage of research and development. The RNA-based methods relevant to the report were identified
on the basis of the specialist literature and assessments by experts and categorised into eight groups:
1. post-transcriptional gene silencing
2. mRNA technologies
3. RNA aptamers
4. long non-coding RNAs (lncRNA)
5. RNA-directed DNA methylation (RdDM)
6. RNA activation (RNAa)
7. circular RNAs (circRNAs)
8. ribozymes

The various RNA-based methods can differ in the type of RNA molecules used, the target molecules to which they bind and/or the mechanism of action (see Figure 1).

The distinction between the different methods is not always clear, and various categories and designations are also used in the literature. The grouping used here is therefore only one of several ways of categorising the different RNA technologies.

1.4 Structure of the report

The first chapters (Chapters 2–5) describe in detail four RNA technologies that are already established with products and/or are considered to have great potential for future applications in therapy or agriculture. The mechanism of action, possible applications and development status for each technology are described.

The relevance of the various methods was determined on the basis of the literature and the assessment of the experts involved (see imprint). Overall, post-transcriptional gene silencing (Chapter 2) and mRNAs (Chapter 3) are currently regarded as the two most relevant approaches. Both have already established themselves with the first therapeutic products and have great potential for further medical applications. Based on the post-transcriptional gene silencing approach, applications for agriculture are also being researched and developed. The first crop protection product has already been registered for authorisation in the USA. Aptamers (Chapter 4) are also an established technology with authorised products. However, no further authorisations have been granted in recent years and research has long been limited to niche applications. Long, non-coding RNAs (Chapter 5) are still at a relatively early stage of development. However, due to their diverse mechanisms of action, their potential is considered to be great, even if many challenges remain.

Chapters 6–9 provide a brief overview of four other RNA technologies that are also being researched and developed but are still at an early stage of development: RNA-dependent DNA methylation can regulate the activity of genes in plants by attaching chemical groups to the DNA; RNA activation causes increased gene activity by binding short RNAs to RNA or DNA molecules; circular RNAs are ring-shaped RNAs with various functions; ribozymes can catalyse chemical reactions.

Delivery methods are an important area of research and development for all RNA technologies in order to bring the RNA molecules specifically to the desired tissues and into the corresponding cells. Since RNA molecules are susceptible to degradation by ribonucleases and have difficulty penetrating cells, they need to be packaged for most applications, e.g. in nanoparticles or liposomes. The successful improvement of the formulation of these particles has been a crucial step that has helped newer RNA...
therapeutics, such as the mRNA vaccine against SARS-CoV-2, to achieve a breakthrough. Research into developing even more efficient and tissue-specific or cell-specific delivery methods is a very active and rapidly evolving field. The final chapter provides an overview of the currently most relevant delivery methods for therapeutic and agricultural applications.

Preparation of the report
The report was compiled by a group of six authors with expertise in the field of RNA technologies under the leadership of the SCNAT Forum for Genetic Research. The entire report was then reviewed by other specialists with different areas of expertise. Details of the people involved can be found in the imprint.

Categories of RNAs

RNAs can be divided into different groups in various ways and labelled accordingly.

They can be categorised into small RNAs (sRNA) and long RNAs (long RNAs) based on their length. Small RNAs have an approximate length of less than 200 nucleotides, long RNAs more than 200 nucleotides. Long RNAs mainly include mRNAs and long non-coding RNAs (lncRNAs).

Furthermore, the structure of RNAs can be labelled as either single-stranded (ssRNA) or double-stranded (dsRNA). Certain RNAs are also classified according to their shape, for example as circular RNA (circRNA) or hairpin-shaped RNAs (e.g. short hairpin RNA, shRNA).

Finally, RNAs can also be classified according to their function. At the highest level, a distinction is made between coding RNA and non-coding RNA (ncRNA). Coding RNAs contain the information for the production of a protein. ncRNAs, on the other hand, take on a variety of other cell functions, for example in the regulation of gene expression, the defence against viruses or the protection of the genome. ncRNAs are further differentiated according to their specific functions. These include, for example, small interfering RNAs (siRNAs), which bind and inhibit specific target RNAs, or ribozymes, which catalyse chemical reactions. However, there is a subgroup of RNAs that are translated and are nevertheless categorised as long non-coding RNAs.
2 Post-transcriptional gene silencing

2.1 Introduction

2.1.1 Antisense oligonucleotides

In 1978, researchers synthesised a small piece of DNA (also known as an oligonucleotide) that was complementary to a short RNA sequence of the Rous sarcoma virus. When the researchers added the piece of DNA to a cell culture, they observed that the virus could no longer spread in the cells. The synthesised DNA sequence had bound the complementary RNA sequence of the virus and thus prevented the virus from replicating. It was later shown that oligonucleotides can also bind the cell’s own RNAs and thereby downregulate genes. This opened up the possibility of influencing viral or cellular RNAs for research purposes or for medical applications. Since then, this technology has been continuously developed to specifically regulate target genes in certain cells (Crooke et al., 2021).

2.1.2 RNA interference

In the 1990s, it was discovered that introducing double-stranded RNAs into a cell can also inhibit the expression of certain genes. It turned out that this mechanism, called RNA interference, occurs in almost all eukaryotic life forms and plays an important role in regulating gene expression, defending against viruses and protecting the genome (Svoboda, 2020). Various types of short RNAs that can regulate genes occur in nature (Svoboda, 2020). They are all non-coding RNAs (ncRNA), which means that their genetic information is not used for the production of a protein. The two most common short, non-coding RNAs are small interfering RNAs (siRNA) and microRNAs (miRNA). Other short ncRNAs often have very specific functions, such as piwi-interacting RNAs (piRNA), which regulate so-called retrotransposons in the gametes. The various short non-coding RNAs differ in their length, origin and the type of target RNAs.

The 21–24 nucleotide (nt) long siRNAs are produced in nature by the cleavage of a long dsRNA into short pieces by the Dicer enzyme (Dicer-like (DCL) in plants). In vertebrates, the original double-stranded RNA (dsRNA) usually originates from a virus or virus-like element (e.g. a transposon). In plants and invertebrates, siRNAs can also be encoded in the genome and regulate their own genes (e.g. trans-acting siRNAs). In addition, siRNAs can be multiplied in plant cells by special polymerases and spread throughout the plant (Svoboda, 2020).

Figure 2: RNA silencing.

A) RNA silencing concept

B) RNA interference pathway

C) microRNA pathway

A) Basic process: non-coding RNA is cleaved into shorter RNA by Dicer and bound by an Argonaute protein (AGO). Together with AGO, the short RNA binds a sequence on the target RNA. The effect can be enhanced by an RNA-dependent RNA polymerase, which amplifies the dsRNA. B) RNA interference. Synthetic dsRNAs or dsRNAs from viruses or from the cell’s own transcripts are converted into siRNAs by Dicer and dsRNA-binding proteins, which lead to enzymatic cleavage of the mRNA when they bind to the target mRNA. C) miRNA in mammals: in the cell nucleus, the pri-miRNA encoded in the genome is truncated to pre-miRNA by the Drosha and DGR8 proteins and then transported into the cytoplasm. There it is shortened to miRNA by Dicer and binds to the target mRNA together with AGO and other proteins. If sequence complementarity is not complete, translation is blocked and the mRNA is degraded (Svoboda, 2020).
Natural miRNAs, on the other hand, are always encoded in the genome and are present after transcription as primary miRNAs (pri-miRNAs), which can be hundreds of nucleotides long. These pri-miRNAs are then processed into 70–80 nt long miRNA precursors (pre-miRNAs) and transported from the cell nucleus into the cytoplasm of the cell, where they are cut by Dicer into 17–24 nt long double-stranded miRNAs (Svoboda, 2020).

Both siRNAs and miRNAs are then bound by a protein from the Argonaute family. Subsequently, one of the RNA strands is removed and the resulting RNA-induced silencing complex (RISC), consisting of Argonaute and other proteins, and single-stranded siRNA or miRNA bind to a specific sequence of the target RNA. If the complementarity between the target RNA and the siRNA is high, the target mRNA is enzymatically cleaved by the RISC complex. This is the normal case with siRNAs, but can also occur with miRNAs, especially in plants. In animals, however, the complementarity of miRNAs with the target mRNAs is usually only partial, which means that the RISC complex binds the mRNA but does not cleave it. As a result, translation is blocked and the mRNA is often degraded (Svoboda, 2020).

Due to their higher complementarity, siRNAs are more specific because they can usually only bind one target RNA. miRNAs, on the other hand, can bind several mRNAs and thus inhibit the production of different proteins simultaneously (Svoboda, 2020).

While in plants both siRNAs and miRNAs play an important role in the defence against viruses and gene regulation, siRNAs are only of secondary importance in animals, especially mammals. This is also shown by the fact that the two types of dsRNA in plants are processed by a complex network of different protein variants. In animals, on the other hand, they are processed in separate processes, whereby that of siRNAs is greatly reduced. Synthetic siRNAs that have been introduced into a mammalian cell are processed by the same factors as miRNAs (Svoboda, 2020).

Short double-stranded RNAs can inhibit not only translation but also transcription (see Chapter 6 ‘RNA-directed DNA methylation’).  

### 2.2 Therapeutic applications

The ability of ncRNA molecules to bind specifically to certain sequences of other RNAs and thereby influence protein synthesis can be utilised for therapeutic purposes in several ways. On the one hand, single-stranded or double-stranded RNA molecules can be produced that penetrate the affected cells and reduce the amount of a target RNA there. This can reduce the quantity of disease-causing proteins. On the other hand, the production of disease-preventing proteins can be increased by binding harmful miRNAs, which reduce the production of the corresponding protein. In addition to miRNAs, long non-coding RNAs can also be considered as targets for regulation (see Chapter 5 ‘Long non-coding RNAs’).

miRNA-based therapeutics are not considered a separate class of molecules but are an umbrella term for siRNAs or ASOs that either bind to miRNAs or replace miRNAs (see 2.2.1.3 ‘miRNA-based therapeutics’).

### 2.2.1 Description of the procedures

#### 2.2.1.1 Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are synthetic, single-stranded sequences with a length of 12–24 nucleotides. They can be DNA, RNA or artificial nucleotides – such as LNA (locked nucleic acids). Ribonucleotides are often modified and combined with artificial nucleotides in order to obtain oligonucleotides with the desired pharmacological properties (see Section 2.2.1.4 ‘Chemical modifications’) (Crooke et al., 2021; Hall et al., 2022).

ASOs can act via different mechanisms. By binding to the target mRNA, some ASOs can initiate its cleavage by the ribonuclease H enzyme (RNase H). This utilises a natural mechanism of the cell, which has the task of eliminating naturally occurring DNA-RNA duplexes, for example during transcription or viral infections. This occurs primarily with DNA-based, modified ASOs or so-called gapmers. Gapmers consist of a DNA sequence surrounded by modified oligonucleotides. ASOs can also be used to modify splicing. During splicing, certain areas are cut out of a precursor form of the mRNA, the pre-mRNA, and are therefore not translated into a protein during translation (see also Chapter 3 ‘mRNA technologies’). This process can be altered by ASOs in such a way that, for example, a stop codon is incorporated into the mRNA, which leads to an early termination of translation and degradation of the target RNA. Or a sequence with a harmful mutation can be skipped so that the mutation is not included in the final mRNA (see Figure 3).

In addition, modified single-stranded RNAs can act like siRNAs and – when incorporated into the RISC complex – lead to enzymatic cleavage of the target RNA (see Section 2.2.1.2 ‘Small interfering RNA (siRNA)’) (Crooke et al., 2021).

However, ASOs can also upregulate gene expression, for example by binding to miRNAs and thus impairing their
function (see Section 2.2.1.3 ‘miRNA-based therapeutics’) or by increasing the translation rate by binding to certain regulatory sequences in the mRNA (Crooke et al., 2021).

The therapeutically less relevant mechanisms of ASOs include preventing translation of the target mRNA by blocking the ribosome or the cleavage of the so-called cap structure at the end of the mRNA. In addition, ASOs can change the length of the so-called poly(A) tail at the other end of the mRNA and thereby influence the stability of the mRNA (Crooke et al., 2021).

2.2.1.2 Small interfering RNA (siRNA)

Small interfering RNAs (siRNAs) are 19–24 nucleotide-long double-stranded RNA molecules. Together with several proteins, they form the RNA-induced silencing complex (RISC). In the RISC complex, one of the strands, called the leading strand, binds a complementary sequence on the target mRNA by base pairing. In most cases, this leads to enzymatic cleavage of the target mRNA (Svoboda, 2020).

Introducing siRNAs into a cell can be used to specifically degrade both coding and non-coding RNAs. This makes it possible to downregulate genes that cause disease due to a mutation or dysregulation. siRNAs, like ASOs, are characterised by the fact that they can bind any RNA whose sequence is known with a high affinity and specificity. Small biomolecules or antibodies that are intended to influence protein function, on the other hand, must be designed to fit correctly into a complex three-dimensional shape, which is difficult or impossible to achieve with many proteins. However, this higher binding capacity also makes it easier for siRNAs to bind to non-target molecules, which can lead to unintended (off-target) effects. In addition, siRNAs are relatively short-lived in the body. In order to improve their pharmacological properties, siRNAs are usually chemically modified for therapeutic applications (Hu et al., 2020) (see Section 2.2.1.4 ‘Chemical modifications’).
To date, four RNA interference (RNAi)-based therapeutics have been approved by the US Food and Drug Administration (FDA); others are in the clinical phase (see Section 2.2.2 ‘State of development’).

2.2.1.3 miRNA-based therapeutics

There are two different strategies for miRNA-based therapeutics: miRNA antagonism and miRNA replacement therapies. The aim of miRNA antagonism (anti-miR) is to suppress or reduce the expression or function of a defective target miRNA. Antagomirs are short single-stranded non-coding RNAs (with a similar structure to ASOs), which bind to the target miRNAs and interfere with their function through complementary hybridisation and steric hindrance. In other words, they prevent the miRNA from binding to the natural target mRNA. In turn, miRNA replacement therapy aims to restore the expression or function of a miRNA. For example, this may be a miRNA that downregulates certain genes that promote the development of cancer. If this miRNA is downregulated, the risk of a tumour developing increases. In replacement therapy, a short, double-stranded RNA molecule (miRNA mimic) is synthesised that resembles the endogenous miRNA. This is then introduced into the cell, where it takes over the function of the natural miRNA (Traber & Yu, 2022). Unlike siRNAs, which are very specific, miRNAs often regulate the expression of several genes. This property makes it possible to use miRNA-based therapeutics to simultaneously influence several critical elements in deregulated cellular processes (Traber & Yu, 2022).

While some anti-miR therapies are already in the clinical phase, miRNA replacement therapies are only in the preclinical phase (see Section 2.3.3 ‘State of development’).

2.2.1.4 Chemical modifications

As RNA is very unstable in vivo and can be rapidly degraded by enzymes (nucleases), it must be chemically modified for many medical applications in order to protect the phosphodiester backbone from nucleases. However, modifications can also have a negative effect on the ability to bind to the target molecule. The search for modifications that improve stability, binding capacity, cellular uptake and other pharmacological properties without reducing binding strength and specificity too much is one of the main focuses of medical research in this area.

