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Isolation and Detection of Carcinogenic Nucleic Acid Adducts

Dickson M. Wambua[1], Amanda L. Brownstone[1],
Charles A. Barnes[3] and Norman H. L. Chiu[1,2,*]
*[1]Department of Chemistry & Biochemistry,
University of North Carolina at Greensboro, Greensboro, NC,
[2]Department of Nanoscience, Joint School of Nanoscience and Nanoengineering,
University of North Carolina at Greensboro, NC,
[3]Department of Chemistry, Iowa State University, Ames, IA,
USA*

1. Introduction

In general, nucleic acid adducts are formed when harmful chemical compounds react covalently with cellular DNA or RNA molecules. With only four natural nucleobases in DNA or RNA, identical nucleic acid adducts can theoretically occur at multiple positions within the human genome or transcriptome. The frequency of nucleic acid adduction is further increased by the reactivity of DNA or RNA to form adducts with many different types of chemicals, which include both exogenous compounds that our bodies have been exposed and endogenous compounds that are generated through normal metabolic activities in our bodies.[1,2] Some exogenous compounds may require metabolic activation prior to the formation of nucleic acid adducts, whereas others may react directly with nucleic acids. If DNA adducts are not effectively removed by the DNA repair mechanism, the adducts can directly interfere with DNA replication and transcription.[3] Similarly, the presence of adducts in RNA molecules can affect their biological functions. From the results of many experimental studies, the association of either DNA adducts or RNA adducts to cancer have already been well established. In the case of DNA adducts, it is widely recognized as the key element for the onset of carcinogenesis. Both DNA adducts and RNA adducts are, therefore, important biomarkers for cancer research, which include the monitoring of exposure to carcinogens, genetic mutation, DNA repair and so on. Similar to the analysis of other cancer biomarkers, both identification and quantification of nucleic acid adducts are required. Prior to the detection of nucleic acid adducts, it is important to ensure the biological or clinical samples are collected and stored properly. Equally important, the isolation of genomic DNA or RNA has to be carried out with high efficiency, which includes the yield and purity of selected material, reproducibility and the rate of sample throughput. For the detection of nucleic acid adducts, the requirements can be divided into the following order:

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* Corresponding Author

1. *Limit of Detection*. First and foremost is the ability to detect the presence of the adduct of interest. The detection signal that corresponds to a specific adduct should be at least two times above the background noise which originates from either the sample matrix or electronic components in an analytical instrumentation.

2. *Selectivity*. The major analytical challenge for the analysis of nucleic acid adducts is that the ratio between unmodified nucleotides and adducts can be as high as $10^{12} : 1$. To achieve an accurate measurement, it is very important to be able to select or separate the adduct of interest from other nucleotides that have not been modified.

3. *Specificity*. For the analysis of nucleic acid adducts, specificity refers to the ability to distinguish the target adduct from the other possible adducts, including different isomers, that may co-exist in the same sample. In the case of mass spectrometric methods, this can be easily achieved by using a mass spectrometer that can provide high mass resolution (e.g. Orbitrap from Thermo Scientific).

4. *Quantitation*. As mentioned above, it is important to be able to accurately determine the amount of target adduct in a biological sample. To achieve absolute quantitation, the construction of a calibration graph with the dilutions of a pure standard is required. This can be a challenge simply because many pure standards of nucleic acid adducts are not available.

5. *Reproducibility*. It is important to ensure the method being used to measure a target adduct is reproducible which includes both intraday and interday reproducibility.

6. *Sample Throughput*. With a wide variety of nucleic acid adducts and their high frequency of occurrence in a genome and transcriptome, the number of assays that are required in a specific study, especially in a clinical related study, can be relatively high. Thus, it is necessary to consider the time which is required to complete every step in the method being used as well as how many samples can be processed in parallel at the same time.

7. *Cost*. For measuring a relatively high number of samples, the cost of running a particular method for each sample can add up to a prohibitive level. In general, the higher the sensitivity of the method, the less sample and reagent are required. Also, the smaller the number of experimental steps is involved in a selected method, the fewer reagents are required.

To address these challenges for analyzing nucleic acid adducts, a number of different methods have been designed and developed.[4,5,6,7,8,9] Among those methods, LC-MS is the most commonly used analytical technique for carrying out the end point measurements of nucleic acid adducts. However, different samples or different adducts often pose their unique challenges. In other words, there is no single analytical technique that can be considered as a universal technique for detecting nucleic acid adducts. Unlike some of the recent publications, in which only the use of one specific analytical technique was discussed, this chapter provides an overview of the entire process for analyzing nucleic acid adducts. This chapter begins with a section on the introduction and challenges of nucleic acid adduct analysis. In the second section, the isolation and initial characterization of genomic DNA or specific groups of cellular RNA are discussed. Various analytical methods that have been developed for the detection of nucleic acid adducts are discussed in the third and fourth section, which also includes the rationale for selecting each detection method. It is, however, not our intention to review the analysis of each specific nucleic acid adduct that have been reported in the literature.

