

1

RNA Extraction, Separation, and Analysis

1.1

The Need to Be Able to Extract, Manipulate, and Analyze RNA

RNA is a powerful biological material. It is an active molecule that is directly involved in performing or controlling many biological functions. Unlocking the role of the various types of RNA present in biological cellular processes is critical to developing new methods of diagnosis and understanding and treating disease. New research is being reported daily that someday will lead to new drugs or methods of treating disease. Indeed, some of the work is so promising that it is hoped that a range of diseases will someday be effectively treated and cured. In any case, understanding RNA and its function is one of the keys to understanding life.

There are many different types of RNA, each of which carries out diverse and important functions in the cell, as shown on the book cover and in Figure 1.1. The need to extract and understand the roles of these RNAs is important not only for an understanding of biology but also for developing therapeutics. This particular figure was chosen as the book cover because it illustrates the complexity and importance of RNA in functional mechanisms within the cell. To name a few, RNAs have been shown to function in transcription (pri-RNA, mRNA), splicing (snRNA), translation (tRNA, tmRNA), and post-transcription regulation (siRNA, miRNA). Each type and function of RNA is described in Chapter 2 of this book, while each pathway is discussed in more detail in Chapter 7.

Unlike DNA—which is quite rugged—RNA is transient. RNA is also fragile, elusive, and difficult to recover. Proteins are expressed from DNA using RNA, with the cell carefully controlling which proteins are active, largely through overseeing the synthesis and degradation of RNA. The expression of DNA through RNA to produce new proteins could not be accomplished if the “old” RNA were still present from previous expressions of proteins; consequently, the cells routinely remove the “old” RNA by degrading it with RNase enzymes. Unfortunately, the RNase that is present for biological cellular function can also make RNA collection difficult, and any procedure employed for such collection and use must take this into account.