2.2.1.4.1 ASOs

The most common modification in ASOs, the replacement of the natural phosphodiester bonds between the nucleotides with synthetic phosphothioate (PS) bonds, prevents cleavage by nucleases and facilitates cell entry for the oligonucleotide. In addition, the stronger binding to serum proteins helps the drug to spread in tissues and organs. The 2′-OH group of ribose, the only site where DNA and RNA differ chemically in the nucleotide chain, is often replaced by a 2′-O-methoxymethyl group (2′-MOE), a methyl group (2′-O Me) or an enclosed ethyl group (2′-Et). Alternatively, the ribose moiety can be linked to LNAs (locked nucleic acids) with an additional C-O bridge to increase the binding affinity to the target RNA. The ribose sugar can also be replaced by a synthetic methylene morpholine ring (phosphorodiamidate Morpholino oligomers, PMOs, also known as Morpholinos), which prevents degradation by ribonucleases. To facilitate uptake in liver cells, the ASOs can be combined with N-acetylgalactosamine (GalNAc). In addition, there are a variety of different chemical modifications for adapting the oligonucleotides to the desired mode of action and route of delivery (Adachi et al., 2021; Hall et al., 2022). It is also possible to insert the sequence into a circular RNA, which is more stable than linear RNA and less likely to be recognised by the immune system (see Chapter 8 ‘Circular RNAs’).

2.2.1.4.2 siRNAs

The first therapeutic siRNAs (e.g. Bevasiranib) were largely unmodified and were administered locally. However, they showed low efficacy and off-target effects. In addition, the innate immune response can be triggered. Therefore, many of the modifications that have proven successful in ASOs are also used for siRNAs, in particular, 2′-O-methyl (2′-OMe) and phosphothioate (PS) linkages, which prevent cleavage by nucleases, and N-acetylgalactosamine (GalNAc), which promotes uptake into certain liver cells (hepatocytes) (Hu et al., 2020).

2.2.2 State of development

2.2.2.1 ASOs

To date, eight ASO-based drugs have been approved by the US Food and Drug Administration (FDA) and four by the European Medicines Agency (EMA). The first, Fomivirsen (FDA: 1998, EMA: 1999, withdrawn in the EU in 2002 and in the US in 2006 for economic reasons) was used for the treatment of cytomegalovirus infections and was an unmodified DNA ASO that binds viral RNA. Eteplirsen (FDA: 2016, EMA: rejected), Golodirsen (FDA: 2019, Viltolarsen (FDA: 2020) and Casimersen (FDA: 2021) are Morpholino oligomers and Nusinersen (FDA: 2016, EMA: 2017) is a 2′-MOE-modified ASO. All of these lead to the skipping of a coding RNA segment (exons) which, if it contains a certain mutation, can cause Duchenne muscular dystrophy. Mipomersen (FDA: 2013, EMA: rejected) for the treatment of homozygous familial hypercholesterolaemia, Inotersen (FDA: 2018, EMA: 2018) for the treatment of hereditary transthyretin-mediated amyloidosis and Volanesorsen (EMA: 2019) for the treatment of familial chylomicronaemia syndrome are 2′-MOE-
modified ASOs that, when binding to mRNA molecules, cause their degradation by RNase H (Crooke et al., 2021). With the exception of Fomivirsen, which was used for viral eye inflammation, all drugs approved to date are aimed at the treatment of rare hereditary diseases; they are therefore used in an area in which there is little competition from small molecule drugs and biopharmaceuticals (e.g. monoclonal antibodies) and therefore a high-price policy is possible (Hall et al., 2022).

In 2020, seven ASO-based drugs were tested in clinical phase III and 31 in phase II. These include drugs for diseases of the nervous system; muscular, cardiovascular, inflammatory and metabolic diseases; eye diseases; various cancers and infectious diseases (Crooke et al., 2021).

Another potential application of ASOs is in the treatment of very rare diseases through personalised medicine. Mi-ласен was developed specifically for the treatment of a single patient by identifying her specific genetic defect and synthesising a PS-MOE oligonucleotide to correct the splicing defect (Hall et al., 2022).

2.2.2 siRNAs
Four RNAi-based therapeutics have already been approved in Europe and the US: Patisiran (FDA & EMA, 2018; Swissmedic, 2019), Givosiran (FDA, 2019; EMA, 2020; Swissmedic, 2021), Lumasiran (FDA & EMA, 2020; Swissmedic 2021) and Inclisiran (EMA, 2020; FDA & Swissmedic, 2021).

Patisiran (Onpattro®) was the first RNAi-based drug to be approved by the FDA in 2018 for the treatment of familial amyloid polyneuropathy. In this disease, amyloid plaques form from misfolded transthyretin (TTR), a protein that is mainly produced in liver cells. Patisiran binds to the TTR mRNA and thereby reduces the formation of amyloid plaques. Givosiran (Givlaari®) is authorised for the treatment of acute hepatic porphyria (AHP). In AHP, toxic substances accumulate in the cell due to overproduction of the protein aminolevulinate synthase 1 (ALAS1). Givosiran binds to the ALAS1 mRNA and thereby reduces the amount of protein. Inclisiran (Leqvio®) is approved for the treatment of familial hypercholesterolaemia and clinical atherosclerotic cardiovascular disease and reduces the enzyme proprotein convertase subtilisin/kexin type 9 (PCSK9) in the liver (Hall et al., 2022; Traber & Yu, 2022). Lumasiran (Oxlumo®) is used to treat primary hyperoxaluria type 1. In this disease, oxalate is deposited in various organs. Lumasiran reduces the enzyme glycolate oxidase (GO), which produces a precursor of oxalate in the liver, thereby lowering the amount of oxalate in the body (Traber & Yu, 2022).

Patisiran consists of two multi-methylated RNA strands enclosed in a lipid nanoparticle that facilitates uptake.
into liver cells (see Section 10.1.2.1 ‘Lipid nanoparticles and liposomes’). The second-generation siRNA drugs – Givosiran, Lumisiran and Inclisiran – no longer contain natural nucleotides and are bound to N-acetylgalactosamine (GalNAc) for increased uptake into certain liver cells (so-called hepatocytes) (Hall et al., 2022).

### 2.2.2.2.1 Challenges

Therapeutic siRNAs must enter the cytoplasm and/or the cell nucleus of the target cells in order to become active. To do this, they have to overcome a number of obstacles. They must remain stable in the blood, i.e. they must not be degraded enzymatically, and they must evade recognition by the immune system. They must then accumulate in the desired tissue and be transported through the cell membrane (Y. Zhu et al., 2022).

In oligonucleotide drug development, attempts are being made to improve transport into cell types and tissues other than the liver by means of chemical modifications (see Section 2.2.1.4 ‘Chemical modifications’). On the other hand, various delivery vehicles – for example made of lipids, lipid-like materials, polymers, peptides, exosomes and nanoparticles – are being developed (see Section 10.1 ‘Medical delivery methods’) (Hu et al., 2020). The conjugation of GalNAc ligands to siRNA is a tried-and-tested method for introducing siRNAs into liver cells. Methods for targeting other cell types have not yet been established but are being intensively researched. Therefore, siRNAs have so far only been delivered intravenously into liver cells (Crooke et al., 2021). Delivery systems that can deliver siRNAs specifically into the central nervous system, eyes, kidney cancer cells and epithelial cells are now at an advanced stage of development (Hu et al., 2020).

### 2.2.3 Undesirable effects

#### 2.2.3.1 ASOs

The undesirable effects that can occur with ASOs depend heavily on their chemical composition, as this can influence the mechanism of action and the interactions with other molecules (see Section 2.2.3.1 ‘Chemical modifications’). A distinction must also be made between off-target effects, which are caused by complementary base pairing outside the target sequence, and non-specific effects, such as immune reactions or interactions with cellular proteins (Jason et al., 2004).

The frequently used phosphothionate (PS) modification in ASOs can induce a non-specific pro-inflammatory response by binding to immune receptors. PS-ASOs can activate both the complement system and the innate immune system and have the potential to have an immunotoxic and cytotoxic effect. However, this risk can be greatly reduced by certain modifications, such as 2’-MOE. On the one hand, the modifications can prevent recognition by immune receptors and, on the other hand, reduce the amount of molecules required for therapy through better pharmacological properties (Crooke et al., 2021). For some PS-ASOs containing 2’-MOE, cEt or LNA modifications, cell-toxic effects have been observed, probably caused by the interaction with cellular proteins. However, the addition of 2’-O-methyl (2’-OMe) greatly attenuated this effect (Shen et al., 2019). In contrast, actual off-target effects due to non-specific binding of RNase H1 are rarely expected if the target sequence has been carefully selected, as high complementarity is required for cleavage (Crooke et al., 2021).

#### 2.2.3.2 siRNAs

siRNAs that are bound in the RISC complex should only bind to a specific target RNA. However, the complex tolerates certain errors in complementarity, which is why sequences that are similar to the target sequence can also be bound. The probability of such an off-target effect can be reduced by bioinformatic analyses and an appropriate design of the siRNAs. Another source of off-target effects is the second strand of the siRNA, the sense strand, which...
should not bind to the target mRNA. It can happen that instead of the intended antisense strand, the sense strand remains in the RISC complex and binds an unwanted RNA. However, this can be prevented by chemically modifying the sense strand so that it is more difficult to load into the RISC complex (Hu et al., 2020).

As with ASOs, siRNAs also harbour the risk of activating the innate immune system. Attempts are also being made here to reduce the probability of an immune reaction through chemical modifications (Hu et al., 2020).

2.3 Applications in plant protection

2.3.1 Description of the procedure

Crops are threatened by a variety of viruses, fungi, bacteria and other microorganisms. Animal pests can also cause major crop losses. In agriculture, these pathogens and pests can be kept in check by suitable soil cultivation, diverse crop rotations and resistant varieties, but they are also combated with biological and synthetic plant protection products. However, these have a relatively unspecific effect, i.e. they also affect non-target organisms and can have a negative impact on ecosystems, biodiversity and other protected goods. Due to the sequence-specific mechanism of RNAi, dsRNAs can be used to downregulate essential genes of pathogens and harmful organisms relatively specifically, thereby weakening or killing them (OECD, 2020; K. Y. Zhu & Palli, 2020; Zotti et al., 2018).

The double-stranded RNAs (dsRNAs) that trigger the silencing effect can either be expressed endogenously (i.e. in the plant) by incorporating corresponding DNA sequences into the plant genome (by producing genetically modified plants), or they can be synthesised in bacteria or cell-free systems and applied externally (exogenously) (Zotti et al., 2018). The endogenous expression and formation of dsRNA is also known as host-induced gene silencing (HIGS). It has the advantage that dsRNAs are present in sufficient quantities in the plant and can either act against pathogens or be taken up by harmful organisms (Koch & Petschenka, 2022). However, plants that express dsRNAs endogenously are transgenic organisms, as foreign DNA is incorporated into the genome. They are therefore not the subject of this report and are not discussed in detail below, but only mentioned where they contribute to the understanding of the individual applications and their development status.

Exogenous dsRNA, on the other hand, is either applied to the plant surface by spraying – in this case it is referred to as spray-induced gene silencing (SIGS) – or introduced directly into the plant interior, e.g. by injection or mechanical inoculation (see Figure 5). For many applications, systemic distribution of the dsRNA in the plant is required (see Section 2.3.3.1 ‘Challenges’). Systemic distribution is facilitated by injecting or mechanically inoculating the dsRNA into the plant, as the dsRNA does not have to penetrate the plant surface first. However, these methods are currently only suitable for use in the laboratory, partly due to the high manual effort involved (Rank and Koch 2021).

2.3.2 Applications

2.3.2.1 Insect pests

Experiments have shown that RNAi in insects can be triggered by dsRNAs when ingested. By expressing the corresponding dsRNAs from the crop plant itself or spraying them onto it, it is possible for the dsRNA to be taken up by insect pests during feeding and to down-regulate certain genes that are essential for the development and physiological processes of these insects. This approach can be used to produce sequence-specific insecticides adapted to specific target insects, which act very selectively and can be quickly customised (see as an example Bachman et al., 2013, 2016). Extensive RNAi screens have now been carried out on a number of pests to identify essential genes that could be suitable targets for this method (Koch & Petschenka, 2022; Zotti et al., 2018).

However, the effectiveness of such dsRNAs has proven to be highly variable. While certain insect orders such as Orthoptera (grasshoppers), Blattodea (cockroaches and termites) and most Coleoptera (beetles) show a strong RNAi response following the uptake of dsRNAs, this response is significantly weaker in Lepidoptera (butterflies and moths) and Diptera (flies). In the Hemiptera (plant lice, cicadas and bugs), the response varies greatly (Huvenne & Smagghe, 2010; Koch & Petschenka, 2022; OECD, 2020).

The effectiveness of the dsRNA is influenced by various factors. These include the stability of the RNA, the efficiency of uptake of the dsRNA by the insects and the expression level of the genes required for the RNAi process (see Section 2.1.2 ‘RNA interference’). The speed at which dsRNAs are processed or degraded by nucleases also has an influence on their effectiveness. RNAs can spread systemically in some insect species, while they remain localised in others (S. Liu et al., 2020). In some insect species that reproduce asexually (especially aphids), RNAi activity with decreasing strength has also been observed in subsequent generations. This is probably due to the direct transfer of dsRNA from the mother to her offspring while she is carrying it (Abdellatief et al., 2015).
When using dsRNA in spray form, it is important to distinguish between two main exposure routes due to the different feeding behaviour of insect pests. RNA active ingredients that are particularly effective against leaf-feeding insects, such as the Colorado potato beetle, must be adapted so that they exhibit high surface stability and adhesion. dsRNAs against sucking insects, on the other hand, must be absorbed into the leaf interior (apoplast/intercellular space) and into the vascular tissue (phloem/xylem). One difficulty is protecting the RNA on the plant from environmental influences such as UV radiation or rain. Both in the plant and in the insect, the RNA must remain stable until it can bind the target mRNA (Christiaens et al., 2020; Koch & Petschenka, 2022). There is also evidence that the dsRNA can be absorbed to a small extent via the outer skin (cuticle) of insects (Christiaens et al., 2018; Hoang et al., 2022; Huang et al., 2019; Romeis & Widmer, 2020).

RNA sprays have already been successfully used under laboratory conditions against various harmful insects and arachnids. These include the ladybird (*Henosepilachna vigintioctopunctata*), the Colorado potato beetle (*Leptinotarsa decemlineata*) and the common pollen beetle (*Brassicogethes aeneus*). However, there is still hardly any reliable data from field trials (Koch & Petschenka, 2022) (see Section 2.3.3 ‘State of development’).

### 2.3.2.2 Viruses

RNAi is an important natural defence mechanism of plants against viruses (see Section 2.1.2 ‘RNA interference’). It is therefore not surprising that dsRNAs have proven to be an effective means of controlling viral pathogens. Both transgene-based and exogenous RNAi methods have shown a very high efficacy of 90% against viruses (Koch & Wassenegger, 2021). It has also been shown that exogenous dsRNAs can spread systemically in the plant, which is crucial for their effectiveness against viruses. However, many of these applications require complex pre-treatment of the plants, for example by mechanically damaging the leaves, whereby a dsRNA abrasive solution is manually rubbed onto the leaves. For this reason, the search is currently on for possible formulations that facilitate cellular uptake (see Section 2.3.3.1 ‘Challenges’) (Rank & Koch, 2021).