2. Methods

2.1 Isolation of genomic DNA and specific groups of cellular RNA

With the advance in the techniques for isolating nucleic acids from cellular samples, the analysis of nucleic acids has become an indispensable tool for studying the biological processes in different types of living organisms. As shown in Figure 1, sample preparation is the most important step preceding any specific type of nucleic acid measurement. For instance, the purpose of proper sampling is to ensure that a representative sample is collected, whose identity and composition is representative of the true *in-situ* abundance. Equally important, the integrity of DNA and RNA should be preserved to ensure trustworthiness and relevance of the data would be obtained in the downstream measurements. This section will cover the current methods for isolating cellular DNA or RNA with high yields and purity as well as the best practice on storing the samples.
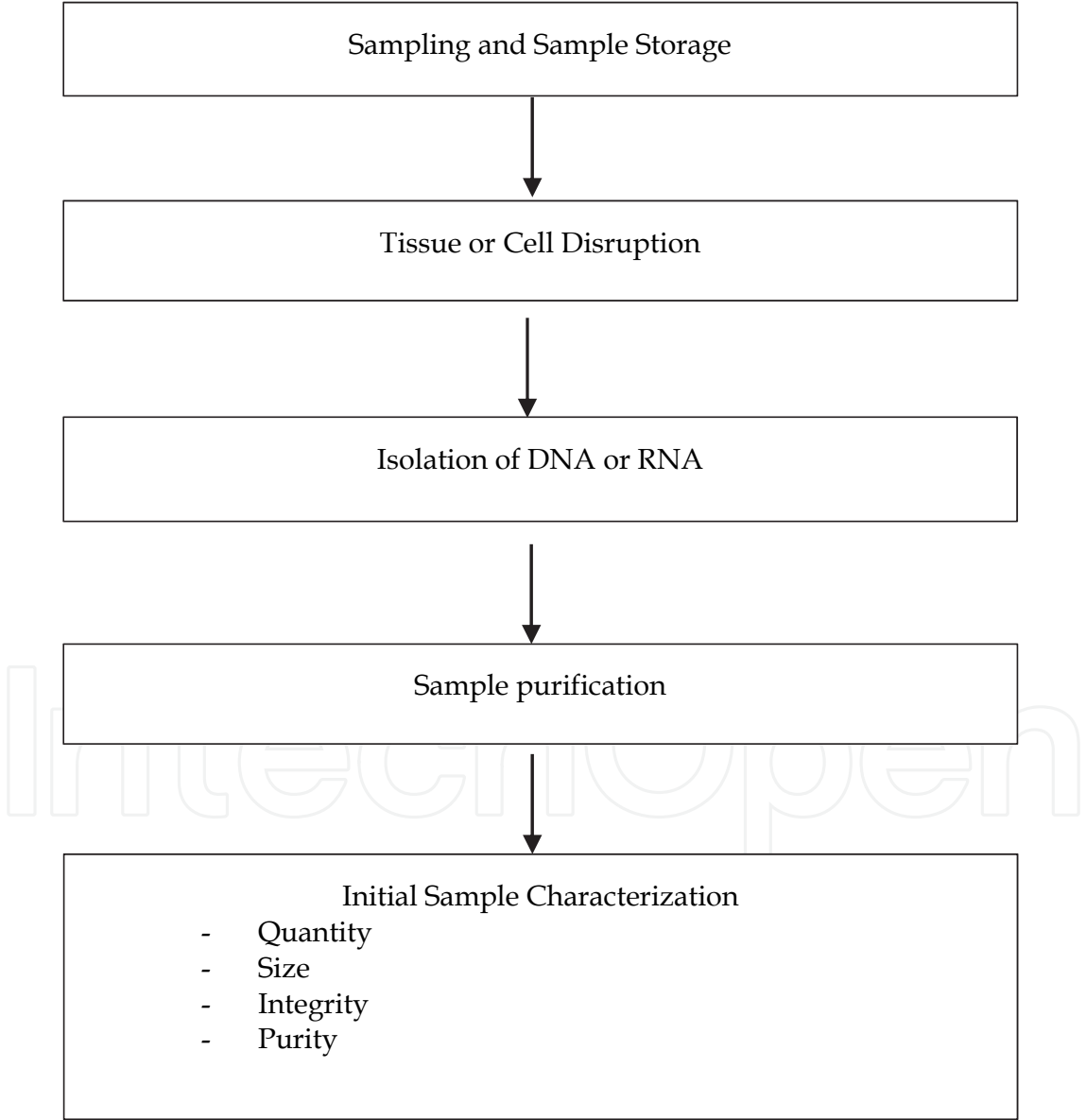

Fig. 1. Schematic diagram of work flow for preparing nucleic acid adduct samples

In general, the isolation of nucleic acids is dependent on the efficiency on disrupting the tissues or cells, the inactivation of nucleases such as deoxyribonuclease (DNase) or ribonuclease (RNase), and the removal of any proteins that may be structurally associated with nucleic acids. Since the quality of any downstream process including the nucleic acid measurements is dependent on the purity of isolated nucleic acids, great care should be taken to avoid any contamination. The most common contaminants include cellular proteins, carbohydrates, lipids, or the presence of RNA in isolated DNA and vice versa.[10] An extensive review on the effects of nucleic acid sample preparations on downstream applications was recently published by GE Healthcare.[11]

The procedures used for sample preparation prior to the isolation of cellular nucleic acids are critical for success. Special care should be taken to prevent sample degradation by nucleases during and after isolation. For example, the degradation of a DNA or RNA biomolecules may hinder a downstream measurement where the nucleic acid is required to bind to a complementary probe such as in microarray analysis. Some indicators of nucleic acid degradation or contamination may include extra products in PCR reactions, failure or reduced success in steps involving nucleic acid modification or restriction using enzymes. A classic signature of nuclease contamination in DNA samples is the characteristic ladder like separation or smear when the DNA is electrophoresed in an agarose gel. In some circumstances, it is necessary to isolate both genomic DNA and RNA from the same sample and this is discussed later in the chapter.[12]

Many commercial kits for the isolation of nucleic acids have been developed but the challenges of sample preparation still remain. These challenges include the availability of very small sample sizes, the ability to eliminate all contaminating materials that may interfere with downstream measurements and the fact that degradation of nucleic acids starts immediately after sample collection. In some cases, samples are difficult to disrupt so as to get to the nucleic acids. It is also challenging to isolate both DNA and RNA from the same sample, to isolate small RNA that are less than 200 nucleotides long, to detect nucleic acids arising from viruses in collected samples, or to detect RNA transcripts which are expressed in low abundance.[13]

2.2 Sampling and sample storage

Prior to the isolation of nucleic acids, it is important to determine which is the nucleic acid of interest (i.e. DNA or RNA), the type of sample that will be collected, and an estimate of how much nucleic acid materials will be required. The limit of detection of the technique to be used for downstream measurements, and its tolerance to contamination should also be considered. All these information are needed for choosing the most suitable isolation method.[14] Nucleic acids have been successfully isolated from tissue, cell culture, bacteria, yeast, fungi, virus, soil, fecal samples, and biofluids such as blood, serum, plasma, lymph, cerebral spinal fluid, ascites, saliva, urine, and amniotic fluid. Following the collection of samples, the general guideline is to begin the process of nucleic acid isolation as quickly as possible. In this way, the possible degradation of nucleic acids will be kept to the minimum. If immediate isolation is not possible, the tissue or cellular samples can be mixed with a stabilizing agent (e.g. RNAlater® from Qiagen) or flash frozen the samples in liquid nitrogen and stored at -80°C. In cases where nucleic acids have to be stored at room temperature for extended periods of time, commercial products such as the FTA Cards technology

developed by Whatman can be used. In brief, the FTA cards contain chemically conditioned cellulose fiber matrices whose composition quickly lyse the cells, inactivate proteins and immobilize the nucleic acids thereby protecting the samples from degeneration. Once the samples have been spotted on the cards, the cards have to be thoroughly dried and can be stored at room temperature for long term archiving. For the FTA cards, the cellular debris is washed away and the nucleic acids remain bound to the card. Whereas, for the FTA Elute, nucleic acids are eluted from the cards using water at 95°C while contaminants remain bound on the cellulose matrix.[15] Isolated DNA samples are best stored at -20°C in 1X TE buffer. If long term storage is required, the isolated nucleic acids should be dissolved in 70% ethanol and stored at -80°C. Prior to storing the samples, the stock solution should be divided into small aliquots which can avoid the degradation of the sample during the freeze and thaw cycles of the stock solution. It is important to make sure that the buffer used to store the nucleic acids is compatible with the downstream experiments such as PCR, mass spectrometry etc.[16]