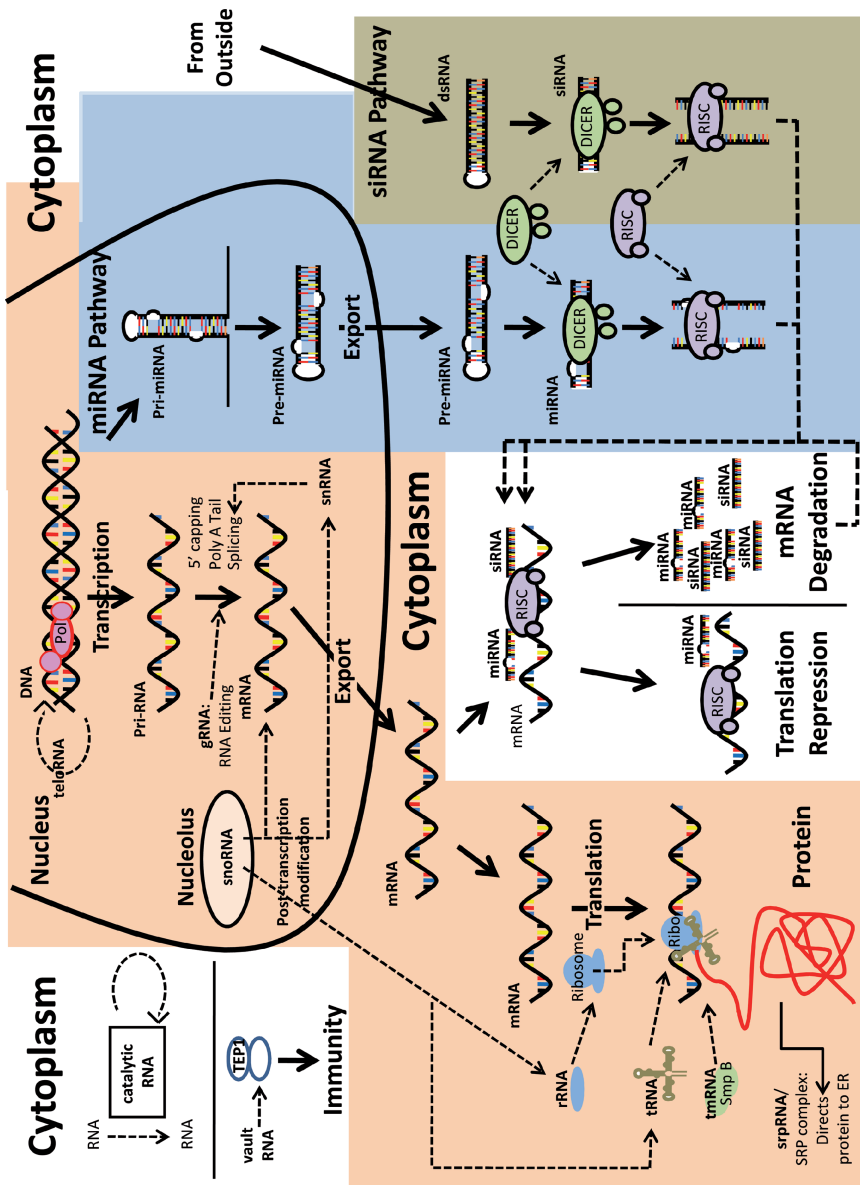


Figure 1.1 The schematic arrangement of different types of RNA, and their functions. These include telomerase RNA (telomRNA), primary RNA (pri-RNA), messenger RNA (mRNA), guide RNA (gRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), transfer messenger RNA (tmRNA), signal recognition particle RNA (srpRNA), micro RNA (miRNA), short interfering RNA (siRNA), vault RNA, and catalytic RNA (ribozyme). The arrows indicated alongside each type of RNA show the cellular functions or processes that they are involved in. Each type and function of RNA is described in Chapter 2, and each pathway is described in more detail in Chapter 7.

Although many of these procedures have been described, it is not the purpose of this book to provide “cookbook” methods for RNA extraction, separation, and analysis. Yes, there is a multitude of kits available for RNA, and there is also a place for cookbook procedures, as these can free the researcher to use kits for what is tedious and automatic and allow them to design experiments that are of real interest. The danger lies in when a kit does not exactly fit the research problem at hand, or may not even be available. While it may be possible to modify an existing kit to do the job, that will not be possible if the investigator does not understand the chemistry employed in the kit, or how the chemistry can be controlled, manipulated, and optimized to fit the situation.

In this book, we describe the chemistry that is used in the various methods and tools. By understanding the basic chemical concepts, and how they apply to the way in which RNA is manipulated, the biologist will be able to better use the routine products and methods that are currently available on the market. The purpose of this book is also to provide a working knowledge in the context of the types of samples, types of RNA, what is important, and how to accomplish the various tasks through a basic chemical knowledge of the separation and analysis systems. With this information, it is possible for the biologist to make adjustments to fine-tune a product or procedure to a particular research need, or to develop entirely new procedures and methods to fit the problem at hand.

1.2

Using Chemical Tools to Solve the Problem of Analysis of Biological Processes

The types of RNA chemicals tools described in this book are concerned with: (i) solid-phase extraction; (ii) liquid chromatography; and/or (iii) chemical reagents, including enzymes. Both, RNA solid-phase extraction and RNA separation by liquid chromatography operate on similar principles—that is, an interaction of the RNA molecules with the solid-phase surfaces, or within functional groups in the extraction or chromatography media. The surface chemistry of the media can be quite similar—or even exactly the same—in the two cases. Both technologies operate on similar methods of chemical interaction of RNA with the surface. Moreover, both of the methods used to control this interaction and collect working amounts of RNA material are similar.

Part of the difference between solid-phase extraction and liquid chromatography relates to whether classes of molecules are separated, or whether higher-resolution separations are performed on individual molecules. Whereas, solid-phase extraction can be used to extract a particular type or class of RNA, chromatography can be used to separate classes or individual molecules. Nevertheless, both technologies operate on similar methods of chemical interaction of RNA with the surface. The methods used to control this interaction and to collect working amounts of RNA material are also similar.

1.3

The Principle of Chromatography and Solid-Phase Extraction

1.3.1

Principle of Chromatography

The Russian botanist Mikhail Tswett coined the term “chromatography” in 1903, the name being derived from the Greek words “chroma” and “graphein”, meaning “color” and “writing” [1]. Tswett described a method for the separation of pigments found in plant leaves by using an open tubular column filled with a dry solid adsorbent of granular calcium carbonate (chalk). To the top of the chalk column, he added an extract of plant material containing its pigments. He then washed the chalk with an organic solvent, which began to flow down through the column by gravity. As the solvent was added to the top of the column, it carried the mixture of plant pigments down the column. Then, as the washing continued, the various pigments began to separate into a series of discrete colored bands, each of which was found to contain a single pigment from the original mixture. Tswett continued to add solvent, so that gradually the regions between the bands became entirely free of pigments and, after a while, all of the bands had been resolved or separated. Tswett then stopped adding the solvent, waited for the solvent to stop dripping, and finally pushed the moist chalk material out of the tube as an intact cylinder. Each pigment could be recovered by cutting the bands apart with a knife and extracting each individual band with an appropriate solvent.