### 2.3.2.3 Fungi and oomycetes

Various HIGS-based studies have shown that the RNAi approach is also effective against phytopathogenic fungi and oomycetes (a group of organisms that are physiologically similar to fungi but not closely related to them). dsRNA sprays have been successfully used in the laboratory against *Fusarium graminearum*, the pathogen that causes fusarium ear blight (Höfle et al., 2020; Koch et al., 2016, 2019; Werner et al., 2020). However, the ability to take up dsRNAs varies considerably between the fungal and oomycete species tested. Necrotrophic fungi, which feed on dead plant cells, appear to react much more sensitively to exogenous RNAs than biotrophic fungi, which can only take up RNAs from the cell interior of their host plants. It is therefore assumed that, depending on their lifestyle, fungi can take up dsRNAs from the extracellular as well as from the intercellular and intracellular space of the plant. As with other RNA-based applications, the

![Figure 5: Mechanism of action of RNAi using an RNA spray as a crop protection agent.](image)

After entering the plant, a long dsRNA is cleaved by DCL into shorter siRNAs, which are taken up by the target organism and lead to the degradation of a target mRNA (Koch & Petschenka, 2022).
often insufficient stability and limited cellular uptake of RNA is an obstacle to the development of anti-fungal RNA sprays (see Section 2.3.3.1 ‘Challenges’).

2.3.2.4 Bacteria
Most bacterial diseases in plants are difficult to combat. The use of antibiotics for direct control is undesirable due to the risk of resistance development and the associated problems in human and veterinary medicine. Therefore, often only preventive measures against bacterial plant diseases are possible. There are currently only a few studies on the use of HIGS against bacterial pathogens, in particular against plant tumours caused by Agrobacterium tumefaciens (Escobar et al., 2001) and against Pectobacterium carotovorum (Mahmoudi & Soleimani, 2019). No trials with SIGS-based methods are known.

2.3.3 State of development
For a long time, RNAi-based crop protection focused on genetically modified, transgenic plants that express dsRNAs endogenously. Their effectiveness has been demonstrated in many studies, and several plant varieties are already ready for the market (Rank & Koch, 2021). The maize MON87411 (SmartStax® PRO) is already authorised for trade (e.g. EU) and cultivation (e.g. USA, China). In addition to several Cry proteins from the bacterium Bacillus thuringiensis, it also expresses a dsRNA that makes it resistant to the corn rootworm (S. Liu et al., 2020).

RNA sprays were long considered uneconomical because the cost of producing synthetic dsRNAs was very high. Approximately 20 g/ha are required for application in the field (Koch & Petschenka, 2022). This is low compared with conventional pesticides, but prices of several thousand dollars per gram make an RNA-based product significantly more expensive. However, thanks to new cell-free synthesis processes, the price has now fallen to less than one dollar per gram. This has increased interest in the development of RNAi-based pesticides (Rank and Koch 2021; Hoang et al. 2022).

To date, extensive field trials have been carried out in the European Union with the dsRNA-based insecticide Ledprona from GreenLight Biosciences™ (product name Calantha™) against the Colorado potato beetle (Leptinotarsa decemlineata). The effect on the mortality of the beetle larvae was comparable to that of the conventional insecticides spinosad and chlorantraniliprole (Pallis et al., 2022). In 2021, GreenLight Biosciences™ submitted an application to the US Environmental Protection Agency (EPA) to register Ledprona as a new active ingredient (EPA, 2021) and received a tolerance exemption in spring. In December 2023, provisional authorisation was granted for three years, which will subsequently be reviewed (EPA 2023). An application for registration in the EU is also expected.

After acquiring the spray-based RNAi platform BioDirect® to control the bee mite Varroa destructor from Bayer/Monsanto, GreenLight Biosciences™ further developed it into a ready-to-use product (pollinator). This was successfully tested in several large-scale trials, which led to the submission of a product label for registration with the EPA (de Schutter et al., 2022).

In addition, GreenLight Biosciences™ has already developed further dsRNA-based plant protection concepts against the fungi Erysiphe necator, Botrytis, Sclerotinia and Fusarium and successfully evaluated them in the field (although not yet published).

2.3.3.1 Challenges
To be effective, dsRNAs must remain stable for a long time under very diverse conditions. dsRNA that is applied to plant surfaces is exposed to rain and UV radiation. dsRNA that is to act systemically throughout the plant must be taken up by the plant and transported to distant tissues. Depending on the application, it must also reach the inside of the plant cells. After uptake by an insect, it must pass through the digestive tract, be absorbed into the cells and exert its effect there. However, RNA is a relatively unstable, short-lived molecule. This means that the sprayed dsRNA often does not reach the target cells or does not reach them in sufficient quantities. As with the medical applications of RNAi, protection against nucleases and extreme pH values as well as the targeted transport of the RNA pose major challenges (Hoang et al., 2022; Rank & Koch, 2021).

Solutions based on nanomaterials are being sought for many of these problems. dsRNA attached to clay nanosheets (BioClay™/LDH) is less easy to wash off (see Section 10.2.3.1 ‘Layered double hydroxide’). ‘Single-walled carbon nanotubes’ (SWCNT) and carbon quantum dot nanoparticles could help to introduce dsRNA into plant cells and protect it from enzyme activity (see Section 10.2.3.6 ‘Single-walled carbon nanotubes (SWCNT)’ and Section 10.2.3.2 ‘Carbon quantum dots’). To improve stability, the RNA can be chemically modified, as in medical applications. The fact that these nanomaterials and chemical modifications can influence the stability and chemical interactions in the plant, the target organisms and in the environment also leads to new potential risks that should be investigated prior to authorisation (Christiaens et al. 2020; Rank and Koch 2021; Hoang et al. 2022; S. Liu et al. 2020; Romeis and Widmer 2020).

Another difficulty is that it is not yet fully understood
As with all plant protection products, the potential impact of RNAi-based plant protection products on non-target organisms – including humans as users and consumers – of an ecosystem must be clarified (P. M. Bachman et al., 2020; Christiaens et al., 2018; Romeis & Widmer, 2020). The decisive factor here is whether other organisms come into contact with biologically active dsRNAs and take them up. This depends, among other things, on how long these molecules remain stable in the environment. A distinction must also be made between effects that are specific to the RNA sequence and non-specific effects such as immune reactions (Romeis and Widmer 2020).

### 2.3.4.1 In the target organism

#### 2.3.4.1.1 Off-target effects

For effective silencing, relatively long dsRNAs are usually used, which are cleaved by the Dicer into several short siRNAs. As the siRNAs do not have to be fully complementary to the target sequence during base pairing, it is likely that not only the target mRNA is bound, but also other mRNAs that contain a similar sequence (OECD, 2020). In the target organism, however, such off-target effects are unproblematic, as they lead at most to further damage to this organism (S. Liu et al., 2020; OECD, 2020).

#### 2.3.4.1.2 Resistances

There are concerns that single base mutations in the target gene present in the insect population could reduce the effectiveness of RNAi and lead to the evolution of resistance (Scott et al., 2013). However, this risk is low if a pool of different siRNAs or long dsRNAs are used to compensate for the non-binding of individual siRNAs. It is also speculated that mutations in molecules that play a role in the RNAi process and viruses that code for RNAi suppressors could give certain insects an evolutionary advantage (Scott et al., 2013). However, as insects are dependent on a functioning RNAi mechanism, it is unlikely that disruption of this mechanism by mutations or viruses would lead to a selective advantage.

However, the greater danger seems to lie in the fact that insect populations become resistant by preventing the uptake of dsRNA via the gut. This mechanism has been demonstrated for resistant populations of the western corn rootworm *Diabrotica virgifera virgifera* (Khajuria et al., 2018) and the Colorado potato beetle (Mishra et al., 2021) which were selected under artificial laboratory conditions. These populations would therefore also be resistant to dsRNA targeting other genes.

#### 2.3.4.2 Effects on non-target organisms

As with all plant protection products, the potential impact of RNAi-based plant protection products on non-target organisms – including humans as users and consumers – of an ecosystem must be clarified (P. M. Bachman et al., 2016; Christiaens et al., 2018; Romeis & Widmer, 2020). The decisive factor here is whether other organisms come into contact with biologically active dsRNAs and take them up. This depends, among other things, on how long these molecules remain stable in the environment. A distinction must also be made between effects that are specific to the RNA sequence and non-specific effects such as immune reactions (Romeis and Widmer 2020).

#### 2.3.4.2.1 Stability in the environment

In general, naked (i.e. unformulated) RNA does not remain stable for long in the environment. Several studies have shown that naked dsRNAs could no longer be detected 48 hours after being applied to clayey soils and after seven days in aquatic systems with different sediment types (P. Bachman et al., 2020). Naked dsRNA that had been sprayed onto the leaves of potatoes was difficult to wash off after drying and proved to be significantly more UV-resistant than on glass surfaces. Even four weeks after spraying, this dsRNA was still biologically active. However, it is unclear whether the dsRNAs actually remained on the surface or whether they had been taken up into the plant (S. Liu et al., 2020).

If formulated dsRNA is used, the formulations (see Section 10.2 ‘Delivery in plant protection’) significantly influence their stability. For example, dsRNAs bound to BioClay/LDH could still be detected on the tobacco plant (*Nicotiana tabacum*) 30 days after spraying, while naked dsRNAs were barely detectable after 20 days (Mitter et al., 2017). It was also shown that carbon quantum dots protect dsRNAs from degradation by RNase A for at least 30 days (Wang et al., 2023).

#### 2.3.4.2.2 Sequence-specific effects

When non-target arthropods come into contact with biologically active dsRNA, it is possible that they take it up and process it using their RNAi machinery. The likelihood of this happening depends strongly on various factors, such as the species or the length of the RNA (Romeis & Widmer, 2020). If the non-target arthropod expresses an mRNA whose sequence is sufficiently similar to the target sequence, unintentional gene silencing may occur. The probability of this can be reduced by choosing suitable sequences for the dsRNAs. When a larger number of insect genomes are decoded and available in the future, potential sequence matches in non-target organisms should be easier to predict bioinformatically. However, purely bioinformatic predictions may overestimate the effects. It is also important to consider whether the non-target organisms actually take up the dsRNAs and whether they have a harmful effect on them. Furthermore, the effects of siRNAs can be positively or negatively influenced by the presence of other naturally occurring siRNAs (S. Liu et al., 2020).
2.3.4.2.3 Sequence-unspecific effects
It is known that dsRNAs can trigger an immune response in mammals, including humans. Whether the uptake of dsRNAs via food and feed plants previously sprayed with RNA has an effect on the immune system is still unclear. However, early reports of the presence of dietary dsRNA in mammalian body fluids and tissues have not yet been confirmed (Kang et al., 2017). This also seems rather unlikely due to the various physical and biochemical barriers that would have to be overcome. Nevertheless, the probability of such undesirable effects increases if the dsRNAs are modified (see Section 2.2.1.4 ‘Chemical modifications’) in order to increase their stability and cellular uptake (S. Liu et al., 2020). Non-specific immune responses are also observed in non-target insects when high doses of dsRNA are ingested or injected (Christiaens et al., 2018; OECD, 2020; Romeis & Widmer, 2020; Weinstock et al., 2006).

2.3.4.3 Contamination
dsRNA for use in RNA sprays can be produced either by chemical synthesis, by in vitro transcription, in bacterial cell cultures or in cell-free systems. In chemical synthesis, in vitro transcription and most bacterial methods, a single strand is synthesised first and then a complementary strand is added. Using RNA bacteriophages – viruses that infect bacteria – dsRNAs can also be synthesised directly. Contamination can occur with all these methods if the dsRNA is not carefully purified. However, this can be prevented by quality control measures. In addition, dsRNA can now be produced in large quantities using cell-free systems at low cost and without residues (Dietz-Pfeilstetter, 2021).
3 mRNA technologies

3.1 Introduction

Messenger RNAs (mRNAs) are single-stranded ribonucleic acids that carry the information for the production of a protein. The protein is produced in the cytoplasm of the cell. The possibility of introducing artificially produced mRNAs into a cell and producing practically any desired protein opens up many potential applications.

The best established mRNA-based application to date are the mRNA vaccines. These introduce an mRNA molecule containing the genetic information for a specific protein of a pathogen into body cells, where the protein is subsequently produced. This protein is then recognised as foreign by the immune system and stimulates an immune response that ultimately leads to immunisation against the pathogen. Compared to conventional vaccines, mRNA vaccines can be produced very quickly using cell-free processes. They can also be quickly adapted to mutations in the pathogen. The method is also suitable for pathogens against which no conventional vaccines are currently available. The method can even be used against cancer cells (Chaudhary et al., 2021).

Another application of synthetic mRNAs is passive immunotherapy. Antibodies have proven to be extremely effective in the treatment of a wide range of diseases – from autoimmune diseases to infectious diseases and various types of cancer. However, their production is very complex and they have to be administered repeatedly. Administering the antibody sequence as mRNAs, which are subsequently translated into proteins in the body, promises simpler production and a longer-lasting effect compared with administration as a protein (Schlake et al., 2019).

An mRNA-based method for replacing or supplementing endogenous proteins that are not produced in the required quantity or have lost their function is also being developed. Here, the corresponding gene product is inserted into the cells as mRNA, where the replacement protein is produced, which then replaces or supplements the endogenous protein (Qin et al., 2022).

This report does not deal with the possibility of modifying the genome using synthetic mRNAs. This involves introducing an mRNA molecule into the cell, which then expresses a protein that can cleave DNA or change bases (genome editing). These include Cas enzymes, TALEN or zinc finger nucleases.

3.2 Description of the procedure

Natural mRNAs fulfil an essential function in the production of proteins in the cell. They are produced in the cell nucleus during transcription by converting the genetic information on the DNA into a precursor of the mRNA, called pre-mRNA. The pre-mRNA is then processed. The resulting mRNA is transported from the cell nucleus into the cytoplasm, where it is read by molecular complexes (ribosomes). Through this process, called translation, various amino acids are assembled into a specific protein according to the sequence information contained in the mRNA (Qin et al., 2022).

During processing in the cell nucleus, certain sequences known as introns are cut out of the pre-mRNA so that only the regions known as exons remain. This step is known as splicing. By cutting different sequences from the pre-mRNA (alternative splicing), different mRNAs can be created from one pre-mRNA molecule. In addition, a structure called a cap is attached to one end (5’-end) of the pre-mRNA and a series of adenine nucleotides – called poly(A) tails – are attached to the other end (3’-end). These protect the RNA from enzymatic degradation by nucleases. They are also a prerequisite for transport out of the cell nucleus and for translation. The sequence that contains the blueprint for the protein – called the open reading frame (ORF) – is flanked by two shorter sequences that are not translated into a protein. These sequences are called untranslated regions (UTR) and are important for the stability and correct function of the mRNA. In particular, they contain binding sites for proteins that influence the stability and transport of the mRNA and initiate translation. In addition, miRNAs can bind in the 3’-UTR and thereby regulate the stability and translation of the mRNA (see Section 2.1.2 ‘RNA interference’) (Qin et al., 2022).