2.3 Sample disruption and deactivation of nucleases

DNA is found within the nucleus of the cell, tightly coil around histone proteins and together forming the chromatin, while RNA is present both inside and outside the nucleus. The cell membrane forms a protective and selectively permeable layer around the cellular contents, protecting them from the extracellular environment. In order to isolate nucleic acids from collected samples, the DNA or RNA has to be liberated from the cells first by breaking down the cell membrane and forms a cell lysate, a process known as sample disruption. Cultured cells can be lysed directly. Whereas, solid samples such as tissue sections have to be physically disrupted. Sample disruption should be carried out in such a manner that the integrity of nucleic acids is preserved. A high degree of cell membrane disruption is desired in order to maximize the yield of isolated nucleic acids and prevent problems in subsequent steps such as clogging of purification columns. After sample disruption and homogenization, there should be no visible particulates unless the sample contained non cellular components such as bones or connective tissue. The cell membranes can be disrupted and homogenized in a variety of ways. These disruption methods are classified as either mechanical or non-mechanical.[17]

2.3.1 Mechanical methods of cell disruption

Mechanical cell lysis methods involve breaking the samples by shearing force under liquid nitrogen. The samples are mechanically ground using equipment such as a French press, sonicator, bead mill or homogenizer.[18] Mechanical disruption methods can lead to complete cell disruption but can also cause heating or foaming of the sample. Mechanical methods are used for samples such as animal tissues that require great force to disrupt.[19]

2.3.2 Non-mechanical methods of cell disruption

Non-mechanical methods involve physical, chemical or enzymatic processes. These methods can be used for samples such as cultured cells that do not require strong disruption methods. Physical ways of disrupting samples include decompression, freeze thaw, osmotic shock lysis, desiccation, or thermolysis. For example, the freeze thaw technique uses

repeated freezing and thawing of cells to form sharp ice crystals within the cells to disrupt the sample.[20] Osmotic shock lysis technique is dependent on the changes of hydrostatic pressure created by a change in concentration of solutes between a semipermeable cell membrane. The osmotic gradient forces water to flow through the cell membrane, and result in breaking the cells apart. Chemical methods use detergents, salts, solvents, or chelating agents to disrupt the cells. For chemical disruption, chaotropic salts such as guanidinium isothiocyanate disrupt cells by providing a less hydrophobic condition in the sample that in return weakens the hydrophobic interactions within the cell membrane, thus breaking the cell membrane apart. Chaotropic salts also possess the ability to denature nucleases and proteins. Detergent chemical based disruption works by disturbing the hydrophobic interactions of phospholipids that are part of the cell membranes. Enzymatic methods utilize lysozymes and proteases to break down the cell membranes and are mainly used to disrupt cell cultures. Enzymatic treatment is typically followed by sonication, and homogenization by overtaxing in a lysing buffer.21
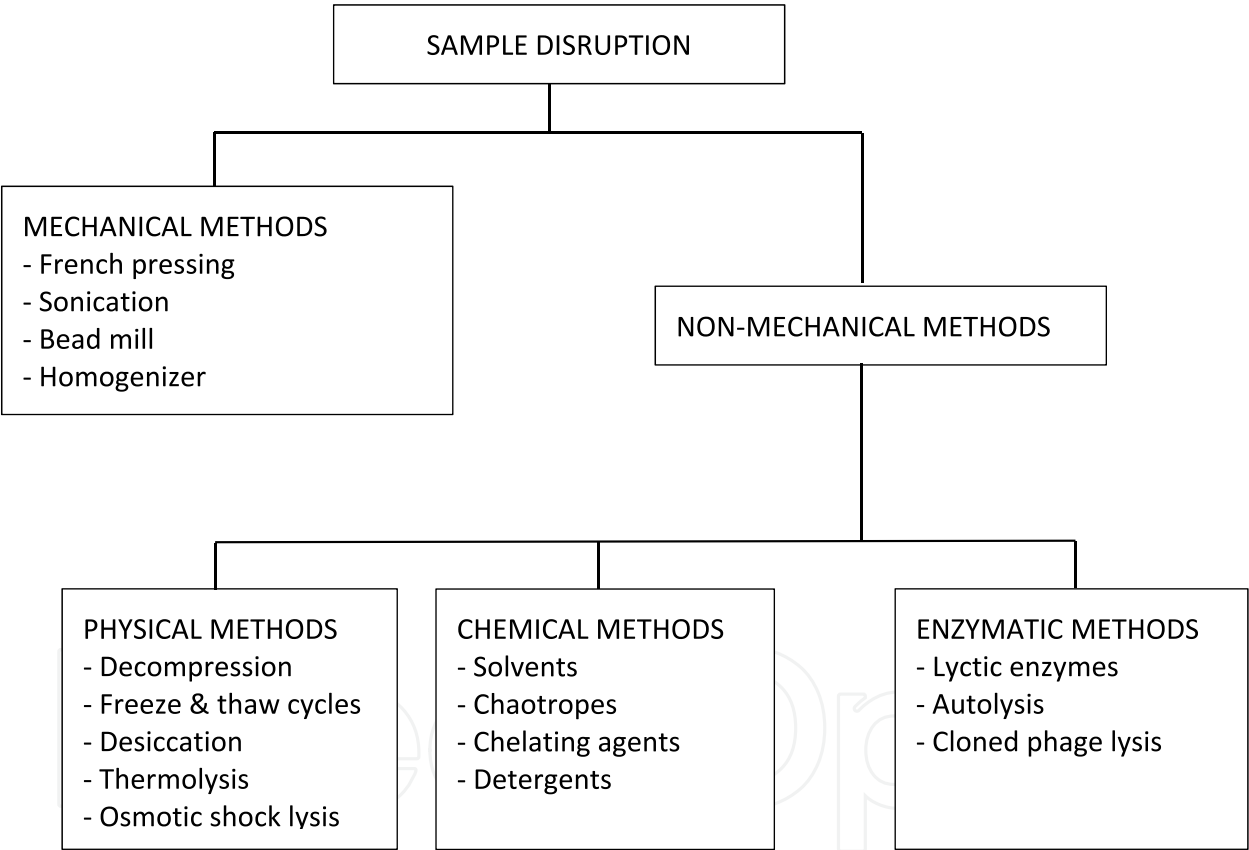

Fig. 2. Summary of sample disruption methods.