Tswett called this process “chromatography”, and was the first to recognize that the separation process was based on a general principle. This was the relative degree by which compounds in solution were either adsorbed to a stationary solid phase of the column or dissolved in the liquid mobile phase. Depending on the liquid phase, a particular compound can partly adsorb to the solid stationary phase and partly dissolve in the liquid mobile phase. Since each compound’s affinity for the column is likely to be different, the compound will be carried through the column by the solvent, and the rate that a compound moves down the column will depend on how tightly the compound sticks to the column, or how well it is dissolved in the mobile phase. As the mathematical equations developed later in this book will show, the rate of travel of any one compound is proportional to the ratio of the amount of compound in solution divided by the amount of compound adsorbed on the column, for any given solvent.

This elution process of washing the compounds down the column continues until all components of the mixture are separated (Figure 1.2). Thus, the compounds which compose a mixture can be separated based on how each compound differs in its attraction to the stationary phase or the mobile phase. When a mixture of compounds is applied to the top of a column and washed down the column, there are some compounds in the mixture that do not “like” the mobile phase but rather “like” the stationary phase and so stick tightly or adsorb to the column material. These compounds either stay on the column indefinitely, or are eluted very slowly from the column. Other compounds that “like” the mobile phase and

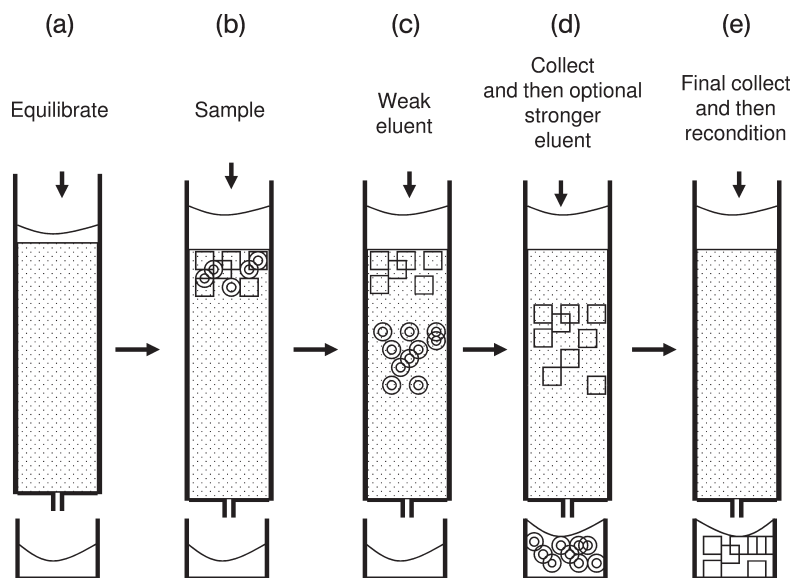


Figure 1.2 The liquid chromatography separation process. (a) The first step is to condition or equilibrate the column to the desired start pH, buffer and/or solvent conditions which ensure that the components are adsorbed into the column. (b) The components of a mixture are added as a concentrated aliquot to the top of the column. (c) Adding eluent to the top of the column starts the separation process. Some components will travel through the column

faster because they are solvated by the eluent and interact less with the column packing. Other components interact more strongly with the packing and travel more slowly through the column. Eluent is added from time to time so that the column is not allowed to become dry (the solvent level should not be allowed to fall below the top of the column bed). (d) Component 1 (circles) is eluted and collected. (e) Component 2 (squares) is eluted and collected.

do not adsorb or stick tightly to the stationary phase will travel down the column and be eluted more quickly.

1.3.2

Mobile Phase Gradient Controls Elution

It is quite easy to adjust and control the rate of travel of a compound through a column. The RNA that elutes from a column can be detected by ultraviolet absorbance and recorded either on a chart recorder or electronically. The assessment of the separations involves an analysis of the peaks. Changing the mobile phase to a solvent that does not dissolve the compound as well as the previous solvent will slow the rate of travel of the compounds through the column and improve the separation, albeit at the expense of taking longer to perform the separation. Conversely, a stronger mobile phase will speed up the movement, but (perhaps) at the expense of some of the peaks running down the column together and coeluting.

It is possible to have the best of both situations, however, by using a gradient of eluent strength. When starting with weak initial eluent conditions, most of the compounds will be adsorbed at the top of the column and remain there, even with continued washing by the mobile phase. Only those compounds that stick weakly to the column will move down the column and be separated. However, if the eluent strength is then slowly increased it will cause more compounds to move down the column. Such an increase in mobile phase strength is accomplished by changing the solvent to one that can either dissolve or attract the compounds more effectively.