For medical applications, the open reading frame together with the two non-translated regions is first synthesised as DNA, which is then transcribed. The poly(A) tail is often synthesised together with the open reading frame but can also be added to the mRNA by a specialised enzyme. The cap structure can be added either during transcription or later with enzymes. The synthetic mRNA is then introduced into the somatic cells, where it is translated into a protein in the cytoplasm of the cell (Qin et al., 2022).

There are various ways in which mRNA can be introduced into body cells. The simplest way is to inject ‘naked’ mRNA – i.e. an mRNA molecule without a coating –
into the muscle, skin or a tumour, for example. However, mRNA, like all RNAs, is unstable and is quickly degraded by enzymes, often before it can develop its primary effect in the target cell. For this reason, various delivery systems have been and are being developed that can efficiently introduce mRNAs into the desired cells. Liposomes or lipid-based vectors such as lipid nanoparticles, which are composed of natural and artificial lipids, are currently the most widely used. In contrast to liposomes, lipid nanoparticles have a core of lipids and molecules with lipid-like properties in which the active ingredients are encapsulated. Research is also being carried out on polymer- or peptide-based nanoparticles or lipid-polymer hybrid nanoparticles. The section that codes for the protein can also be incorporated into the genome of a virus, which then introduces the genetic information into the cell (see Section 3.4.5 ‘Challenges’ and Section 10.1 ‘Medical delivery methods’) (Gupta et al., 2021).

3.3 Applications

3.3.1 mRNA vaccines

Conventional vaccines contain an attenuated or killed pathogen – or parts of it – which is recognised by the immune system after vaccination and thus leads to immunisation against the pathogen (Krammer, 2020). This procedure has proven to be very successful for a number of infectious diseases, such as smallpox or poliomyelitis. However, the method has also been shown to have limited or no effect on many pathogens. This is particularly the case when these are able to mutate quickly and thus evade the immune response. As the development of new conventional vaccines takes a relatively long time, a reaction to a new or mutated pathogen can only occur with a time delay.

In mRNA vaccines, no pathogens are administered, only the blueprint for certain proteins of the pathogen. These proteins, known as antigens, are then produced in the body’s cells through translation of the mRNA and recognised as foreign by the immune system (see Figure 6).

mRNA vaccines have various advantages over conventional vaccines. They can be produced, scaled up and customised very quickly in a cell-free process, which makes it possible to react quickly to mutations. An mRNA can also carry the information of several proteins simultaneously, and it does not integrate into the genome, which is located in the cell nucleus. As the proteins are produced in the body’s own cells, the glycosylation also corresponds to that of natural proteins. In addition, mRNA vaccines have the potential to activate the immune system not only against pathogens, but also against cancer cells (Chaudhary et al., 2021).

Antigen-presenting cells, such as dendritic cells, play an important role in recognising antigens and the subsequent immune response. They take up antigens and present them to the B and T cells of the immune system. Instead of injecting the mRNA into the muscle, it is therefore also possible to remove dendritic cells from the patient and insert the mRNA directly into these cells. The dendritic cells are then injected under the skin, where they trigger the immune response. This approach is much more complex than the direct injection of mRNAs, but has proven to be potentially effective, especially in vaccinations against cancer cells (Qin et al., 2022).

A rough distinction can be made between three categories of mRNA vaccines: 1) non-nucleoside-modified mRNAs, 2) nucleoside-modified mRNAs and 3) self-amplifying non-nucleoside-modified mRNAs. Non-nucleoside-modified mRNAs contain only the four canonical bases A, C, G and U. In nucleoside-modified mRNAs, one or more canonical bases have been replaced by modified bases to prevent the mRNA from being recognised by the innate immune system and to improve translation. These are mainly pseudouridine, 1-methylpseudouridine or 5-methylcytidine. Self-amplifying mRNAs differ from conventional mRNAs in that they not only contain the genetic information for the antigen, but also for several proteins that are needed by viruses to replicate their RNA. This allows the mRNA to replicate itself after being introduced into the cell. Therefore, very little initial mRNA is sufficient to achieve immunisation in this process. In addition, dsRNA structures are formed during amplification, which are recognised by the innate immune system and thus enhance the effect of the vaccine (Pardi et al., 2018).

3.3.2 Antibodies

For some years now, antibody-based therapies have been among the most promising medical interventions against a number of diseases that were previously difficult to treat. They have proven to be effective against viral and bacterial infections, autoimmune diseases and various types of cancer. This form of therapy uses so-called monoclonal antibodies that specifically bind to a certain section (epitope) of a target molecule, thereby blocking it in a targeted manner. The antibodies are usually produced in cell lines from mice or humans that have come into contact with the antigen. Alternatively, there are also so-called recombinant monoclonal antibodies. These can also be produced in vitro without prior immunisation and therefore more quickly. However, these production
methods are very time-consuming and the purified antibodies must then be constantly stored and transported under refrigeration until they are administered (Lu et al., 2020). This time-consuming and expensive production process can be avoided by introducing only the genetic information in the form of mRNA into the body cells and producing a certain amount of antibodies there for a short time, which can then perform their function in the body (Qin et al., 2022).

### 3.3.3 mRNA-based protein replacement therapies

In mRNA replacement therapies, cellular proteins that are not produced in sufficient quantities or are not regulated correctly are replaced or supplemented. This is done by introducing the mRNA of the corresponding protein into the cell and expressing it. Replacement therapies are particularly suitable for the treatment of rare genetic metabolic diseases and hereditary diseases such as muscular dystrophies or cystic fibrosis (Xiao et al., 2022).

### 3.4 State of development

#### 3.4.1 Vaccines

The possibility of using mRNAs as a vaccine was considered more than 30 years ago. The first promising trials were carried out in animals in the early 1990s and in humans in the early 2000s (Pascolo, 2021). However, despite these early successes and all the potential benefits, this technology was long considered to be unpromising. This was mainly due to the fact that the mRNA molecules were considered too unstable to be used effectively as medical devices. A further difficulty was achieving adequate stimulation of the immune system. During the SARS-CoV-2 pandemic, mRNA vaccines finally established themselves as the most promising approach within a short space of time and proved their effectiveness. Not least because of this success, research into mRNA vaccines against other pathogens and cancer cells has also increased significantly in recent years (Chaudhary et al., 2021).

![Figure 6: How mRNA technologies work.](image)

mRNAs are packaged in nanoparticles and taken up into the cell by endocytosis. The mRNA is released from the endosome and the nanoparticle is dissolved. The mRNA is translated in the cytoplasm and the resulting protein either remains in the cytosol, is integrated into the cell membrane or is secreted (Bhat et al., 2021).
3.4.1 Infectious diseases
So far, mRNA vaccines have only been authorised against the SARS-CoV-2 coronavirus. The first two mRNA vaccines received emergency use authorisation from the US Food and Drug Administration (FDA) in 2020 and were also approved in the EU and Switzerland shortly afterwards. Both the Comirnaty® vaccine developed by BioNTech™ and the Spikevax® vaccine from Moderna®, contain the mRNA of the spike glycoprotein of SARS-CoV-2. The mRNAs were embedded in lipid nanoparticles and the uridine bases were replaced by N1-methylpseudoouridine. Since then, several billion vaccine doses have been administered worldwide (Qin et al., 2022). Vaccines adapted to new virus variants from these manufacturers have already been authorised or are still in the clinical test phase. As of February 2024, in addition to the vaccines from BioNTech™ and Moderna®, the self-amplifying non-modified mRNA HGCO19 from Gennovo in India, ARCoV (PLA Academy of Military Science / Walvax Biotechnology® / Suzhou Abogen Biosciences™) in Indonesia and SYS6006 (CSPC Pharmaceutical Group™) in China have been approved. Four others are in clinical phase III and six in phase II. Vaccines against cytomegaloviruses, influenza viruses and the human respiratory syncytial virus (RSV) are also in clinical phase III. Vaccinations against a number of other viruses, including the human immunodeficiency virus (HIV), rabies and Eppstein-Barr, as well as against various bacteria and parasites are still in the preclinical or early clinical phase (vfa, 2023).

3.4.1.2 Cancer
mRNA vaccines for cancer treatment have been investigated in preclinical and clinical studies and have shown promising results in various types of cancer. To date, nine clinical trials with mRNA vaccines against cancer cells have been completed. 17 additional phase I and II trials with lipid-coated mRNA are ongoing or in the process of recruiting subjects. In July 2023, Merck and Moderna announced the start of a Phase III trial of their personalised mRNA-4157 vaccine against melanoma (Carvalho, 2023). mRNA-based vaccinations with dendritic cells are ongoing or in the process towards. Both the Comirnaty® vaccine developed by BioNTech™ and the Spikevax® vaccine from Moderna®, contain the mRNA of the spike glycoprotein of SARS-CoV-2. The mRNAs were embedded in lipid nanoparticles and the uridine bases were replaced by N1-methylpseudoouridine. Since then, several billion vaccine doses have been administered worldwide (Qin et al., 2022). Vaccines adapted to new virus variants from these manufacturers have already been authorised or are still in the clinical test phase. As of February 2024, in addition to the vaccines from BioNTech™ and Moderna®, the self-amplifying non-modified mRNA HGCO19 from Gennovo in India, ARCoV (PLA Academy of Military Science / Walvax Biotechnology® / Suzhou Abogen Biosciences™) in Indonesia and SYS6006 (CSPC Pharmaceutical Group™) in China have been approved. Four others are in clinical phase III and six in phase II. Vaccines against cytomegaloviruses, influenza viruses and the human respiratory syncytial virus (RSV) are also in clinical phase III. Vaccinations against a number of other viruses, including the human immunodeficiency virus (HIV), rabies and Eppstein-Barr, as well as against various bacteria and parasites are still in the preclinical or early clinical phase (vfa, 2023).

3.4.2 Antibodies
Most mRNA-based antibody therapies are still in the preclinical phase. Only one antibody against the chikungunya virus is currently undergoing clinical testing. Antibodies against the Zika virus, HIV and human RSV have been successfully tested in mice. The monoclonal antibody trastuzumab, which has been approved against certain types of breast and stomach cancer, was successfully administered to mice as mRNA (Qin et al., 2022).

3.4.3 mRNA-based protein replacement therapies
Intensive research is currently being conducted into protein replacement therapies. In particular, therapies are being sought for heart and lung diseases, haematopoietic disorders, orthopaedic diseases, neurological diseases, metabolic diseases and cancer. However, the vast majority of these therapies still have preclinical status. The only two mRNAs that are in the clinical phase code for the vascular endothelial growth factor (VEGF) to regrow blood vessels in the heart and for the cell surface protein ‘cystic fibrosis transmembrane conductance regulator’ (CFTR) for the treatment of cystic fibrosis (Qin et al., 2022).

3.4.4 Veterinary medicine
Although many of the diseases against which mRNA vaccines are being developed can be transmitted by animals, there are as yet no applications of mRNA technologies in veterinary medicine. Unlike in human medicine, no mRNA vaccines have been approved for the vaccination of domestic and zoo animals against SARS-CoV-2, but protein vaccines and attenuated viruses (Sharun et al., 2021). An experimental vaccine against rabies is currently under development and has already been successfully tested in mice and dogs (Li et al., 2022). The progress made in the development of mRNA vaccines for humans and the successful trials with various vaccines in laboratory animals suggest that this technology will also be increasingly used in veterinary medicine in the future (Aida et al., 2021).

3.4.5 Challenges
Transporting the mRNA to and into the desired cells remains a major challenge. mRNA – like all single-stranded nucleic acids – is negatively charged and therefore has difficulty penetrating the negatively charged cell membrane. It is also very unstable in cells and is degraded within a few hours. Even if it has successfully penetrated the cell, there is a high probability that it will be trapped in an endosome, a membrane-enclosed organelle, and thus deactivated. Suitable delivery systems consisting of liposomes or polymers protect the mRNA from degradation, promote uptake by the cell and allow the mRNA to escape from the endosomes. However, these systems must not be harmful and must not trigger an immune response. In this way, mRNAs can now be reliably and specifically
introduced into various liver cells (hepatocytes, Kupffer cells and endothelial cells). Lipid nanoparticles, which are composed of various lipids with different properties, are most frequently used for this purpose. Nanoparticles made of biodegradable polymers and cationic nanoemulsions can also be used (see Section 10.1 ‘Medical delivery methods’) (Qin et al., 2022).

Non-endogenous mRNAs, in particular by-products of in vitro production, which may be present in the preparations (such as dsRNA), are recognised by various receptors of the innate immune system. On the one hand, this can hinder protein production and thus the effectiveness of immunisation. On the other hand, with mRNA vaccines, activation of the innate immune system is also an important factor for the activation and maturation of dendritic cells, which ensure effective and long-lasting immunity. The challenge is therefore to optimise the immunostimulatory properties of the mRNA in such a way that a reaction of the immune system is triggered without negatively affecting translation and without causing severe side effects. The choice of transport system can also influence the immune response. For example, there are lipid nanoparticles with cyclic lipids that trigger a specific immune response (Pardi et al., 2018). When administered intramuscularly, this activation of the immune system has been achieved without serious negative side effects in both nucleoside-modified and non-modified COVID-19 vaccines (Barbier et al., 2022).

The previously authorised mRNA vaccines must be stored deep-frozen at −90 °C to −60 °C and only have a relatively limited shelf life. Even if the shelf life has now been improved, the storage requirements restrict the areas of application. Here, too, it is expected that stability at higher room temperatures can be improved by means of improved delivery methods, such as lyophilised liposomes (Barbier et al., 2022).

3.5 Undesirable effects

Before the coronavirus pandemic, most mRNA technologies had only been tested on animals, and many of the clinical trials are still at an early stage. After the outbreak of the pandemic, a lot of resources were invested globally in vaccine research and development and the approval processes were accelerated, which enabled clinical trials and subsequent approval of the vaccines within a short period of time. An important aspect in the development of mRNA agents concerns the immune response: with mRNA vaccines, this must be optimised so that it is strong enough for immunisation while keeping side effects to a minimum. In contrast, mRNA-based antibodies and protein replacement therapies require the lowest possible immune response in order to minimise side effects (Barbier et al., 2022). Serious side effects were rarely observed in the context of vaccination against SARS-CoV-2. However, more cases of myocarditis, Bell’s palsy, cerebral venous and sinus thrombosis, Guillain-Barré syndrome and a number of other disorders of the vascular and immune system have been recorded. As these also occur more frequently after infection with SARS-CoV-2, it is speculated that the spike protein encoded on the mRNA is responsible for this (Trougakos et al., 2022). Anaphylactic reactions have also occurred. It is assumed that these are due to the presence of the polymer polyethylene (PEG) in the lipid envelope (Chaudhary et al., 2021). New formulations (liposomes, LNP, polyplexes) of mRNA (modified or unmodified) and alternative injection sites (e.g. subcutaneous) are being tested to further improve mRNA vaccines.
4 RNA aptamers

4.1 Introduction

As early as 1967, it was shown that RNA coding for a protein, which in turn replicates the RNA, can evolve in a cell-free medium through random mutations and subsequent selection and thus be optimised for specific requirements (Mills et al., 1967). In 1990, a method called SELEX (systematic evolution of ligands by exponential enrichment) was developed (see Section 4.2.3 ‘SELEX’). Under this method, a series of selection steps can be used to generate RNAs that bind very specifically to a molecule thanks to their three-dimensional structure (Tuerk & Gold, 1990). In the early 2000s, natural RNAs (riboswitches) were discovered in bacteria that recognise certain metabolites and regulate metabolism in this way (Nudler & Mironov, 2004).