Pathological samples are often formalin-fixed and paraffin embedded (FFPE). If FFPE samples have to be used for the isolation of nucleic acids, proper deparafinization using an organic solvent like xylene should be done first.22 As a general rule, it is advisable to use the mildest disruption method possible to avoid mechanical breakdown of nucleic acids, perform the isolation within the shortest period of time by using pre-chilled equipment and use nuclease inhibitors. The choice of purification method will depend on the desired quality and purity of nucleic acids as well as the yield required for downstream analysis.

Disruption of frozen samples is best done in liquid nitrogen rather than letting the samples thaw for the same reasons of limiting nuclease activity.[23]

Usually one of the most important steps towards achieving high quality of nucleic acids is the inactivation of nucleases immediately after the sample is disrupted. Naturally occurring nucleases in the sample can degrade nucleic acids and therefore, during lysis, it is advisable to add a nuclease inhibitor into the lysing buffer. After sample collection, the samples can be flash-frozen using liquid nitrogen or homogenized in the presence of nuclease inactivation solution. Placing sample in a lysing buffer such as guanidinium isothiocyanate can inactivate nucleases. Chelating agents such as EDTA that sequester Mg^{2+} ions which are required for nuclease activity can also be used to prevent nucleic acid degradation by nucleases during storage. Treatment of the sample using proteinase K digests and degrades all proteins, including nucleases. The breakdown of proteins reduces the viscosity of samples and helps in subsequent steps such as filtration.[24] Moreover, nucleases are ubiquitous and can be found in glassware as well as on our fingers. Thus, care should also be taken to avoid contaminating the samples with exogenous nucleases. The use of a clean nuclease-free working area and wearing gloves should always be part of the experimental procedure. Likewise, glassware should be sterilized at 250 °C for two hours.[25]

2.4 DNA isolation techniques

Since the first DNA isolation by the Swiss physician Friedrich Miescher in 1869, numerous protocols have been developed. DNA isolation begins with cell membrane disruption, removal of histone proteins, cell membrane debris and other biomolecules like RNA, lipids and proteins. The common DNA isolation methods are either solution based isolation, silica based methods or anion exchange. Solution based isolation methods use organic solvents such as phenol-chloroform mixture followed by ethanol precipitation. Silica and anion exchange based techniques utilize DNA binding media that is in the form of membranes or solid phase such as spin columns. Many commercial DNA isolation kits are available in the market. The kits use varying protocols and the time for nucleic acid isolation can range from a few minutes to few hours depending on the protocol that has been used in the kit. The kits typically contain all the required solvents and buffers.[26] Numerous studies have been done to compare various kits based on hands-on- time, cost per DNA isolation, ease of use, the yield and quality. However, when setting up a new project, it is always advisable to evaluate different kits for their suitability for the sample of interest from which DNA is to be isolated.[16]

2.4.1 Solution based extraction techniques

The most common solution based extraction technique is the phenol-chloroform based method which has been extensively used with great success. The phenol-chloroform extraction technique depends on phase separation. The lysed samples are mixed with a phenol-chloroform solution where DNA or RNA partitions in the aqueous phase depending on the pH and salt concentrations of the solutions. If the solution is neutral or basic, RNA partitions in the organic phase or in the interface together with proteins, while DNA partitions in the aqueous phase. Centrifugation is done to separate the two phases and residual phenol can be removed by extracting the purified sample using chloroform. The

procedure is completed by precipitating the nucleic acid from the aqueous phase using ethanol. This technique is inexpensive, however, it involves the use of phenol which is toxic. The presence of residual phenol in the extracted DNA can impact negatively on downstream analysis that may involve the use of enzymes. Phenol is neurotoxic and can cause severe burns and therefore care must be taken when using it.[27]

2.4.2 Silica based extraction techniques

The silica based techniques that are used by most current methods offer a fast and robust method for DNA purification. This technique is dependent on the simple fact that DNA adsorbs onto silica surfaces in the presence of high concentrations of chaotropic salts. The silica can be in the form of two different formats: particles that are coated on magnetic beads or silica columns. Many commercial kits using the silica based technique are available, some of which are automated. The buffers used in cell lysis can be chosen in such a manner that during adsorption, only DNA adsorbs onto the silica while all other biomolecules such as RNA and proteins remain in solution. Cellular debris and other biomolecules are washed out using ethanol while the DNA is retained on the silica surface. The DNA is eluted using low salt buffer such as Tris-EDTA (TE) or water. Relatively pure DNA can be obtained using silica based technique; however, treatment of the purified DNA with RNase to remove residual RNA may sometimes be helpful. Care should be taken to wash out all the ethanol to make sure that it does not interfere with downstream applications e.g. presence of ethanol in DNA samples can cause the sample to "float" out of gel electrophoresis wells causing a loss of sample.[28]

2.4.3 Anion exchange chromatography

This purification method relies on the interaction between the negatively charged phosphate backbone of DNA and positively charged functional groups found on an anion exchange resin. The sample is loaded under low salt concentration to allow DNA to bind. A medium strength buffer is used to wash off RNA, proteins and other cellular metabolites. The DNA is then eluted using high strength buffer. Additionally, alcohol precipitation can be used for desalting as well as concentrating the isolated DNA. Since the DNA is eluted with high salt buffer, most downstream applications will require the sample to be desalted. Plasmid DNA is routinely precipitated with ethanol; however, ethanol precipitation of genomic DNA makes it harder to be redissolved. If desalting or concentration of genomic DNA is required, it is best done using isopropanol precipitation or desalting columns.[29]

2.5 Characterization of isolated DNA

Because DNA absorbs at 260nm while protein absorbs at 280 nm, the UV absorbance ratio at A_{260}/A_{280} is generally accepted as a measure of the nucleic acid purity from the contamination of any cellular proteins. A ratio of 1.8 and above is considered as good quality. Organic compounds and chaotropic salts usually absorb at 230nm and therefore the A_{260}/A_{230} ratio can be used to evaluate the presence of residual contaminants from the extraction of DNA. A value of 1.5 and above is considered to be good. A measure of turbidity at 320nm can also be used to evaluate presence of contaminants. The quality of DNA can be assessed by using gel electrophoresis, which provides valuable information on