Increasing the mobile phase strength can be carried out in a stepwise manner, by adding aliquots of increasingly strong eluents to the column in succession, with each aliquot addition moving the compounds faster and faster down the column. This procedure is known as a “step gradient” process, because each increase in mobile phase strength is performed as a step. It is also possible, by using the appropriate instrumentation, to increase the gradient gradually—a process known as a “continuous gradient”.

1.3.3

Different Types of Column and Eluent Chemistries

It was some time after Tswett’s original work before the significance of these discoveries were realized. However, as further investigations were carried out [2–6] it was found that liquid chromatography could be used for many different types of application, including the separation of proteins, organic and inorganic ions, small organic molecules, carbohydrates, and amino acids. The separation of large biological molecules such as nucleic acids came later with refinements in column chemistry and instrumentation.

RNA is one of the most difficult materials to separate under chromatographic conditions. One reason for this is that RNA is an extremely large molecule, and the differences between types of RNA are almost insignificant. RNA molecules are also susceptible to contamination, which causes them to precipitate or to be irreversibly adsorbed to the tubing, frits, columns, or other components of a chromatographic system. Finally, biology works against RNA Chromatography, in that RNA is degraded very quickly in nature and is stabilized only with great difficulty. This includes maintaining stability of the sample before separation, of the RNA molecules during the separation process, and of the collected RNA material when the separation has been completed. The problem here is that ribonuclease (RNase) enzymes are ubiquitous, and their control or removal is necessary to prevent RNA destruction.

The adsorbent contained in the column is based on a core substrate onto which the functional groups that provide column selectivity are bonded. The substrate is chosen based on its uniformity, size, chemical and mechanical stability, and on the ease of attaching appropriate functional groups to the particle surface. Solid-phase substrate materials are usually based on silica, polymer or agarose core

particles. Due to problems of inertness and pH stability, a polymer substrate is usually used for the column media in RNA Chromatography, while RNA affinity chromatography will mostly use agarose-based substrates.

Depending on the types of compound to be separated and the desired separation mechanism, ion-exchange, reverse-phase or affinity chemical groups may be bonded to these core substrate particles. All of these groups have been used to separate RNA.

The control of this selectivity is achieved by using different types of eluent to wash compounds down the column, including salts, buffers, acids, bases, and different organic solvents. Each type of separation is based on a particular eluent mobile phase being matched with a column stationary phase, so as to create the correct balance of interaction between the two phases. The selectivity is based on whether a sample compound prefers to be adsorbed onto the column, or to be desorbed and dissolved by the eluent. Specific examples of different combinations of columns and eluents for RNA separation are described in the various chapters and sections of this book. Step gradients or linear gradients, which can be used to alter the eluent composition as the separation is proceeding, can provide additional control on whether a compound moves down the column, or not. It is often advantageous first to retain the sample compound on the column and to wash away all of the impurities. Then, by changing the conditions to increase the dissolving power of the eluent (i.e., by increasing the eluting power of the eluent mobile phase), the sample compounds can be desorbed from the stationary phase and moved down the column.

The classical gravity flow column can serve as a powerful means of liquid chromatography separation. For this, the separation material is made into a slurry with buffer and packed into a column under pressure. For a good separation, it is very important that the resin bed is consistent; in addition, care must be taken to maintain a uniform column when adding sample or buffer to the top of the column, so as to not disturb the column packing. The column must first be “conditioned” by allowing the solvent to run freely through the column, under gravity. Care must be taken not to allow the column to run dry, as this will introduce air into or physically disturb the packed bed and cause an inconsistent liquid flow through the column. (If this were to happen, the liquid would flow around the air pockets, such that any packing material within the pocket would not be exposed uniformly to the sample and eluent.)

After having conditioned the column with solvent, the sample is added to the column top; the liquid mobile phase is then added to the column top and allowed to run through and drip out at the bottom. In some columns, the flow rate can be controlled by using a stopcock. Initially, any nonretained materials will be washed through the column with a wash solvent, after which an elution solvent can be added. If the sample components are colored, a visual record can be used to track the separation and to collect the materials. In other cases, the fractions of eluent can be collected from the end of the column and analyzed, normally plotting the sample concentration versus fraction number; in this way, a chromatogram is constructed.

Under a given set of conditions, a particular sample component will always be found in a certain volume fraction of the eluent, the next compound can be found in the next fraction, and so on. For example, compound A is always located in the 3–5 ml eluent fraction, compound B in the 5–7 ml fraction, and compound C in the 7–11 ml fraction. Thus, under predetermined conditions, even noncolored components of mixtures can be separated, collected in a single fraction, and then analyzed using spectroscopy, titration, and so on, to determine the amounts of each component in the original mixture.