4.2 Description of the procedure

RNA aptamers are short (10–120 nt), non-coding, single-stranded RNA sequences (RNA oligonucleotides). Unlike non-coding RNAs in post-transcriptional silencing (see Section 2.1.2 ‘RNA interference’), they do not bind to other ribonucleic acids through base pairing but recognise and bind a specific target molecule through their three-dimensional configuration, similar to monoclonal antibodies. This can either activate or inactivate the target molecule. In addition to RNA aptamers, there are also DNA aptamers and peptide aptamers. In the following sections, aptamers always refer to RNA aptamers.

4.2.1 Binding to target molecules

The aptamers bind to their target molecules through van der Waals forces, hydrogen bonds, electrostatic interactions, stacking of flat aromatic ligands and shape complementarity. Small target molecules are integrated into the aptamer, while large target molecules, such as surface proteins, have the aptamer inserted into the structure of the molecule (Kohlberger & Gadermaier, 2021).

4.2.2 Aptamers vs antibodies

Aptamers have a similar function to antibodies but have certain advantages over them that make them particularly interesting for therapeutic and diagnostic applications. RNA aptamers can fold back into their functional structure even after several denaturations (i.e. the loss of their structure). As a result, they remain stable for several years even at room temperature under sterile conditions. Antibodies, on the other hand, are much more sensitive to temperature; they have to be stored cold (2–8 °C) and denature irreversibly at excessively high temperatures. Aptamers can be chemically synthesised. In addition, an in-vitro SELEX process usually only takes 2–8 weeks. This makes them cheap to produce and there are hardly any differences in quality between different batches. They can also be chemically modified relatively easily in this process (see Section 4.2.4 ‘Chemical modifications’). While the production of antibodies requires a strong immune response against the target molecule, aptamers can be optimised for a wide range of targets, from small molecules to cells, and hardly trigger an immune response when unmodified. This means that aptamers can also be obtained against molecules that are evolutionarily conserved and therefore do not trigger an immune response in animals. Aptamers are significantly smaller than antibodies and can penetrate many tissues better than antibodies. They have a high affinity and specificity towards the target molecule (Chen et al., 2015). In contrast, the affinity of antibodies depends on the number of binding sites (epitopes) on the antigen, and different antibodies can bind the same antigen (Kohlberger & Gadermaier, 2021).

4.2.3 SELEX

Aptamers that bind to specific molecules are selected using a process called SELEX. The target molecule is incubated together with $10^{14}$–$10^{16}$ random single-stranded RNA oligonucleotides (called the aptamer library). These oligonucleotides usually consist of 40–100 nucleotides with random regions in the centre and constant sequences at the ends with binding sites for amplification by the polymerase chain reaction (PCR). The non-binding oligonucleotides are then washed away, while the binding oligonucleotides are reverse-transcribed, i.e. converted into DNA, and then amplified by PCR. These DNA oligonucleotides are then transcribed and the resulting RNA oligonucleotides are used as a library for the next selection run. In several cycles, the aptamers that have a particularly high affinity and specificity for the target molecule are filtered out. At the end of the process, they are finally sequenced. In addition, aptamers that bind an undesired molecule can also be selected (counter selection). No prior knowledge of the target molecule is required for the SELEX process (Kohlberger & Gadermaier, 2021).
SELEX is often combined with other methods to optimise the specificity for certain target molecules or the efficiency of the process (e.g. Capillary Electrophoresis-SELEX, Microfluidic-SELEX or Atomic Force Microscopy-SELEX) (Y. Zhang et al., 2019).

One further development that has attracted particular attention is Cell-SELEX. Cell-SELEX is used to select aptamers that bind to surface proteins of living cells in vitro. The method is based on the fact that the target cells (e.g. tumour cells) have a specific expression pattern of the surface proteins. Aptamer selection is first carried out on a negative control (usually healthy cells). The unbound aptamers are then used as a pool for SELEX with the target cell line. Aptamers can also be specifically selected that are taken up by the target cells when they bind a surface protein (cell-internalisation SELEX) (Sola et al., 2020).

For in vivo SELEX, the aptamer libraries are injected into cancerous or pathogen-infected animals, usually mice, and then those aptamers that have bound to the affected tissue are amplified (Sola et al., 2020).

### 4.2.4 Chemical modifications

Aptamers can be modified to make them more specific and more affine for the target molecules and optimised for in vivo applications. They are therefore very rarely used unmodified in therapeutic applications (see Section 2.2.1.4 ‘Chemical modifications’).

#### 4.2.4.1 Nuclease activity

Unmodified aptamers often remain active in vivo for only a few minutes because they are very quickly digested by nucleases. In order to extend their half-life in the body, most aptamers are chemically modified so that they are no longer recognised by nucleases.

Aptamers can be chemically modified during selection (In-SELEX) or after selection (Post-SELEX). In-SELEX has the advantage of ensuring that the RNA remains functional despite modification. However, special polymerases are required for amplification because the modified RNA is not recognised by natural polymerases. Pegaptanib (Macugen®), a drug against macular degeneration and the first authorised aptamer therapeutic, was modified in this way. Post-SELEX, the nucleotides at the ends of the aptamer (5’ or 3’ position) are usually modified (Chandola & Neerathilingam, 2019).

Another way to avoid hydrolysis by nucleases is to use L-enantiomers of the ribonucleotides (called L-RNA aptamers or Spiegelmers). Here, a mirrored version of the target molecule is synthesised. This L-molecule is used as the target molecule in the SELEX process. The D-oligonucleotide that binds best is then sequenced and synthesised with L-nucleotides. The resulting L-aptamer should bind the natural D-molecule as strongly as the D-aptamer binds the L-molecule. Since natural nucleases can only bind D-oligonucleotides, L-aptamers are protected from hydrolysis (Vater & Klussmann, 2015).

#### 4.2.4.2 Aptamer diversity

Another reason for chemical modifications is the increase in oligonucleotide diversity, which increases the probability that an aptamer has a high affinity to the target molecule. This can be achieved either via the addition of hydrophobic side chains (SOMAmers), via non-natural nucleotides or via glycosylation (Byun, 2021).

#### 4.2.4.3 Renal filtration

Due to their small size, aptamers are quickly filtered out of the blood by the kidneys and excreted. This can be prevented by attaching large molecules – such as polyethylene glycol (PEG), liposomes, proteins, cholesterol or various nanomaterials (Kohlberger & Gadermaier, 2021).

### 4.3 Applications

#### 4.3.1 Therapies

##### 4.3.1.1 Target inhibition

Aptamers can bind to a target molecule and have either an activating (agonistic ligand) or inactivating (antagonistic ligand) effect. An aptamer that binds and deactivates the HIV Tat protein was developed as early as 1990. Since then, the catalogue of agonistic and antagonisticaptamers has grown steadily. In 2004, the first aptamer therapeutic (Pegaptanib) was approved for the treatment of age-related wet macular degeneration, a serious eye disease. This aptamer binds a specific isoform of vascular endothelial growth factor (VEGF) and thus prevents it from binding to the VEGF receptor. In all nine RNA aptamer drugs that have been clinically tested to date (Pegaptanib, EYE001, ARC19499, ARC1905, REG1, Nox-E36, Nox-A12, Nox-H94 and BT200), the aptamer binds a signalling molecule (Byun, 2021).

However, aptamers are also being developed that can prevent a virus from entering a cell. To do this, they bind to proteins that are necessary for attachment to the cell. They can also prevent the replication cycle of the virus by binding to proteins that are essential for replication (e.g. capsid proteins). Furthermore, they can compete with viral mRNA for an RNA polymerase binding site (Zou et al., 2019). An RNA aptamer-based drug against SARS-CoV-2 is also under development, which contains several RNA
aptamers that are attached to a gold nanoparticle and bind the cell surface protein ACE2 at various sites. This prevents the virus from binding to ACE2 and thus entering the cell (Sun et al., 2021, 2022).

4.3.1.2 Targeted drug delivery

One of the greatest challenges in the development of cell-specific or tissue-specific therapeutics is ensuring that the active substances reach the target cells and are taken up by them. Cell-SELEX can be used to select aptamers that bind surface proteins typical for certain cell types (see Section 4.2.3 ‘SELEX’) and are taken up by the cell after binding. However, these methods are still at an early stage of development. There are two ways in which aptamers can fulfil this function:

1. Aptamers can be conjugated with a drug (aptamer-drug conjugates (ApDC)) or complexed. In ApDCs, the two molecules are connected by a linker molecule. This can be selected so that it becomes unstable under certain chemical conditions or can be cut by an enzyme, thus releasing the drug. This should make it possible to use the drug in a specific cell compartment or in the acidic environment of a tumour. ApDCs are being tested to deliver cytotoxins or radioisotopes specifically into cancer cells and thereby improve chemotherapy and radiotherapy. ApDCs are also being developed that modulate the immune system and could be used for immunotherapy (G. Zhu & Chen, 2018).

2. Aptamers can also be linked to liposomes that transport the active ingredient. When the aptamer is taken up into the cell, the liposome fuses with the cell membrane and releases the cargo molecule (see Section 10.1.2.1 ‘Lipid nanoparticles and liposomes’). Finally, aptamers can also guide viral vectors to a specific target (Chandola & Neerathilingam, 2019).

Figure 7: SELEX.

First, a library of aptamers is synthesised. The aptamers are then brought into contact with the target molecule. The aptamers that bind the target most strongly are then selected. These are amplified and used for the next run until the binding is strong and specific enough. The resulting aptamers are sequenced (Kohlberger & Gadermaier, 2021).
4.3.2 Diagnostics

Due to their similarity to antibodies, aptamers have the potential to replace them in various diagnostic applications (ELISA, affinity chromatography, etc.). Their high stability, flexibility and affinity make them particularly suitable for patient-oriented laboratory diagnostics (point-of-care instruments) and test kits for analyses in field research (Y. Zhang et al., 2019).

Using SELEX with whole pathogens or purified proteins as targets, aptamers have been selected that target a range of different bacteria (Saad & Faucher, 2021), viruses (e.g. SARS-CoV) (Kim & Lee, 2021) and parasites (e.g. Tryptanosoma cruzi) (Ospina-Villa et al., 2020). Cell-SELEX can also be used to develop new cancer biomarkers (Yan & Levy, 2018).

The most widely used aptamer-based biosensors (aptasensors) are so-called duplexed aptamers (DA), which are composed of two hybridised aptamers. One of the two aptamers can bind the target molecule as well as the other aptamer. When this happens, the aptamers separate and an optical or electrochemical signal is generated that can be measured (Munzar et al., 2019).

With a few exceptions, all of these technologies are in the development phase and are not yet ready for the market (see Section 4.5 ‘State of development’).

4.3.3 Applications in agriculture

There are as yet no applications in plants. However, initial experiments with RNA aptamers are underway in basic research. For example, it has been shown that RNA aptamers can be expressed as transgenes in plant cells and can inhibit protein function (Abdeeva et al., 2019).

4.4 Unintended changes and effects

Aptamers have a short half-life in the body and a high specificity. No off-target effects due to binding to non-target molecules have been observed to date. There are also no known interactions with other drugs. In toxicological studies, no activation of the immune or complement system has been observed with unmodified aptamers to date (Kovacevic et al., 2018). However, chemical modifications or binding to other large molecules (conjugations) can trigger an immunogenic or cytotoxic effect. For example, LNA (locked nucleic acids) – nucleic acids in which the sugar portion is chemically modified in a non-natural way – have shown strong hepatotoxicity. 2’-fluoropyrimidine-modified RNA aptamers lead to cytotoxicity and induce the expression of interferon-beta in human cancer cells in vitro. PEG, which is added to many aptamers to achieve a longer half-life in the blood, can trigger allergic reactions in rare cases (Chandola & Neerathilingam, 2019). In Phase IIb clinical trials of the aptamers pegnivacogin and anivamersen conjugated with PEG, one per cent of subjects experienced allergic reactions to PEG, and clinical trials with the anticoagulator Reg1 were discontinued due to severe allergic reactions (Povsic et al., 2016).

Because the expression levels of the surface proteins are unknown in Cell-SELEX, it is possible that aptamers are selected against certain surface proteins that are also frequently present on healthy cells and that these are therefore also targeted. This risk can be minimised by several rounds of negative selection (Chandola & Neerathilingam, 2019). If the aptamers are bound to liposomes, additional liposome-specific adverse effects may occur (see Section 10.1.2.1 ‘Lipid nanoparticles and liposomes’).

4.5 State of development

Aptamers are used relatively widely in basic research. However, only a few aptamer-based drugs have made it into the clinical phase. Pegaptanib (Macugen®), the only RNA aptamer drug available on the market to date, received FDA approval for the treatment of macular degeneration in 2004. After a successful market launch, demand quickly declined due to competing drugs and it became a niche product. Since then, no aptamer-based drug has reached the market. Especially after the failure of the anticoagulant Reg1 in phase III trials, only a few aptamer drugs have reached the clinical phase. There are various reasons for this. The pharmacokinetic properties are relatively difficult to control. Aptamers can degrade, be excreted or be involved in metabolic processes, which can strongly influence the duration of the effect. To prevent degradation by nucleases and rapid excretion via the kidneys, aptamers are chemically modified, but this can have a negative impact on tolerability (see Section 2.3.4 ‘Unintended effects’). Another important reason is that aptamer binding to the target molecule and therapeutic effects are often not reproducible, which reduces confidence in the technology (Yan & Levy, 2018). The selection of aptamers is highly dependent on the conditions during the SELEX process. Even small differences in pH, the temperature of the buffer solution or the duration of the incubation can influence the selection. The conditions in the SELEX process also differ greatly from those in the natural environment of the molecule. In particular, the presence of ions or molecules with which the aptamer interacts electrostatically can strongly influence the structure, binding affinity and specificity. Therefore, a high binding affinity in vitro does not
guarantee that a similarly high affinity will be achieved in vivo (Byun, 2021).

In recent years, the use of aptamers for the targeted delivery of drugs has sparked interest (see Section 4.3.1.2 ‘Targeted drug delivery’). However, the aptamer-nanoparticle compounds are rapidly degraded in vivo. It is assumed that the nanoparticles are recognised by the immune system, that the nanoparticles aggregate or that the aptamers are cut enzymatically (Byun, 2021).