the integrity of isolated DNA. More vigorous isolation techniques can lead to degradation of DNA, which is evident on the gel as a smear of low molecular weight DNA.[30] DNA quality can also be characterized by using the Agilent 2100 analyzer[31] or real time polymerase chain reaction (qPCR).[32] DNA quantitation is commonly done by UV absorbance. Spectrophotometers such as the NanoDrop developed by NanoDrop Technologies are capable of utilizing as little as one microliter of sample for absorbance measurements with a large dynamic range spanning three orders of magnitude. Usually the conversion factors for 1 absorbance unit at 260nm are 50μg/mL for double stranded DNA, 33μg/mL for single stranded DNA, and 20-30μg/mL for oligonucleotides. Although most quantification work on nucleic acids is done using UV absorbance, this method has some sensitivity and accuracy limits. A_{260} measurements can be altered by contaminants such as free nucleotides leading to erroneous quantification. Fluorescent dyes that selectively bind DNA have also been used for the characterization DNA. In spite of the fact that fluorescent dyes are more expensive than using UV absorbance techniques, the benefits of using fluorescent dyes outweighs their cost drawback. The dyes have a high affinity for DNA and exhibit increased fluorescence enhancement after binding. This enables quantification of very small amounts of DNA. The sensitivity can be more than 10,000 times greater than UV absorbance measurements. Furthermore, a great advantage of fluorescent dyes over UV is that measurements are not affected by the presence of free nucleotides or proteins. Commonly used dyes include PicoGreen and OliGreen for DNA.

2.6 Potential pitfalls of DNA isolation

Residual cellular RNA can be found in the samples of isolated DNA. This is more common when samples are from organs that exhibit more transcriptional activities such as the tissue samples of liver or kidney. Since RNA also absorbs UV light at 260 nm, the presence of any RNA in DNA samples adversely affect the quantitative UV measurements. The presence of RNA in isolated DNA samples also interferes with sequencing and reduces the efficiency of amplification. RNA contamination can be eliminated by treating the sample with RNase to degrade the RNA. The presence of proteins in isolated DNA samples can be the result of incomplete digestion by proteases and interfere with the mobility of DNA during gel electrophoresis as well as altering the kinetics of other enzymatic reactions. Residual organic solvents such as ethanol, phenol, chloroform etc. will negatively affect most downstream procedures while salts will slow down or inhibit enzymatic activity such as restriction enzymes. Furthermore, just because a sample contains only DNA is not absolute proof of good quality. This is because during isolation, the sample can be contaminated with DNA from other sources. Such external DNA will give a false impression during DNA quantitation and interfere with amplification procedures.[33]

2.7 RNA isolation techniques

Total RNA can be divided into several groups of RNA which include messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), mitochondrial RNA (mtRNA) and many groups of small RNA such as microRNA (miRNA), and piwi-interacting RNA (piRNA). On average, about 80% of total RNA is made up of rRNA while 1-5% is mRNA. Expression of mRNA can vary greatly from cell to cell and can range from a few copies per cell to several thousand.[34] If poly-adenylated mRNA is required, it is preferably isolated from total RNA by

capturing the poly(A) tail onto oligo(dT) primers. The same approach can also be used to isolate mRNA directly from the lysate without necessarily isolating the total RNA. Similar to the isolation of DNA, the requirements for both quality and quantity of isolated RNA would determine the choice of isolation method. For example, RNA isolated for use in microarrays and RT-PCR needs to be of high purity and free from contaminants such as salts, phenol, ethanol etc. whereas methods such as Northern blotting may be more forgiving for contaminants. When purifying RNA, it is very important that it is prevented from degradation by both endogenous and exogenous RNases. Many commercial kits for RNA isolation have been developed, some of them are designed to isolate specific group of RNA, e.g. mRNA.

The most common solution based technique for RNA isolation is the guanidinium, phenol-chloroform ethanol precipitation. This technique was first reported by Chomczynski and Sacchi in 1987 and later revisited in 2006 by the same authors.[35] Over time, the method has been widely used and modified. Basically, it operates on the principle that RNA can be separated from DNA if an acidic solution containing sodium acetate, phenol, chloroform and guanidinium isothiocyanate is used. Guanidinium isothiocyanate is a very effective protein denaturant and when used in buffers, it provides an RNase-free environment. Phenol dissociates nucleic acids from proteins while chloroform denatures both proteins and lipids. Chloroform also makes nucleic acids less soluble in phenol and maintains the separation of the aqueous and the organic layer. Mixing the lysate with the acidic solution results in phase separation with the RNA partitioning in the upper aqueous layer while the DNA, lipids and protein remain either in the interphase or in the lower organic phenol layer. The upper aqueous layer is retrieved and the total RNA recovered through precipitation with isopropanol.

2.7.1 Sequence specific RNA isolation

To capture RNA whose sequence is known, biotinylated DNA probes consisting of a sequence complimentary to the known portion of the RNA to be isolated can be used. The probe-RNA complex is then isolated using streptavidin coated magnetic beads through the interaction of biotin and streptavidin. The beads are pulled out of solution using a magnet, and the supernatant containing cellular debris and contaminants is removed. The captured RNA is then eluted from the beads for downstream applications.[36]

2.7.2 Isolation of mRNA

To isolate mRNA, a sample containing total RNA is loaded in an oligo(dT) spin column. In high salt conditions, the 3' poly(dA) tail of mRNA hybridize to oligo(dT) that have been immobilized on the column. The contaminants are washed off with a high salt buffer and a low salt buffer. The mRNA is then eluted using a low ionic strength buffer. Alternatively, magnetic beads coated with poly(dT) beads can be added to total RNA sample. The beads are captured with a magnet and the contaminants are removed. The bead complex is then washed and mRNA are eluted. This technique is also useful for concentrating mRNA samples.[37]

2.7.3 Isolation of Small RNA with <200 nucleotides (nt)

The most widely used techniques for total RNA isolation are phenol or silica based. These methods were designed and optimized for isolation of high molecular weight RNA because

small RNA molecules were thought to be unimportant. As such, these traditional methods do not retain most of the small RNA fraction and are therefore not suitable for the isolation of small RNA (<200nt). Since small RNA only makes up a small fraction of total RNA, the use of total RNA as starting material for any detection method can potentially lower its sensitivity and/or specificity. Small RNA such as microRNA, siRNA, piwiRNA, rasiRNA and others have been shown to perform important biological functions. In order to isolate these RNA, the traditional methods have been redesigned or completely new methods have been developed. Initially, separation and enrichment of small RNA from total RNA was done by size fractionation using size exclusion columns or by using 12-15% denaturing polyacrylamide gel electrophoresis. These techniques however only yield minute amounts of small RNA. The need to isolate larger amounts of small RNA has led to development of the new methods. Commercially available kits for small RNA isolation include PureLink from Invitrogen (Carlsbad, CA, USA)38, miRNeasy Mini Kit from Qiagen (Valencia, CA, USA)39 and mirVana that is produced and sold by Ambion (Austin, Texas, USA)40 among others. The mirVana kit makes use of organic as well as solid phase isolation. RNA is first isolated from the lysed sample using acidic phenol-chloroform solution that separates RNA from proteins, DNA and other cellular debris. The isolated RNA can be further purified for small RNA by immobilizing them onto a glass-fiber filter. A low ionic strength buffer is used to wash the filter before small RNA is eluted with a relatively low ethanol concentration (25%).[41]