Classical gravity flow liquid chromatography can serve as a powerful separation technique, employing very simple tools. On the negative side, however, the technology can be slow and awkward to use and, frankly, it can also be very boring to watch the drops emerge from a column while ensuring that it does not run dry during any part of the process. Thankfully, the many advances in automation described later in this chapter make chromatography much more accessible to the investigator!

1.3.4

The Principle of Solid-Phase Extraction

The classical liquid chromatography technique described by Tswett is related to solid-phase extraction, but with some important differences. Similar to chromatography, the process of solid-phase extraction involves dissolving the sample in a solvent and passing this through a column containing solid-phase material. Importantly, solid-phase extraction does not rely on chromatography partitioning phenomena (partially sticking and unsticking from the column surface) for separation. Rather, the extraction–separation principle is based on what is sometimes called an either “on” or “off” process of separation, and which generally results in a much more crude purification.

The solid-phase extraction process is shown in Figure 1.3. The first step is to condition the column, when the solid-phase surface is prepared to accept interaction with the sample. This may include treatment at a particular pH with a buffer to convert the surface or functional groups on the solid into a particular chemical form. Alternatively, the conditioning may simply involve using a solvent to wet and clean the surface.

In the next step, the sample containing the compound of interest in a sample matrix is applied to the column. The compound is attracted to the surface with specific binding, and is adsorbed to the column. The selectivity for the sample compound is often very high, with conditions being selected such that all of the material is adsorbed while the matrix mostly passes through the column. The conditions are selected so that matrix materials do not adsorb to the surface (although some may adsorb to a slight degree); this is known as “nonspecific binding” because the selectivity is not high for the matrix material. In contrast, other matrix materials may have a fairly strong, more specific bond with the surface, although the adsorption is still much weaker than that of the sample compound of interest. These materials must be removed in the next step, of washing.

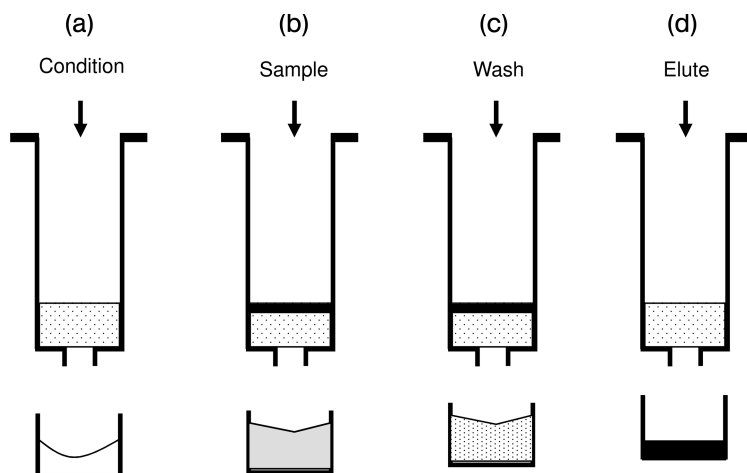


Figure 1.3 The solid-phase extraction process. The column may be a spin column, gravity column, or a vacuum column. (a) The first step is to condition the column. The solid-phase surface is prepared to accept interaction with the sample by equilibration with the appropriate start pH, buffer and/or solvent conditions. (b) In the next step, the sample containing the sample compound of interest in a sample matrix is applied to the column. The sample compound is attracted to the surface with specific binding and adsorbs “on” to the column. The column is usually small, and many of the matrix materials pass through directly through it. (c) The remainder of the matrix materials are removed in the washing step. A strong wash is used if a very pure product is desired, but a weaker wash is used if a high yield is desired. (d) A solvent or buffer is introduced in the elution step, releasing the sample “off” of the column. The purified material is then collected.

If some of the matrix material has a weak affinity for the surface, it may be removed by using a washing solution containing a competitive agent, although of course the wash must be weak enough so as not to remove the sample compound of interest. For the elution step, a solvent is introduced which releases the sample material from the column so that it can be collected in a purified state. In some cases, sample purity may be sacrificed in order to obtain higher yields by using a very weak washing solution; however, if sample purity is preferred, then a stronger washing agent must be used and the yield sacrificed.

Use of the partitioning technique of liquid chromatography allows even compounds with small differences in selectivity for the solid surface to be separated. In addition, by using the on/off technique of solid-phase extraction a particular compound, or class of compounds, can be separated from other classes of material.