Aptamer-based instruments for diagnostics and environmental analyses (e.g. detection systems for microbes in water samples) are of great interest to both research and industry because aptasensors are relatively easy, quick and cheap to produce. However, one obstacle to their use in diagnostics is the lack of standardised protocols. Aptamers generated by the same process against the same target molecule can differ in their primary structures, binding affinities, specificities and other chemical parameters. Therefore, a protocol developed for one aptamer cannot be used for another. However, standardised kits and protocols – based on well-characterised aptamers – are essential for use in human diagnostics (Byun, 2021). For this reason, aptamer biosensors have so far mainly been used in basic research. No RNA aptamer biosensor has yet been approved for clinical use. In Indonesia, however, the DNA aptamer-based SARS-CoV-2 diagnostic kit ‘Aptamex’ has been on the market since 2022, and the company SomaLogic offers a SOMAmer-based proteome assay for non-clinical research with the ‘SomaScan’ platform (Grand View Research, 2023).
5 Long non-coding RNAs

5.1 Introduction

Long non-coding RNAs (lncRNAs) are all RNAs that are more than 200 nucleotides long and do not code for a protein. This broad definition means that this class includes a very diverse collection of transcripts. As of February 2024, more than 20,000 lncRNAs are known in the human genome (Frankish et al. 2021). Although no biological function is yet known for most of these lncRNAs, it is becoming increasingly clear that some lncRNAs play an important role in the regulation of various cellular processes (Esposito et al., 2022; S. J. Liu et al., 2017; S. Zhu et al., 2016).

lncRNAs exhibit a broad spectrum of possible interaction mechanisms because, thanks to their length, they not only bind other ribonucleic acids by base pairing but can also form various three-dimensional structures (see Figure 8). In this way, they are able to form three-dimensional structures similar to siRNAs or miRNAs (see Chapter 2 ‘Post-transcriptional gene silencing’), in order to bind certain mRNAs by base pairing and influence their stability or translation into proteins. However, they can also bind complementary DNA sequences, forming either a so-called triplex structure with both DNA strands or a loop known as an R-loop. Finally, they can also bind to certain proteins due to their three-dimensional structure (Mercer et al., 2022; Statello et al., 2020).

LncRNAs are formed in a similar way to mRNAs. The reading of DNA by a protein known as RNA polymerase produces a transcript, which is usually spliced and receives a cap structure at one end and usually a poly(A) tail at the other end (see Section 3.2 ‘Description of the procedure’). Unlike mRNAs, however, not all lncRNAs are transported from the cell nucleus into the cytoplasm (Statello et al., 2020).

![Figure 8: Functional mechanisms of lncRNA.](image)

A) Bind and sequestrate proteins  B) Bind enzymes as co-factor
C) Target and modulate mRNA stability  D) Modulate mRNA translation
E) Triplex-mediated DNA interactions  F) R-loop DNA interactions

IncRNAs remaining in the cell nucleus can interact with the DNA or with proteins involved in transcription. In this way, they activate or suppress the transcription of certain genes. Many of the genes known to date that are regulated in this way are important for different developmental phases, such as certain phases of embryonic development in animals or flowering in plants. In some cases, they cause a change in the structure and function of chromosomes in the cell nucleus. The IncRNA XIST, for example, induces the inactivation of one of the two X chromosomes in female mammals. By interacting with mRNAs, IncRNAs can regulate the splicing of mRNAs in a similar way to short non-coding RNAs (siRNAs and miRNAs). IncRNAs also regulate various proteins that are responsible for DNA repair and the control of cell division (Statello et al., 2020).

In addition, many IncRNAs are also localised in the cytoplasm or in organelles. By binding to mRNAs, for example, they can alter their stability and translation rate or influence the transmission of signals within the cell. Other IncRNAs bind miRNAs or proteins and reduce their availability (Noh et al., 2018).

The various functions of IncRNAs in the regulation of essential cellular processes – such as development, DNA repair and cell division – are the reason why some incorrectly regulated IncRNAs can contribute to the development of cancer. These play a role in particular in the formation of metastases, immune escape, changes in cell metabolism and vascularisation in tumours. They are also important in diseases of the vascular and nervous system and in diabetes (Mercer et al., 2022).

5.2 Description of the procedure and applications

As IncRNAs can regulate cellular processes, they also have the potential to be used as active substances themselves. However, the size of the molecules makes it difficult to deliver them into the cell; furthermore, they may activate the immune system. This could be prevented by isolating the functional regions and constructing a shorter RNA with the same properties. This has already been achieved in the mouse model with the IncRNA NRON, which can slow down bone loss in osteoporosis (Mercer et al., 2022).

5.3 State of development

So far, IncRNA-based therapies have only been tested in preclinical studies. Even though various mouse studies have already shown that IncRNA-based therapies are possible in principle, no candidate has yet made it into the clinical phase (Mercer et al., 2022).

5.3.1 Challenges

The transport of long RNAs in sufficient quantities into the right tissues and cells, and additionally into the desired cell compartments, has proven to be difficult. In addition, off-target effects are relatively common. Studies in animals are complicated by the fact that IncRNAs are relatively poorly conserved evolutionarily and therefore IncRNAs in experimental animals sometimes differ greatly from their counterparts in humans. In such cases, it is often necessary to genetically modify model organisms accordingly. In preclinical cancer studies, human tissue is also transplanted into model organisms or artificial organs, so-called organoids. (Mercer et al., 2022).

5.4 Possible adverse effects

Due to their length, the risk of IncRNAs binding unintended RNA or DNA sequences is relatively high. As IncRNAs are normally only present in small quantities, there is also a risk that too high a dose could lead to the binding of proteins that are needed for other cellular tasks. In addition, the IncRNAs can enter non-target cells. It is still unclear how strongly the human immune system reacts to IncRNAs (Mercer et al., 2022).
6 RNA-directed DNA methylation

6.1 Introduction

In plants, short non-coding RNAs can regulate gene expression not only after transcription, as described in Chapter 2 ‘Post-transcriptional gene silencing’, but also before transcription. This process is known as RNA-directed DNA methylation (RdDM). Methyl groups are added to the DNA, making genes located in this DNA region less accessible to proteins and therefore less likely to be transcribed. This process is often used naturally to suppress genes called transposons, which can otherwise jump around in the genome and influence other genes. However, it can also regulate genes that are involved in the development or reproduction of the plant. Finally, RdDM can also be a response of the plant to stress. DNA methylation can be inherited over several generations (Erdmann & Picard, 2020). How DNA is remethylated in humans and other animals is still relatively poorly understood.

6.2 Description of the procedure

As in RNA interference, a double-stranded RNA is first truncated and then bound by a protein from the Argonaute family (AGO). In contrast to post-transcriptional gene silencing, however, the RNA-AGO complex does not bind a functional mRNA, but an RNA that is currently being transcribed and is therefore in the vicinity of the complementary DNA sequence. After binding, this complex recruits various proteins that methylate DNA in this region (see Figure 9). The methylations can also lead to changes in proteins linked to the DNA (so-called histones). This modifies the structure of the chromosomes and can make the DNA in this region less accessible to proteins (Erdmann & Picard, 2020).

Figure 9: RNA-mediated DNA methylation in Arabidopsis thaliana.

a) A DNA sequence is transcribed by polymerase IV. b) The enzyme RDR2 synthesises a second strand for the single-stranded RNA. c) The dsRNA is processed into siRNAs by DCL3 and methylated by HEN1. d) Together with AGO4, the siRNA binds a Pol-V transcript and recruits the protein DRM2, which methylates the DNA sequence (Kumar & Mohapatra, 2021).
6.3 Possible applications and state of development

The RdDM process can be used to suppress certain genes specifically and over several generations without changing the DNA sequence. This can be achieved, for example, by integrating a short RNA sequence specific for the target gene into a virus. The virus then introduces the short RNA sequence into the plant cells.

So far, the flower colour of petunias and the fruit ripening of tomatoes have been changed in this way. However, no application is yet ready for the market (Erdmann & Picard, 2020).

DNA methylation patterns can also be modified using the CRISPR-Cas9 system. This uses a Cas9 protein that has been modified so that it cannot cleave DNA molecules. Instead, it can be linked to proteins that methylate the DNA. By binding a specific DNA sequence, the guide RNA brings the complex into the vicinity of the DNA region that is to be methylated (Papikian et al., 2019). This system is also being tested in human cell cultures in order to suppress cancer-causing genes in cancer cells (McDonald et al., 2016). However, this technique, known as ‘epigenetic editing’, differs significantly from the natural RdDM described above.
7 RNA activation

7.1 Introduction

In order for cells to function correctly, a large number of genes must be regulated so that the right proteins are produced in the right quantity at the right time in the right place in the cell. Many diseases can be traced back to faulty gene regulation and the resulting overproduction or underproduction of certain proteins. Correcting these incorrect regulations therefore lends itself to targeted therapies. Chapter 2 ‘Post-transcriptional gene silencing’ presents methods that use short pieces of RNA, called small interfering RNAs (siRNAs) and antisense oligonucleotides (ASOs), to reduce or completely switch off the activity of genes. On the contrary, short RNA oligonucleotides can also increase the transcription of a gene. These processes are called RNA activation (RNAa), corresponding to RNA interference (RNAi), and the short non-coding RNAs involved are called small activating RNAs (saRNAs). This utilises a natural regulatory mechanism of the cell (Tan et al., 2021).

7.2 Description of the procedure

saRNAs are chemically synthesised, -stranded RNA oligonucleotides with a length of 21 nucleotides. Like siRNAs, saRNAs are also bound by a protein of the Argonaute family (usually AGO2) and an RNA strand is then removed (see Section 2.2.1.2 ‘Small interfering RNA’). The saRNA and AGO protein are imported together into the cell nucleus, where the saRNA binds to a specific DNA sequence by base pairing. This is a so-called promoter sequence. Promoter sequences are essential for regulating and initiating transcription of a specific gene. After binding to the promoter, the saRNA and the AGO protein together with other proteins form the RNA-induced transcriptional activation (RITA) complex, which promotes transcription of the gene. In addition, certain proteins that interact with the DNA, so-called histones, are often modified in such a way that the gene can be read more easily (see Figure 10). However, what exactly happens during the process of RNA activation and which proteins are involved is still only partially known (Portnoy et al., 2016).

![Figure 10: RNA activation.](image)

The saRNA is taken up into the cell as dsRNA by endocytosis and bound to AGO2. An RNA strand is then removed. After leaving the endosome, the saRNA and AGO2 bind a DNA sequence and, together with RHA, CTR9 and DDX5, form the RITA complex, which leads to an increase in transcription of a gene (Tan et al., 2021).
7.3 Applications and state of development

A number of saRNAs have already been tested in cell cultures or animal models. In most of these studies, attempts have been made to increase the expression of tumour-suppressing genes in cancer cells. This has resulted in a slowdown in tumour growth in various types of cancer. The most advanced is the development of the saRNA MTL-CEBPA, which activates the CEBPA gene that is downregulated in many cancer cells. Phase Ia and Ib clinical trials have been carried out on patients with myeloid leukaemia. Treatment with lipid nanoparticle-coated MTL-CEBPA was successful. A phase II trial is planned for 2022 (Tan et al., 2021).

In principle, saRNAs can be developed for all genes for which a promoter sequence is known. Bioinformatic methods can be used to identify the target sequence and design the saRNA. In addition to the aforementioned cancer therapies, treatments for diseases of the nervous system or metabolism caused by the dysregulation of individual genes are promising (Tan et al., 2021).

As with siRNAs, chemical modifications are also used for saRNAs in order to increase stability and reduce the immune response. Lipid nanoparticles, hybrid nanoparticles and GalNac are used for cell-specific delivery (see Section 2.2.1.2 ‘Small interfering RNA’) (Tan et al., 2021).

7.3.1 Challenges

Since saRNAs are chemically and structurally very similar to siRNAs, similar challenges arise with regard to the stability and specificity of the molecules (see Chapter 2 ‘Post-transcriptional gene silencing’).
8 Circular RNAs

8.1 Introduction

Circular RNAs (circRNAs) are single-stranded RNAs that are produced in the cell primarily during splicing of pre-mRNAs to mRNA (see Section 3.2 ‘Description of the procedure’) and are relatively stable in the cell. They can code for proteins as well as be non-coding. CircRNAs differ from long non-coding RNAs (lncRNAs) and messenger RNAs (mRNAs) in that their ends are not terminated by cap and poly(A) structures but bind to each other covalently. In human cells, repetitive sequences in particular form circular structures. Although the first circular RNAs had already been discovered in the 1970s, it was only in the last ten years that researchers realised how frequently this class of RNAs occurs in the cell (C. X. Liu & Chen, 2022).

Since then, it has been discovered that non-coding circRNAs can interact with other RNAs as well as with DNA and proteins. As a result, they play very diverse roles in gene and cell regulation (see Figure 12). Like lncRNAs, some circRNAs can bind DNA segments and form so-called R-loops (see Chapter 5 ‘Long non-coding RNAs’). These loops influence the replication, repair and/or transcription of DNA. By binding to proteins, they can influence various cellular processes. The translation and splicing of mRNAs can also be regulated by circRNAs. circRNAs, which have several binding sites for certain proteins or miRNAs (see Chapter 2 ‘Post-transcriptional gene silencing’), are known as protein or miRNA sponges. They can reduce the concentration of these proteins or miRNAs in the cell. In addition to non-coding circRNAs, there are also protein-coding circRNAs (C. X. Liu & Chen, 2022).

Figure 11: circRNA-based medical applications.

A) RNA aptamer B) miRNA sponge C) Protein sponge D) Circular antisense RNA E) Immune response boosters (not part of the report) F) Immune response inhibitors (not part of the report) G) Translatable circRNAs H) circRNAs as targets of therapy (C. X. Liu & Chen, 2022).
8.2 Description of the procedure and applications

Because circRNAs are more stable in the cell and are less frequently recognised by the immune system than linear RNAs, they are of particular interest for medical applications. Artificial circular RNAs are being tested for various applications. The ends of RNA aptamers can be joined after synthesis, which preserves their activity for longer (see Figure 11 A). circRNAs can also be designed to contain multiple binding sites for specific miRNAs or proteins, thereby serving as molecular sponges that bind these molecules and thus reduce them in the cell (see Figure 11 B and C). Circular antisense RNAs function in a very similar way to linear ASOs and modulate the stability, structure and activity of mRNAs through binding (see Figure 11 D and Section 2.2.1.1 ‘Antisense oligonucleotides’). Some circRNAs can also modulate the innate immune system by activating or blocking certain receptors (see Figure 11 E and F). In addition, in vitro-transcribed, protein-coding circRNAs have the potential to serve as a more stable alternative for mRNAs (see Figure 11 G). Since some circRNAs can contribute to the development of cancer, they are also potential targets for therapies. In addition, circRNAs are over-expressed or under-expressed in certain diseases, which could allow them to serve as biomarkers for these conditions (see Figure 11 H) (C. X. Liu & Chen, 2022).

circRNAs can be produced either in vitro or in vivo. In in vitro synthesis, a linear RNA is first produced and its ends are joined together either chemically or biochemically using enzymes or ribozymes (see Chapter 9 ‘Ribozymes’). In the in vivo method, the genetic material is introduced into a cell on a plasmid, where the RNAs are expressed and circularised by natural cellular mechanisms (Obi & Chen, 2021).