2.8 Characterization of isolated RNA

Similar to the characterization of isolated DNA, the traditional method for the quantification of RNA is by using UV absorbance measurements. A diluted sample of RNA is measured at 260 and 280nm. Using the Beer-Lambert law which assumes that the relationship between absorbance and concentration is linear, the concentration of RNA can be calculated. An absorbance of 1.0 using A_{260} measurement translates to an RNA concentration of ~40μg/mL. The A_{260}/A_{280} ratio is used as a measure of RNA purity since proteins absorb at A_{280}. A ratio of 1.82:1 is accepted as a measure of highly purified RNA. In the presence of DNA contamination, UV measurements can overestimate the RNA concentration because DNA also absorbs at 260nm, however, if DNA contamination is suspected, the sample should be treated with RNase-free DNase to eliminate the DNA. The quantity of RNA can be determined by using fluorescent dyes such as RiboGreen and fluorometric measurements. This method is less sensitive to free nucleotides and proteins but can be inaccurate in the presence of DNA contamination. The integrity of isolated RNA is often measured by using denaturing gel electrophoresis. When total RNA sample is run on a denaturing gel stained with ethidium bromide, the 28S and 18S rRNA show up as intense bands. The 28S and 18S rRNA band intensity ratio can be used as a measure of the integrity of RNA. A ratio of 2:1 (28S:18S) is usually accepted as a measure of intact RNA. Smeared 28S and 18S coupled with a ratio below 2:1 is an indication of RNA degradation. Highly degraded RNA samples appear as a smear in the region of low molecular weight. For limited amounts of RNA samples, other RNA staining dyes that are more sensitive such as SYBR Gold and SYBR Green can be used.[42] A more recent method is the Agilent 2100 Bioanalyzer that uses a microfluidics-based platform in conjunction with capillary electrophoresis and fluorescent dyes to perform both qualitative and quantitative measurements. The sample size for the 2100 Bioanalyzer can be as low as 1μL of 10ng/μL RNA.[43]

2.9 Potential pitfalls of RNA isolation

The presence of RNase causes degradation of RNA and will affect all downstream applications that require intact RNA. The presence of polysaccharides especially in liver and muscle tissues can affect RNA isolation and lead to low yields. Polysaccharides also inhibit RT-PCR, though, the mechanism of interference is not known. Residual genomic DNA in isolated RNA interferes with RT-PCR, microarrays and even nuclease protection experiments. The DNA contaminants can compete for PCR primers or other probes for the detection of RNA and lead to false positive signals. DNA is easily removed from the isolated RNA by digesting it with DNase. The DNase can be easily removed before downstream experiments are performed. Residual EDTA and solvents used to isolate RNA can chelate Mg2+ ions that are needed by polymerases in PCR and prevent cDNA synthesis. They can also interfere with microarray experiments. Carryover salts from buffers and other solutions slow down reverse transcriptase and cDNA synthesis. Alcohol, chloroform and phenol that may be carried over during isolation affect most biological experiments by inhibiting enzymatic activity.

3. Detection and quantificaton of DNA adducts

Referring to Figure 3, depending upon the type of biological samples, nucleic acid adducts can exist at the genomic level (i.e. intact DNA) or individual nucleotide adducts, which result from biodegradation and have been excreted into biofluids (e.g. urine).

For the analysis of genomic DNA, the majority of developed methods require breaking down the genomic DNA into monomeric nucleotides or dinucleotides. This can be accomplished by either chemical hydrolysis or enzymatic digestion as shown in the schematic diagram in Figure 3. With the chemical hydrolysis of genomic DNA, if an acidic compound is used, it may induce the depurination of DNA and/or the hydrolysis of DNA adducts. Thus, the alternative enzymatic approach is more widely used to digest genomic DNA. To ensure a complete digestion of genomic DNA, a combination of different nucleases, for instance exonuclease and endonuclease, is often used. Following the digestion of genomic DNA, the approach for analyzing DNA adducts is the same for all types of biological samples, i.e. DNA adducts are separated from a mixture of nucleotides prior to their specific detection.

The removal of unmodified nucleotides can greatly enhance the specificity of the subsequent detection of DNA adducts. This is because, in many studies, the ratio of unmodified nucleotides to nucleotide adducts can be as high as $10^{12} : 1$. As indicated in Figure 3, the separation of DNA adducts is usually achieved by using one or a combination of chromatographic techniques. Often times, this part of an experimental procedure for analyzing specific DNA adducts is referred as enrichment of DNA adducts or a solid-phase extraction of DNA adducts. After the separation of DNA adducts, there are a number of different analytical techniques that have been chosen for carrying out the end point measurements of specific DNA adducts as shown in Figure 3. In general, the end point measurements can be divided into two groups, namely with or without the use of reporting labels. The use of reporting labels, for instance ^{32}P radioactive isotope, can achieve lower limit of detection (LOD). On the other hand, the direct adduct measurements, i.e. without the use of any reporting labels, can achieve higher specificity and also allow the molecular structure of adducts to be determined. The selection of a specific analytical technique is