Solid-phase extraction is very often performed using so-called “spin columns”, which are operated under centrifugal force. Typical spin column bed sizes range from 100 μ l to perhaps 0.5 ml, with the smaller columns being the most popular. Spin columns of several milliliter bed sizes are sometimes used when large sample sizes are processed. Normally, flow through the spin column is

rapid, and it is necessary to spin the column at relatively slow speeds to direct the sample, wash, and elution solvents through the column. A 5000 r.p.m. spin rate is typical. After each spin the column must be removed and placed in a fresh receptacle before the next solvent is applied to the column. The packing materials are very similar to those used in chromatographic columns. Another approach is to use columns that can either fit onto a vacuum manifold or act as gravity columns. For these types of application, kits are available that include the extraction column and all necessary buffers, prepared in suitable concentrations and volumes.

1.4

RNA Chromatography

Starting in the late 1960s, liquid chromatography changed suddenly and drastically with the development of improved theories and instrumentation. As a result, liquid chromatography was re-named as high-performance liquid chromatography (HPLC). As a process, HPLC became fully commercialized during the late 1960s and early 1970s, when high-performance columns of silica packing materials with bound layers of organic alkyl groups were introduced, along with improved automated pumps and detectors. These new packing materials had a small particle size (average 10 μ m), which allowed them to be packed uniformly into small stainless-steel columns. Such columns permitted materials to be separated into very tight bands, which in turn allowed more bands to be separated and smaller elution volumes to be used. In addition, the new instrumentation allowed the use of gradient elution technology to improve the separations.

Liquid chromatographic instrumentation systems employ a pump, an injector, a column, and a detector:

- The pump allows the eluent fluid to be pumped automatically through the column in precise fashion, with uniform and minimal pulsations, accurately, and under high back-pressure conditions (normally 1000–2000 p.s.i.).
- The injector allows the precise and accurate injection of samples into the eluent fluid stream entering the column.
- The detector allows the continuous detection and automatic recording of the separated material eluting from the column.

While developments in the instrumentation are largely responsible for the success of HPLC, the key to the system functioning well is the column, in combination with the interactions of the mixture components with the column and the eluent. To date, most major breakthroughs in chromatographic research have been achieved with the column. Today, the trend is towards even smaller particles, with separation column packings being typically 2 μ m in size. In fact, the newest columns contain no particles at all, but are composed simply of a rod of continuous porous polymer monolith filling a small capillary column.

Both, DNA Chromatography and RNA Chromatography were first described by Guenther Bonn, Christian Huber, and Peter Oefner in 1993 [7–9]. By using ion-pairing, reverse-phase chromatography, this group obtained rapid, high-resolution separations of both double-stranded and single-stranded DNA. Moreover, the separations were usually performed in less than 10 min and, in many cases, a resolution of fragments that differed by only a single base pair in length was achieved. This form of HPLC analysis is largely (though not entirely) based upon the unique separation properties of a nonporous, polystyrene–divinylbenzene polymer bead that has been functionalized with C18 alkyl groups. In this process, an alkylammonium salt (usually triethyl ammonium acetate; TEAA), is added to the eluent to form neutral ion pairs when a DNA sample is introduced into the HPLC instrument. The DNA and RNA fragments are first adsorbed onto the column, and then separated with a gradient of water and acetonitrile, with smaller fragments being eluted from the column first, followed by the larger fragments.

The same technology was also shown to be effective for the separation of single-stranded and double-stranded DNA and RNA. Depending on the type of eluent used, the single-stranded separations are based on differences in the size, polarity and shape of the molecule. By changing the ion-pairing reagent to be more non-polar, the separation can become mostly sized-based. Temperature is an important parameter for single-stranded separations, especially for RNA, while double-stranded DNA separations are mostly size-based. Double-stranded RNA separations are based on the size and shape of the RNA molecule. A commercially available RNA chromatograph is shown in Figure 1.4.



Figure 1.4 A commercial RNA chromatograph based on a high-performance liquid chromatogram designed for the separation and collection of RNA. (Illustration courtesy of Transgenomic, Inc.).

In general, the ion-pairing reagent used is an amine cation salt which forms a nonpolar ion pair with the phosphate anion group of the nucleic acid. TEAA—the most frequently used amine cation salt—is able to pair with the nucleic acid fragments to form nonpolar ion pairs that subsequently adsorb to the neutral nonpolar surface of the column. Acetonitrile is then gradually added to the eluent so as to decrease its polarity until the TEAA/nucleic acid ion-pair fragments are desorbed from the column. In gradient elution, the concentration of acetonitrile pumped through the column is increased gradually as the separation proceeds; under these conditions, the smaller fragments will elute first, followed by fragments of increasing size in line with the increase in acetonitrile concentration.