8.3 State of development

Artificial circRNAs are a very new technology that is still in its early stages. Most applications have so far been developed and tested in cell cultures. However, since circRNAs are similar to other RNAs for which there is already better existing research (e.g. siRNAs, aptamers or mRNAs) it can be assumed that, depending on their functional design, many findings from research into these RNAs can be transferred (C. X. Liu & Chen, 2022).
9 Ribozymes

9.1 Introduction

In the 1980s, it was discovered that not only proteins but also RNA can catalyse chemical reactions. These RNAs were named ribozymes after enzymes. The reaction spectrum of naturally occurring ribozymes is relatively narrow: they mainly catalyse the formation of proteins and the cleavage or ligation of RNA. However, the repertoire of possible binding partners and the reaction spectrum has since been significantly increased by the development of synthetic ribozymes (Balke & Müller, 2019).

9.2 Description of the procedure

Ribozymes for therapeutic applications can be produced either by in vitro selection or by rational design for a specific function. In vitro selection is performed using the SELEX process, which was developed for aptamers. This involves selecting from a pool of random pieces of RNA those that have the desired properties (see Section 4.2.3 ‘SELEX’). In rational design, natural ribozymes are specifically adapted to a new function. However, this requires a great deal to be known about the other molecules involved in the catalysed reaction. So far, three ribozymes derived from viruses have been used for this purpose: Hammerhead (HH), Hairpin (Hp) and Hepatitis Delta Virus (HDV). These three ribozymes can be modified in such a way that, similar to siRNAs, they cleave specific RNA sequences and thereby inactivate them. However, they can also be used to remove harmful mutations by alternative splicing of a pre-mRNA and thereby restore protein function (see Section 3.2 ‘Description of the procedure’). It is also possible to repair other RNAs, such as mRNAs, by exchanging mutated sequences. These applications of ribozymes that have RNAs as target molecules do not alter the genetic material. However, it is also possible in principle to use ribozymes to exchange certain DNA sequences and thus alter the genome (Balke & Müller, 2019).

9.3 Challenges

As with all RNA-based therapeutics, stability, transfer into the cell and recognition by the immune system are hurdles to efficacy. As with ASOs or siRNAs, ribozymes also use 2’-O-methyl, 2’-fluoro or 2’-O-methoxyethyl modifications or artificial nucleotides such as LNAs (locked nucleic acids) to avoid enzymatic cleavage and recognition by the immune system. This can also be achieved using ribozymes from L-enantiomers of natural RNA. A further difficulty is the ion concentration inside cells, which is not ideal for the function of ribozymes and requires complex optimisation (Balke & Müller, 2019).

9.3.1 Possible applications and state of development

Ribozymes have already been successfully used in cell cultures and animal models to combat viruses, including HIV and hepatitis B and C. Promising results were also obtained in the treatment of various cancer cells. However, all subsequent clinical trials were cancelled between 2002 and 2009 due to a lack of efficacy or the risk of serious side effects, at the latest in phase III. Since then, hardly any research has been carried out in this area. On the one hand, this has to do with the difficulties in the application of ribozymes (see Section 9.3.1 ‘Challenges’), but also with the development of RNAi and CRISPR-Cas as methods for gene regulation and gene editing respectively. These new methods are considered more effective in these areas of application and the corresponding constructs are easier to produce than ribozymes. However, ribozymes could still be suitable for certain applications such as the repair of mRNAs (Balke & Müller, 2019).
All the RNA technologies presented are based on the fact that the resulting constructs can perform their function in certain cells – or bind to them. However, RNA molecules are very unstable and susceptible to enzymatic degradation. In addition, they cannot penetrate cell membranes due to their negative charge. In plant cells, the cell walls represent an additional barrier. The difficulty of introducing RNAs into the target cells has long prevented the successful use of these technologies outside the Petri dish. Only the development of delivery methods that enable the RNAs to be transported to the target site and penetrate the target cells has helped these technologies to achieve a breakthrough.

10.1 Medical delivery methods

For medical use, RNA-based therapeutics must fulfill a number of requirements. They must enter the targeted tissue without being absorbed by other tissues or triggering too strong an immune response; they must interact with the correct cell type in the target tissue; and they must be taken up into the target cells (Paunovska et al., 2022).

10.1.1 Naked RNA

In principle, RNA can be injected ‘naked’, i.e. without a coating, under the skin, into the muscle or into the cerebrospinal fluid. Small RNAs, such as siRNAs, can be chemically modified for stabilisation and combined with molecules that bind to receptors on the cell surface and thus enable cell entry. In particular, conjugation with N-acetylgalactosamine for entry into liver cells has proven successful, but other small biomolecules, lipids, antibodies or aptamers can also be used. However, siRNAs are often recognised by the immune system and degraded or excreted via the kidneys, which greatly reduces their effectiveness (see Section 2.3.3.1 ‘Challenges’). Naked mRNA can also be taken up into the cell via an as yet unknown mechanism. A method has also been developed in which mRNA is injected directly into dendritic cells in vitro to stimulate the production of antigens (Xiao et al. 2022).

10.1.2 Non-viral vectors

Research into alternative medical delivery methods began as early as the 1970s. These should make it possible to stabilise RNAs and other biologically active molecules – such as cancer drugs – and protect them from degradation by enzymes. Spherical lipid nanoparticles have proven to be particularly promising.

10.1.2.1 Lipid nanoparticles and liposomes

Lipid nanoparticles (LNPs) and liposomes are spherical particles that are made up of lipids (fat-like substances) and can enclose molecules. They are taken up into the cells via the process of endocytosis. In the process, they are enclosed in another double lipid layer, called an endosome, from which they must escape again in order to release the transported molecules into the cytoplasm, where the RNA becomes functional (see Figure 12). Lipid nanoparticles are currently the most important delivery method for RNA therapeutics. They have already been clinically tested and approved in various formulations for the delivery of siRNAs and mRNA vaccines.

Lipids consist of a water-attracting head group and a water-repellent tail group and are found in all membrane systems in cells. Due to their properties, they line up in an aqueous environment with the head group facing outwards and the tail group facing inwards and form spherical structures consisting of one or two lipid layers. The lipid layers can consist of different types of lipids. The ratio between the size of the head and tail groups, the charge of the head group and the saturation of the tail group of these lipids determine which structures are formed. In nature, the small single-layer micelles and the larger double-layer liposomes occur, which form a cavity. Hydrophilic molecules can be transported in this cavity. Hydrophobic molecules can be trapped in the double lipid layer. Liposomes can have one (unilamellar vesicles) or several double lipid layers (multilamellar vesicles) and a diameter of 20 to over 1000 nanometres. The size of the liposomes has an influence on how quickly they are recognised by the immune system. Naturally occurring lipids are either negatively or neutrally charged. The type and strength of the charge depends on the head groups (Paunovska et al., 2022; Tenchov et al., 2021).

Positively charged (cationic) lipids are suitable for the delivery of RNA active substances. As RNAs are negatively charged, they interact with aqueous environments through hydrophobic and electrostatic interactions. However, positively charged lipids have been shown to be harmful to body cells. Therefore, today ionisable lipids are mainly used, which are positive in an acidic environment but become neutral under physiological conditions.
in the body. This allows the mRNA to bind to the cationic lipids during production in an acidic environment. After injection, the lipids are neutral until they become positively charged again in the endosome at a lower pH, allowing the RNA to enter the cytosol. The pH in the cytosol is then higher again and the lipids are uncharged. In contrast to negatively or dipolar charged lipids, which also occur naturally, both positively charged and ionisable lipids are produced synthetically. In addition, various auxiliary substances are also used, such as cholesterol, to simplify cell entry and increase stability. The addition of the polymer PEG to lipids serves, among other things, to stabilise the lipid shell and bypass the immune system. Paradoxically, however, PEG can also trigger a strong immune reaction (see Section 3.5 ‘Undesirable effects’). The composition of the envelope can be optimised for different applications and forms of administration (intravenous, intramuscular, subcutaneous, etc.) and target tissues (Zak et al., 2021).

10.1.2.2 Polymer-based nanoparticles

Polymer nanoparticles are another way of introducing RNAs into cells. These consist of chains of similar units that can be chemically modified. This makes it relatively easy to change the electrical charge, stability and molecular weight of the particles. A frequently used carrier material is the biodegradable polyester polylactide-co-glycolide (PLGA), which is also used in implants and surgical sutures. As PLGA is neutral, positive chemical groups are added so that it can interact electrostatically with the negatively charged RNA. Other polymers such as polyethyleneimine (PEI), polylysine (PLL) and poly(beta-amino ester) (PBAE) are already positively charged. However, PEI and PLL are poorly tolerated and must be chemically modified before use (Paunovska et al., 2022). The poor compatibility of cationic particles can also be circumvented by using ionisable polymers such as amopolyester (APE). Polymer nanoparticles, similar to lipid nanoparticles, are taken up into the cell by endocytosis. However, they have the advantage that they can bind larger RNA molecules and that they can more easily escape from the endosome and release the RNA into the cell interior (Gupta et al., 2021).

10.1.2.3 Cell-penetrating peptides

Cell-penetrating peptides are a group of linear and branched peptides consisting of five to 30 amino acids that can penetrate the cell membrane. What they have in common is that they have a positive charge and are both water-attracting and fat-attracting. These properties enable them to be taken up into the cell, probably via endocytosis. By being linked to an active substance either by covalent bonding or complexation, they can transport it into the cell interior. In order to improve the physical and chemical properties of the peptides, they are often modified with polymers (e.g. PEG) or fatty acids. Cell-penetrating peptides are mainly used for the delivery of siRNAs (Falato et al., 2021).

The siRNA can be covalently bound to the peptide by means of a separable compound. This compound, for example a disulphide bond, can be separated in the cytoplasm after transport through the cell membrane, whereby the siRNA is released from the peptide and can bind to an AGO protein. However, such binding is difficult to achieve in practice, and the efficiency of silencing has so far been insufficient in most experiments (Falato et al., 2021). In contrast, the complexation of the negatively charged siRNA with the positively charged peptide is relatively simple and does not require a chemical reaction or purification. However, the rather weak binding can lead to instability and premature release of the siRNA due to electrostatic interaction. It is therefore important to find the right ratio between peptide and RNA. The structure of the peptide used is also important for the stability of the complex. This is why experiments are being carried out with different peptides. In experiments, this method showed some promising results both in vitro and in vivo (Falato et al., 2021). By using the right peptides, it has even been possible to introduce siRNAs into certain cell types of the brain (Youn et al., 2014).

Cell-penetrating peptides can also be complexed with lipids to form lipid-peptide hybrid nanoparticles. Similar to the lipid nanoparticles described above, these can enclose an siRNA and transport it into the cell interior (Falato et al., 2021).

The method shows promising research results and there have been several clinical trials with cell-penetrating peptides since 2000. However, none of the approximately 1,850 known cell-penetrating peptides have yet been approved for therapeutic purposes (Tripathi et al., 2018). This is probably due, among other things, to the often still insufficient stability in vivo, lack of specificity and immune reactions (Falato, Gestin, and Langel 2021).

10.1.3 Applications and state of development

As early as the 1970s, attempts were made to use nucleic acids packed in nanoparticles as medicines. At first, polymer nanoparticles were used for this purpose. Later it was shown that it was possible to coat RNAs with cationic liposomes. However, these particles tended to aggregate or were recognised and broken down by the immune system. It was not until around 2010 that most of the problems associated with the use of lipid
nanoparticles were solved. Patisiran, the first drug with siRNAs encapsulated in lipid nanoparticles, was approved in both the USA and Europe in 2018. Another major step was taken in December 2020 with the FDA approval of Comirnaty® and Spikevax®, two mRNA vaccines against SARS-CoV-2. The vaccines were developed by Pfizer and Moderna respectively and both use lipid nanoparticles. In addition to these two authorised vaccines, a number of other SARS-CoV-2 vaccines are in development (Gupta et al., 2021). There are also mRNA vaccines being developed against various other viruses, including all influenza viruses (Arevalo et al., 2022). Several mRNA vaccines are already in the clinical phase (Lorentzen et al., 2022) as well as siRNAs, miRNA mimics and ASOs against various types of cancer (Gupta et al., 2021). Research is also being conducted into new ways of administering mRNAs, such as through nasal sprays (Xiao et al., 2022).

10.1.3.1 Challenges
For many functions, it is essential that the RNAs reach the desired tissues and cells. However, nanoparticles tend to accumulate in the liver when administered intravenously, which limits the applications of the technologies. Research is therefore being carried out into methods that make it possible to direct the nanoparticles into the desired tissues and cells. Targeting can be either passive or active. With passive targeting, the size and composition of the particles are selected so that they remain in the blood circulation for a long time without accumulating in the liver or being excreted or recognised by the immune system (e.g. through the binding of PEG to the nanoparticle surface). On the other hand, the formulation of the particles also enables them to be transported into the target tissue by the cell’s own mechanisms. This is mainly achieved by certain proteins attaching to the surface of the particles. Recognition by the immune system can lead to the particles being transported to lymphatic organs such as the spleen. This removes the particles from circulation, but an immune reaction against components of the particles is still possible. Active targeting uses molecules that bind to surface proteins of the target cells. This binding enables the nanoparticles to subsequently enter the cell interior by endocytosis (Paunovska et al., 2022). For the treatment of tumour cells, for example, tumour-specific antibodies are attached to the nanoparticles (Zak et al., 2021). Crossing the blood-brain barrier is particularly difficult for RNA applications in brain cells (Xiao et al., 2022). Nusinersen is therefore injected directly into the spinal fluid of the spinal column (Claborn et al., 2018). Research is also being conducted into ways of briefly weakening the blood-brain barrier using ultrasound (Kovacs et al., 2017) or to modify ASOs so that they can diffuse through the blood-brain barrier (Relizani et al., 2017).

10.1.4 Undesirable effects
In addition to isolated severe side effects, mRNA vaccinations often also resulted in strong immune reactions, which can be attributed primarily to the lipid components (Klimek et al., 2021). These could be reduced by improving the composition of the liposomes. Among other things, attempts are being made to combine ionisable lipids with exosomes and cell membranes of human cells (Xiao et al., 2022). The choice of injection site also plays a role.

10.2 Delivery in plant protection
Plant cells are surrounded by a solid cell wall and also protected from the outside by a layer of wax. These obstacles have so far made the delivery of RNA in plants considerably more difficult and are one of the reasons why research has long been focused on transgenically expressed RNAs for plant protection. However, this approach is not dealt with in this report. The above-mentioned obstacles must be overcome when administering exogenous RNA.

RNA sprays are used to spray double-stranded RNAs onto the plant surface. If the RNA is to be absorbed by a chewing insect, it is sufficient for the RNA to remain on the leaf surface. There it must withstand UV radiation and rain. However, if stinging-sucking insects or viruses or fungi are to be reached, systemic dissemination in the plant is required. To do this, RNAs must first overcome the cuticle, a water-impermeable, waxy coating of the leaf, and enter the epidermis. Alternatively, dsRNA can also be administered via the roots or by injection (Zotti et al., 2018). In either case, the molecules must then penetrate the cell wall and the cell membrane. The RNA must remain stable and must not be degraded by nucleases. If the RNA is to be effective in an insect, it must also remain stable in the intestinal tract (Hoang et al., 2022). The formulation and method of delivery are therefore of paramount importance (Christiaens et al., 2020; K. Y. Zhu & Palli, 2020).