ultimately determined by the physiochemical properties of DNA adduct and its abundance in the biological samples. More details on each analytical technique that have been used to detect DNA adducts are covered in subsections 3.1.1 to 3.1.5. Readers are advised that the aim of this section is to provide an overview of the current methodologies for DNA adduct analysis that have been used in the last five years and is not intended to cover all the reports in the literature. Several excellent reviews on the detection of specific DNA adducts are available in the literature.[44,45]
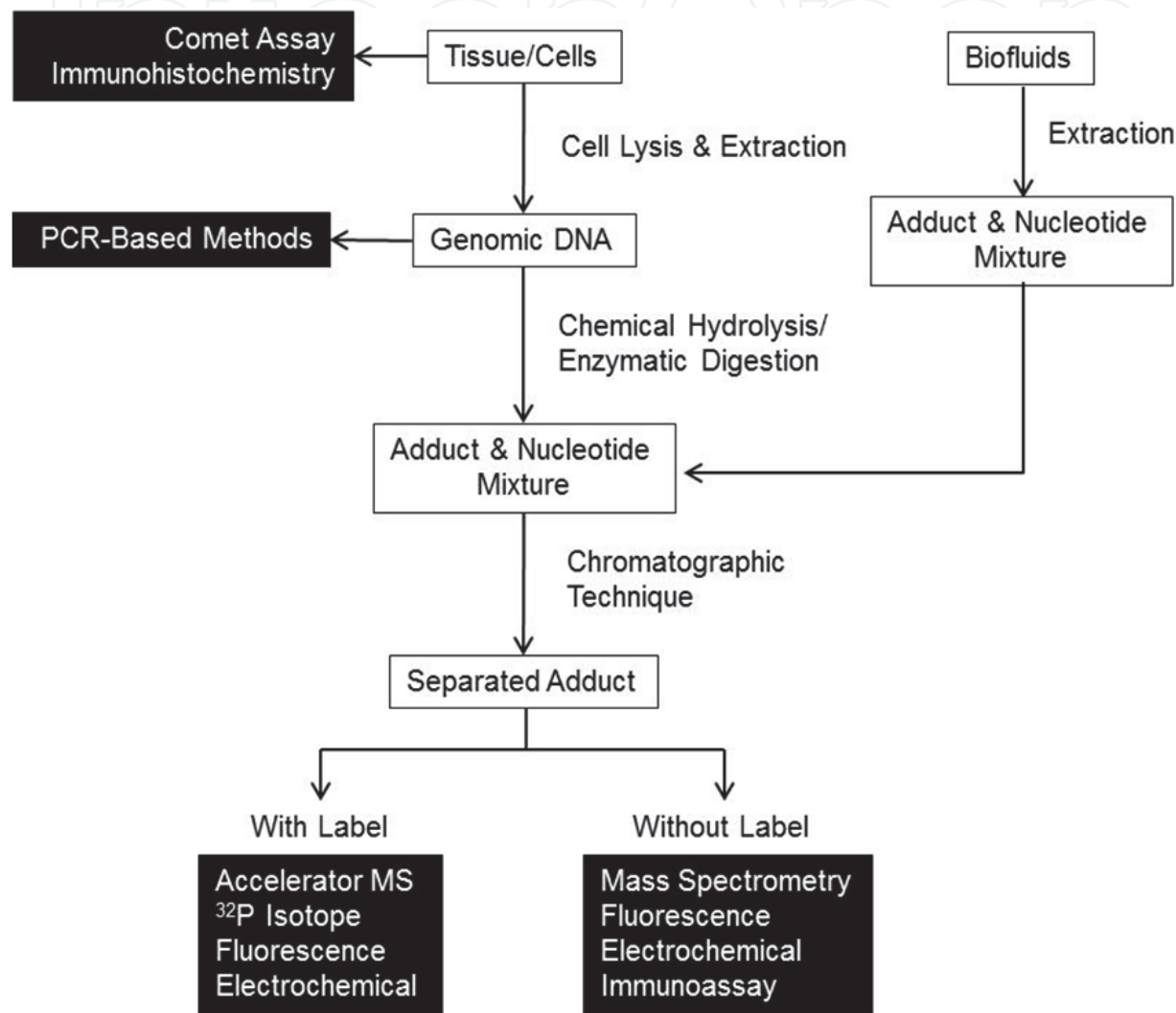

Fig. 3. Schematic diagram of different approaches for analyzing DNA adduction.

It is important to realize that the digestion of genomic DNA into its monomeric nucleotides is equivalent to erasing all the DNA sequence information, thus the locations of adduction within the genome can no longer be determined. In order to determine the locations of DNA adduction, genomic DNA has to be analyzed. There are a number of polymerase chain reaction (PCR) based methods that have been developed which allow us to determine the locations of adduction. In general, these methodologies rely on using specific enzymatic activities or chemical hydrolysis methods to create unique reaction products that correspond to each location of DNA adduct. For instance, an adduct-containing DNA fragment can be cleaved at or near the adduct by a DNA repair enzyme. The resulting

fragments are amplified by using PCR. By sequencing the PCR amplicons, the locations of adduct can then be determined. With the use of PCR, very small amounts of starting material are required. However, the drawbacks of this approach include the specificity of the methods for breaking down the genomic DNA at or near the location of an adduct, the lack of information on the adduct identity, and the inability to determine the location of adducts that are close or next to each other. Overall, the PCR-based methods are the only approach that can be used to determine the location of adducts but may not be universally applicable.

With a wide variety of DNA adductions, there is no single method that can be used to analyze every possible DNA adduct. Before spending time and efforts to develop and/or validate a method, the extent of DNA damage can be evaluated by a non-specific method called comet assay. As indicated in Figure 3, the starting materials for comet assay are tissue or cellular samples. In the comet assay, cells are first embedded in an agarose gel. The cells are then lysed under a condition that induces DNA to supercoil and link to the nucleus matrix. With the presence of DNA damage, it is easier to electrophoretically pull the DNA out from the cell nucleus. By using microscopic fluorometry to monitor the migration of DNA, which forms a tail resembling a comet, the extent of cellular DNA damage can be evaluated. The higher the fluorescence intensity in the tail, the higher the extent of DNA damage. Alternatively, for determining the presence of DNA adducts on a tissue section, the approach of immunohistochemistry can be used, providing a specific detection antibody against the adduct of interest is available.

3.1 Accelerated Mass Spectrometry (AMS)

AMS has generally been regarded as the most sensitive method available in the detection of DNA adducts with a limit of detection (LOD) ranging from ~1-10 adducts per 10^{12} nucleotides.[46] In AMS, the abundance of specific radioisotopes is measured. For measuring biological samples, the most relevant isotopes are ^{14}C and ^{3}H. The sensitivity of AMS is high enough that even very small amounts of exogenously applied radioisotopes can be detected, thereby allowing AMS to be used in human studies.[47] The sample preparation protocol involves the extraction of cellular DNA which can be either analyzed as intact DNA or subjected to enzymatic digestion into nucleotides. This is followed by the separation with HPLC to isolate the adduct of interest. Sample analysis usually involves combustion into elemental carbon or CO_2 for ^{14}C or titanium hydride for ^{3}H. However despite the aforementioned advantages, AMS has several disadvantages as well. Before the adduct measurements, the subject or host has to be exposed to radioactive isotopic-labeled precursors. As such, biomonitoring studies are often prohibited. Since the chemical structure of the adduct is lost due to its decomposition into the elemental components, there is a requirement to compare the chromatograms of synthetic adducts (if available) with that of the sample, to accurately identify the adduct. Significant emphasis must be placed on sample purity to prevent the false positive signals from any radioactive background noise. Currently, there are a limited number (5-10) of AMS spectrometers worldwide that are specifically setup for biological sample analysis.[48] To increase the viability to analyze samples from humans in terms of possible exposure to unlabeled carcinogenic/genotoxic compounds, postlabeling methods have been developed to derivatize DNA adducts of interest with ^{14}C or ^{3}H isotopes.[46]