RNA nucleic acid fragments are extremely large molecules relative to what can normally be separated by a chromatographic process. For example, the average molecular weight of one base pair of RNA is approximately 660 g mol^{-1} , while a double-stranded, 100 base-pair RNA molecule has an approximate molecular weight of 66 kDa. Typically, 1000 base pair fragments, and up to 2000 base fragments, can be separated using RNA Chromatography.

Although, in this process it is possible to use a very rapid gradient program (e.g., 3–5 min), such a rapid elution program will be possible only at the expense of a lower resolution of the peaks. However, in many cases this will be adequate as the mixture to be separated is not complex. A slower gradient process, of 20–30 min, would produce the highest resolving conditions and the greatest separation of peaks, but clearly under the penalty of a longer analysis time.

One of the most powerful features of RNA Chromatography is that material can easily be purified by collecting it directly from the detector effluent. Notably, the purification of biological samples that might include several types of a particular nucleic acid would be desirable; an example of the detection and subsequent purification of different RNA types from a cell extract is described later in this chapter.

While it is possible to perform such collections by hand, they would normally be accomplished using an automated fragment collector and controlling software, with samples collected into either single vials or large, 96-well plates. In this respect, it is important to account for the dead volume in the tubing which connects the detector to the fragment collector, so as to ensure that the desired peak has actually been collected. The measurement of recovery is performed by taking a small portion of the recovered peak, reinjecting and measuring the area, multiplying this by the ratio of total collected volume to reinjected volume, and comparing this value to the area of the original peak. A typical recovery would be about 80% of the injected material. An additional point is that materials such as RNA may be degraded (via an enzymatic process) after their collection; higher recoveries may not be possible due to a loss of material during the concentration process, whether through precipitation or the plating of material on surfaces.

One very important parameter in fragment collection is the careful execution of the collection times as, invariably, there will be a lag between the time at which

the peak is detected and when the fragment reaches the collector probe. Although a timed collection is the most reliable method, unless the timing is correct it is easy to miss some—or even all—of the peak. It is also important to appreciate that there is a dead volume in the tubing from the detector cell outlet to the tip of the deposition probe, and that if this volume is too large it might destroy the resolution of the separation and even result in cross-contamination of the peak of interest with neighboring peaks. It is also important that the probe is cleaned between the collection of peaks, although this is normally carried out automatically by the fragment collector.

1.5

Enzymatic Treatment of RNA and Analysis

RNA has a clear function in every major biological process, including DNA replication, protein translation, and the regulation of gene expression. RNA is an ancient molecule, with much evidence pointing towards it being the first molecule significant for life in the prebiotic world. In recent years, research interest in RNA has been analogous to a chromatographic separation, with periods of great activity followed by some of waning interest. Nonetheless, the new discoveries seem always to “stoke the fire”. The first wave of interest in RNA was its role in DNA replication, where short RNA primers—called Okazaki fragments—are required by the enzyme DNA polymerase to copy the DNA template. This was followed by the discovery that tRNA serves as the adapter molecule that forms the bridge between genotype and phenotype. The genetic code embedded in a gene is translated into protein using tRNA, which has specificity for the three-nucleotide codon denoted by the messenger RNA while, at the same time, being recognized by the enzymes that charge them with amino acids. A flurry of interest developed during the late 1980s with the discovery of the catalytic ability of certain RNAs, and this soon led to the discovery of many catalytic RNAs—called ribozymes—and the formation of the RNA World Hypothesis, which states that the first molecules with biologically significant features were RNAs. Most recently, the discovery that RNAs could regulate gene expression at the translational level has unveiled yet another area of biological complexity which might, in time, open the doors to some intriguing and potential concepts of the mechanisms of drug therapy.

With all of the diversity associated with RNAs themselves, and in the processes that they serve, an underlining theme that unites all RNAs is that they have *structure*. In fact, it is the ability of RNA to fold into three-dimensional (3-D) structures that allows it perform such a wide range of functions. Unlike DNA, which usually exists as a double-stranded helix, RNA is normally single stranded. Although its folding mechanism is not completely understood, RNA can form helices, loops, and bulges that provide it with an ability to form long-range intramolecular interactions that give the molecule its shape. Moreover, by understanding the shapes of

the various RNA molecules – and how they correlate to function – it should become possible not only to understand many fundamental biological processes but also to provide a basis for rational drug design.