10.2.1 Naked RNA
Double-stranded RNA can be sprayed onto the leaves in a solution. It remains intact on the leaf surface for different lengths of time depending on the environmental conditions and the nature of the leaf. Naked RNA can enter the cell interior, especially if the RNA solution is sprayed at high pressure. However, uptake is not very effective and depends on the target plant. In order to avoid the obstacle of the cuticle, the leaves are therefore often mechanically inoculated in the laboratory before application. This means that the dsRNA solution is rubbed onto the leaves
by hand so that the walls of superficial cells break open and absorb the RNA. However, this method is not suitable for large-scale application in the field (Rank & Koch, 2021).

10.2.2 Mini cells

Escherichia coli bacteria can be made to produce small cells that contain proteins, lipids and RNAs, but no chromosome, by switching off the so-called min genes. These minicells cannot grow or divide. If the bacterium also produces dsRNAs, these can be packaged into minicells. Enclosed in minicells, the dsRNAs are protected from degradation by nucleases (Islam et al., 2021).

10.2.3 Nanoparticles

Synthetic nanoparticles are already used for the delivery of RNAi into animal cells (see Section 10.1.2.2 ‘Polymer-based nanoparticles’). However, these particles have a diameter of 100 to 200 nanometres and are therefore 10 to 20 times too large to diffuse through the cell wall. As in animal cells, the nanoparticles must be able to enter the cell by endocytosis, then leave the endosome again and release the RNA into the cell interior. Particles of various sizes and shapes have been developed for this purpose, although most of them are still in the early stages of development (Schwartz et al., 2020; Shidore et al., 2021).

10.2.3.1 Layered double hydroxide

Layered double hydroxide (LDH), also known as BioClay or NanoClay, is a nanomaterial consisting of layers of a positively charged magnesium alloy. The nanolayers bind RNA and can be sprayed onto the leaves when dissolved in water. Initially, aluminium was used as the metallic component (MgAl-LDH). Because aluminium has been suspected to be toxic, it has since been replaced by iron (MgFe-LDH). This alloy is considered non-toxic and biodegradable (Jain et al., 2022). The acidic environment on the leaf surface, which occurs when the carbon
dioxide released by the plant at night reacts with water from the humidity in the air to form carbonic acid, helps to break down the NanoClay and leads to the release of the dsRNA onto or into the plant. In tobacco plants, dsRNAs bound to NanoClay showed activity against PMMo virus for at least 20 days, compared to only five days after spraying naked dsRNAs (Shidore et al., 2021).

10.2.3.2 Carbon quantum dots (CQDs)
Carbon quantum dots are carbon nanoparticles that can form a complex with RNA by attaching positive organic compounds. This protects the RNA from enzymatic degradation by nucleases and optimises uptake by the cells. A nanocomplex with siRNA has an average diameter of 3.5 nanometres and can diffuse through the cell wall. Initial trials with siRNA in Nicotiana benthamiana showed a good silencing effect. However, it turned out that small nanocomplexes that could diffuse well through the cell wall were poorly taken up by the cell and, conversely, large complexes could only diffuse poorly through the cell wall (Schwartz et al., 2020). Quantum dots synthesised from PEG are considered harmless. However, the concentration required for this application and the toxicity at this concentration still need to be evaluated (Shidore et al., 2021).

10.2.3.3 Gold nanoparticles
Gold particles with a diameter of less than 20 nanometres can penetrate the cell wall. Modified DNA strands that can bind siRNAs are attached to the surface of the particles. Whereas rod-shaped particles are taken up into the cell by endocytosis, this is not the case with spherical particles. However, both forms can release the bound siRNAs into the cell (H. Zhang et al., 2021).

10.2.3.4 DNA nanostructures
In nature, DNA molecules form double helix structures. However, it is possible to design DNA strands in such a way that they form other structures – for example a tetrahedron. In addition, binding sites for RNA, DNA or proteins can be incorporated. It has been shown that siRNAs bound to such DNA nanostructures can be taken up by cells. The efficiency of uptake depends on the size and mechanical stiffness of the structure. The smaller and stiffer it is, the easier it is taken up. Surprisingly, the siRNAs trigger different silencing processes depending on the structure to which they were bound (H. Zhang et al., 2019).

10.2.3.5 Chitosan nanoparticles (CNPs)
Chitosan is the deacetylated form of the biopolymer chitin, which is found in nature primarily in the exoskeleton of insects and in some fungi. The positively charged chitosan forms a very stable complex with RNA, which protects it from enzymatic degradation. CNPs are biodegradable and non-toxic (Shidore et al., 2021). They have also been shown to protect against the acidic environment in the gut of insects (Kolge et al., 2021).

10.2.3.6 Single-walled carbon nanotubes (SWCNT)
SWCNTs are nanotubes made of carbon, which are produced by rolling up a graphene layer at a specific angle. Due to their small diameter of less than 20 nanometres, they can enter plant cells through the cell wall. siRNAs can be absorbed by the SWCNTs by detaching the sense strand from the antisense strand and binding the two strands separately to the SWCNT. In the cell, the strands detach from the nanotube and form a double-stranded siRNA. Concerns about toxicity in mammals limit the applicability of SWCNTs, especially in edible plants (Shidore et al., 2021).

10.2.4 State of development
Nanoparticles or microcells have the potential to significantly increase the stability and specificity of exogenous RNA and thus lower two of the biggest hurdles for the successful application of RNAi in crop protection. So far, however, most of the applications of the technologies described above have only been proof-of-concept experiments that show that the methods work in principle. These experiments have been carried out in isolation and on a small scale. However, there is little data on how these preparations behave under real conditions and how long they remain intact in nature (Rank & Koch, 2021).

So far, little is known about why RNA sprays are effective on some plants and not on others. There is also a lack of knowledge about how the various nanoparticles are absorbed into the plant and what unintended effects they may trigger (Rank & Koch, 2021).
RNA technologies: mechanisms of action, applications and forms of delivery


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**Glossary**

**Antigen**
Protein (or part thereof) of a pathogen that activates the immune system.

**Antibody**
Protein that binds specifically to a certain target molecule → antigen.
They have an important function in the immune response of vertebrates.

**Antisense oligonucleotide (ASO)**
Short, single-stranded RNA sequence with a length of 12–24 nucleotides. ASOs are produced synthetically to regulate gene expression as pharmaceuticals. ASOs bind to their target RNA and thereby influence it in different ways.

**Arthropods**
Arthropods are a phylum of the animal kingdom. Arthropods include insects, millipedes, crustaceans and arachnids.

**Aptamer**
Single-stranded RNA or DNA oligonucleotide or peptide that can bind to other molecules due to its three-dimensional structure.

**CRISPR/Cas**
Genome editing tool used to modify DNA at a specific location. The CRISPR/Cas complex consists of RNA molecules and a protein (enzyme).

**Denaturation**
Loss of the functional structure of a molecule, e.g. at high temperatures.

**DNA**
‘Deoxyribonucleic acid’: a macromolecule consisting of a chain of deoxyribonucleotides (adenine, guanine, cytosine and thymine). In all organisms, genetic information is present in the form of DNA.

**dsRNA**
‘Double-stranded RNA’: naturally occurring dsRNAs play an important role in RNA interference and thus gene regulation in almost all eukaryotes. Synthetically produced dsRNAs for gene regulation are developed or utilised as pharmaceuticals (in the form of → siRNA) or for agricultural applications.

**Enantiomer**
Enantiomers are molecules that have the same two-dimensional structure but are mirror images of each other in their spatial structure.

**Eukaryotes**
Living organisms whose cells have a nucleus, especially animals, plants and fungi.

**Exon**
Coding section of the mRNA that is retained during splicing, while → introns are cut out of the pre-mRNA.

**Genome editing**
Process for the targeted modification of genetic material at a specific location. The most common genome editing tools include CRISPR/Cas, TALEN and zinc finger nucleases.

**Host-induced gene silencing (HIGS)**
A form of post-transcriptional gene silencing in plants. The DNA sequence for a → dsRNA is inserted into the plant genome, resulting in a transgenic plant. The plant cells then express the corresponding dsRNA themselves (endogenously).

**Intron**
Section of the → pre-mRNA that is cut out during splicing – in contrast to exons – and is not contained in the mRNA.

**Liposome**
Spherical particle with a shell consisting of a double layer of lipids. Liposomes can be used to enclose molecules such as RNA and transport them into the cell interior.

**LNAs**
‘Locked nucleic acids’: nucleotides in which the sugar molecule has been chemically modified to increase stability.

**miRNA**
‘MicroRNA’: short, double-stranded RNA sequence with a length of 17–24 nucleotides, which can naturally regulate gene expression through RNA interference.

**mRNA**
‘Messenger RNA’: RNA that carries the genetic information for the construction of a specific protein.
<table>
<thead>
<tr>
<th><strong>Non-coding RNA (ncRNA)</strong></th>
<th>RNA that contains no genetic information for the production of a protein.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nuclease</strong></td>
<td>Enzyme that breaks down nucleic acids by catalysing the hydrolysis of the bonds between the individual nucleotides.</td>
</tr>
<tr>
<td><strong>Nucleoside</strong></td>
<td>Compound of a nucleobase and a sugar molecule. Phosphorylation turns the nucleoside into a nucleotide, the basic building block of nucleic acids.</td>
</tr>
<tr>
<td><strong>Nucleotide</strong></td>
<td>Basic chemical building block of nucleic acid. A nucleotide consists of one of four specific nucleic bases, a sugar molecule and at least one phosphate group.</td>
</tr>
<tr>
<td><strong>Off-target effect</strong></td>
<td>Unintended effect outside the target sequence.</td>
</tr>
<tr>
<td><strong>Oligonucleotide</strong></td>
<td>Short DNA or RNA sequence of a few nucleotides (usually up to 10–200 nucleotides).</td>
</tr>
<tr>
<td><strong>Phosphorothioate (PS)</strong></td>
<td>In order to stabilise RNAs, the natural phosphodiester bonds between the nucleotides are replaced by synthetic phosphorothioate bonds, particularly in the case of → ASOs.</td>
</tr>
<tr>
<td><strong>Polyethylene glycol (PEG)</strong></td>
<td>PEG is a water-soluble polymer that has many applications in medicine, research and industry. It is usually categorised as non-toxic but can trigger allergic reactions.</td>
</tr>
<tr>
<td><strong>pre-mRNA</strong></td>
<td>Preform of mRNA that is produced directly after transcription. The pre-mRNA is modified in various ways and further processed into → mRNA. The modifications include the addition of the poly(A) tail and the cap structure at both ends of the pre-mRNA as well as splicing.</td>
</tr>
<tr>
<td><strong>pre-miRNA (precursory miRNA)</strong></td>
<td>Preform of → miRNA, which is created by processing → pri-miRNA. (see pri-miRNA).</td>
</tr>
<tr>
<td><strong>Primer</strong></td>
<td>Sequence that serves as the starting point for DNA replication.</td>
</tr>
<tr>
<td><strong>pri-miRNA</strong></td>
<td>Preform of miRNA that is produced during transcription. It is significantly longer than the miRNA and is bundled together in a loop.</td>
</tr>
<tr>
<td><strong>Ribosome</strong></td>
<td>Complex of RNA and proteins that catalyses the translation and thus the production of proteins in the cells.</td>
</tr>
<tr>
<td><strong>RNA</strong></td>
<td>‘Ribonucleic acid’: a macromolecule consisting of a chain of nucleotides. In contrast to DNA, RNA contains the base uracil instead of thymine and has an additional hydroxyl group on the sugar-phosphate backbone.</td>
</tr>
<tr>
<td><strong>RNA activation (RNAa)</strong></td>
<td>Method for increasing the transcription of a gene.</td>
</tr>
<tr>
<td><strong>RNA aptamer</strong></td>
<td>Short, non-coding, single-stranded RNA that binds specifically to a target molecule due to its three-dimensional shape.</td>
</tr>
<tr>
<td><strong>RNA interference (RNAi)</strong></td>
<td>Mechanism for regulating gene expression that occurs naturally in almost all eukaryotic organisms. In RNAi, small RNA sequences bind to their complementary target RNA and inhibit the production of the corresponding protein.</td>
</tr>
<tr>
<td><strong>saRNA</strong></td>
<td>‘Small activating RNA’: short, chemically synthesised, double-stranded RNA that binds specifically to a target sequence in the genome and thereby causes increased transcription via ‘RNA activation’.</td>
</tr>
<tr>
<td><strong>shRNA</strong></td>
<td>‘Short hairpin RNA’: short, double-stranded RNA with a hairpin-shaped structure. shRNAs are processed into → siRNAs in cells and thus play a role in RNA interference.</td>
</tr>
<tr>
<td><strong>siRNA</strong></td>
<td>‘Small interfering RNA’: short, double-stranded RNA sequence with a length of 21–24 nucleotides, which can regulate gene expression both naturally through → RNA interference and in synthetic form as pharmaceuticals. siRNAs bind complementary target RNA and thereby inhibit it.</td>
</tr>
<tr>
<td><strong>Spiegelmers</strong></td>
<td>Spiegelmers are enantiomers of aptamers.</td>
</tr>
<tr>
<td><strong>Splicing</strong></td>
<td>Removal of certain sequences (→ introns) from the pre-mRNA. Splicing is an important step in the further processing of pre-mRNA into mRNA. If different sequences are cut from the pre-mRNA by splicing, different mRNAs and thus different proteins can be produced.</td>
</tr>
<tr>
<td><strong>Spray-induced gene silencing (SIGS)</strong></td>
<td>A form of gene silencing in plants. The dsRNA is sprayed onto the plant from the outside (exogenously) using a spray.</td>
</tr>
<tr>
<td><strong>sRNA</strong></td>
<td>‘small RNA’: short, non-coding RNA with an approximate length of less than 200 nucleotides.</td>
</tr>
<tr>
<td><strong>Transcript</strong></td>
<td>RNA sequence read (transcribed) from the genetic information.</td>
</tr>
<tr>
<td><strong>Transcription</strong></td>
<td>Overwriting the DNA in pre-mRNA, the preform of mRNA.</td>
</tr>
<tr>
<td><strong>Translation</strong></td>
<td>Translation of mRNA into amino acids and thus proteins.</td>
</tr>
</tbody>
</table>
SCNAT – network of knowledge for the benefit of society

The Swiss Academy of Sciences (SCNAT) works at regional, national and international level for the future of science and society. It strengthens the awareness for the sciences as a central pillar of cultural and economic development. The breadth of its support makes it a representative partner for politics. The SCNAT links the sciences, provides expertise, promotes the dialogue between science and society, identifies and evaluates scientific developments and lays the foundation for the next generation of natural scientists. It is part of the association of the Swiss Academies of Arts and Sciences.

The Genetic Research Forum of the Swiss Academy of Sciences (SCNAT) focusses on developments in genetic research and their impact on society. The forum promotes dialogue between scientists, decision-makers and the public.