1.5.1

Polyacrylamide Gel Electrophoresis

One of the most powerful methods used in the analysis of RNA is that of gel electrophoresis. In this technique, highly crosslinked and uniform gel matrices are easily generated, with very long gels being capable of separating very small differences between molecules. In fact, the separation ability of polyacrylamide gels allows the resolution of molecules that vary in length only by single nucleotides. In gel electrophoresis, the RNA sample is loaded into the gel at one end, and an electrical current is applied that causes the RNA to move through the gel. For this, the RNA sample mixture is loaded into the gel at one end, and an electrical potential is applied to both ends of the gel. The negatively charged cathode electrode is applied to the sample end of the gel, and the positively charged anode to the far end. When a potential of 1–3 kV is applied, the negatively charged RNA fragments travel through the gel toward the anode. Smaller RNA fragments travel more rapidly through the gel, causing the individual fragment types to be separated on the basis of their size. Polyacrylamide gels have the ability to resolve RNA fragments having only single nucleotide variations in length.

Over the years, a multitude of research groups has used gel electrophoresis to great effect. Typically, a nondenaturing gel can be used to determine if a protein interacts with a particular RNA, for example, in a gel-shift assay. Likewise, RNA splicing events may be analyzed by monitoring (via gel electrophoresis) the extent to which a full-length substrate undergoes splicing to shorten the RNA. Gel separation may also be used, with great sensitivity, for the enzymatic end-labeling of RNA substrates with radioactive phosphate. An alternative choice might be to perform a primer extension reaction, where a primer is annealed to the target RNA, after which the 3'-end and a reverse-transcriptase enzyme are utilized to create a cDNA copy of the RNA. For added sensitivity, radioactive primers or radioactive nucleotide triphosphates can also be used to label the cDNA.

1.5.2

RNA Structure Probing with Ribonuclease Enzymes

As information has accumulated regarding the ability of RNA to fold into 3-D structures, gel separation methods have become very useful. Enzymatic digestion using nonspecific ribonucleases can be used to acquire information as to how an RNA folds and which ligands bind to the RNA by utilizing “footprinting” experiments. Chemical modification agents and primer extension can also be used to determine structural information. As this field matures, new computational tools used to interpret the data tools are being developed.

1.6

Content and Organization of This Book

The tools used to extract, separate and analyze RNA depend not only on the type of RNA being studied but also on its origin. In providing information on this topic, the various types of RNA identified are listed and described in Chapter 2, while the chemical interactions of RNA with solid-phase materials are outlined in Chapter 3. An understanding of these interactions, which include ion pairing, ion exchange, affinity or chaotropic interactions, should in turn allow the manipulation of RNA to be optimized. The various applications of RNA, and methods for its extraction, are described in Chapter 4, while Chapter 5 includes detail of how the RNA Chromatographic process works, and how it compares with classical gel electrophoresis. The various applications of RNA Chromatography are discussed in Chapter 6, together with its general principles and applications, and explanations of *how* and *why* these separations are performed. While many of the details of RNA Chromatography are provided throughout this book, a host of theoretical and practical information is also included in the Appendices.

The different approaches to using chemicals and enzymes in the analysis and interrogation of individual RNAs are described in Chapter 7. One major biological feature of RNA is its ability to fold into 3-D structures, the examination of which is based on: (i) the use of enzymes to cleave the RNA; and (ii) the separation techniques (e.g., gel electrophoresis or chromatography) used to resolve the cleavage products. Today, it is possible to reconstruct the cleavage products and hence to piece together a crude 3-D map of RNA, based on the sites available for, and those protected from, cleavage. This also allows structural information to be acquired when the 3-D structures have not been solved, or to be used in conjunction with the 3-D structures to gain more insight. Further information may also be acquired by studying the cleavage of RNA or RNA–protein complexes, so as to identify the sites of RNA–RNA and RNA–protein interactions.

The Appendices of this book are targeted at those readers wishing to learn more of the theory of liquid chromatography and its practical applications. In Appendix A1, details of the discovery of chromatography and the fundamental concepts that govern its use are outlined. An understanding of these concepts might also help the user to improve separations or to develop new separation conditions. Although mathematical equations describing the fundamental phenomena have been included here, it is important for the new user not to get “bogged down” in these equations if they appear irrelevant; rather, simply read the sections, note the concepts, and then come back when you have gained more experience, and can glean more from the information.

Appendix A2 describes the “nuts and bolts” of HPLC, as well as some details of the various components and functions of the instruments. This information should provide an understanding of how the samples are introduced into, and analyzed by, the instruments. Finally, Appendix A3 outlines the use of HPLC for the separation of nucleic acids. The methodology dictates that the instrument is absolutely free from metal oxide contamination; the reasons for this,

and how an instrument can be cleaned and maintained, are listed in this appendix.

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