3.2 ^{32}P radioisotope labeling

The ^{32}P-postlabeling method is useful in analyzing a vast array of DNA adducts (e.g. polycyclic aromatic hydrocarbons, aromatic amines, etc.) and is also highly regarded with respect to sensitivity (LOD of 1 adduct/10^{10} nucleotides). Following the isolation of genomic DNA, the sample is enzymatically digested into 3'-mononucleotides or less commonly dinucleotides. Enrichment procedures call for the use of either immunoaffinity chromatography or *n*-butanol as a means to extract adduct from unmodified nucleotides. The extracted nucleotide adduct is then dephosphorylated using nuclease P1 treatment. Phosphorylation (or ^{32}P-postlabeling) is then performed by the specific activity of T-4 polynucleotide kinases. This produces 3',5'-bisphosphates which are subsequently separated by using a chromatographic technique such as high performance liquid chromatography (HPLC), two-dimensional thin layer chromatography (2D-TLC), or gel separation. Resolution is greater for HPLC but sensitivity is lower than

TLC. Thus, for quantitative analysis, TLC is more common. The initial standardization protocols for quantitation purposes in the 1990s were found to be only qualitative. More recently, a method that overcame some of the shortcomings of earlier trials was reported.[49] With efficient isolation of DNA adduct and labeling, quantitation of specific adducts is possible. A set of standardized protocols are now instituted and allow quantitation of several types of DNA adducts (aromatic amines, PAHs, methylating agents).[50] Nevertheless, the required large quantities of radioactive isotopes and the lengthy time necessary for completing the labeling procedure are problematic. Also, structural characterization of DNA adduct is not possible, thus rigorous co-chromatography against synthetic or previously identified adducts is required to identify DNA adducts.

3.3 Mass spectrometry

With the advances in mass spectrometry, the applications of mass spectrometry to analyze DNA adducts have continued to grow in the recent years. A number of excellent reviews have been published.[51] Following the isolation and digestion of genomic DNA, the analysis of DNA adducts can be carried out by using either gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS). In the case of GC-MS, the less volatile adducts have to be derivatized prior to the GC-MS measurements. Also, owing to the thermal decomposition, the analysis of adducts are often limited to nucleobases. With the development of soft ionization techniques like electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), liquid chromatography has been successfully coupled to mass spectrometry. This allows the accurate mass of specific adducts to be measured as well as their molecular structure can be determined by using tandem mass spectrometry (MS/MS). Specifically, following the elution of an adduct from a LC column and the ionization of adduct, the precursor (or parent) ion of the adduct is selected by the first mass analyzer. The precursor ion is then fragmented by a process called collision-induced dissociation (CID). After that, all the fragment ions which originated from the selected adduct are separated and detected in the second mass analyzer. The molecular structure of selected adduct including the type and position of adduction can be determined with high accuracy by analyzing the MS/MS data. The structural information is very desirable as it may provide insight into the metabolism of a genotoxic agent before adduction. Additionally, small sample sizes are possible with the LC-MS method. This is in

contrast to other methodologies for determining molecular structures, such as nuclear magnetic resonance (NMR) spectroscopy which requires larger sample sizes and higher concentration of adduct. For the quantitation of adducts, the choice of instrumentation is a mass spectrometer that is equipped with a triple quadrupole mass analyzer. To ensure sufficient high sensitivity is achieved, nano-electrospray ionization and/or the selected reaction monitoring (SRM) mode in a triple quadrupole mass spectrometer are recommended. Recently, a two-dimensional linear quadrupole ion trap mass spectrometer operating in a constant neutral loss scanning mode with subsequent triple stage MS was utilized in the detection and quantitation of five different DNA adducts.[52] This technique was demonstrated to be capable of screening various carcinogenic adducts at low LOD (~1 adduct per 10^8 nucleotides) with small quantities of DNA (~10 μg). This is an order of magnitude more sensitive than the best available immunoassays and close to that of ^{32}P-postlabeling method. Several reports have been published in which ESI MS has been coupled to capillary electrophoresis (CE) or nanoLC, thereby providing the capability to examine adducts at low levels. For example, an LOD of ~5 adducts per 10^9 nucleosides with a linear dynamic range between 70 amol and 70 fmol has recently been reported.[53]

3.4 Fluorometric and electrochemical detection

Following the chromatographic separation of adducts, their detection can be achieved by fluorescence, electrochemical (EC), or electron capture detection (ECD) providing the adducts have either intrinsic or chemically induced properties to generate the corresponding signals. In general, the sensitivity of these techniques are often in the range of 1-10 adducts per 10^8 nucleotides and require ~20-100 μg of genomic sample. If pure standards are available, structural characterization as well as the quantitation of adducts are possible. However, the number of adducts which have the required intrinsic physiochemical properties are limited. Also, the derivatization (or labeling) of adducts which lack the intrinsic properties to generate the corresponding signals is labor intensive and the yield is usually low. These techniques have been used to detect adducts with inherent fluorescence property (e.g. PAH and aflatoxin B_1) or electrochemical characteristics (e.g. 8-oxo-7,8-dihydroguanine). Providing the adduct has a derivatizable group in comparison to unmodified DNA, the derivatization of adduct with a volatile ligand, such as pentafluorophenyl, would allow the use of GC which is coupled to an electron capture detector, whose sensitivity can be as low as zeptomole.[54] Similar sensitivity can also be achieved by using laser induced fluorescence (LIF) providing the phosphate group of a nucleotide containing the adduct has been derivatized with an appropriate fluorophore.

3.5 Immunoassay

As mentioned earlier, antibodies can be used to detect DNA adducts in a wide variety of tissue samples (immunohistochemistry). The same antibody can be applied to the detection of digested or excreted DNA adducts in various biofluids by using an enzyme linked immunosorbent assay (ELISA) assay or an equivalent immunoassay. Various antibodies against adducts with either high or low molecular weight are available. However, in clinical studies, the specificity of antibodies to recognize adducts is relatively poor. This is partly due to the fact that DNA adducts are not separated from the other unmodified nucleotides or cellular components before being measured by immunoassay. To address this technical

issue, trapped in agarose DNA immunostaining (TARDIS) assay has been developed. An extensive review of this topic has been discussed.[55] In general, monoclonal antibodies are preferred over polyclonal antibodies with regards to specificity but polyclonal antibodies can provide higher sensitivity (1 adduct per 10^8 nucleotides). The overall assay sensitivity is dependent on the affinity of antibody for the adduct of interest and the detectability of reporting label being used.

4. Detection and quantificaton RNA adducts

In comparison to the DNA adduct analysis, the number of reports on RNA adduct analysis are significantly less. This could be partly due to the fact that, unlike the genomic DNA, cellular RNAs are not used as templates to generate any biological molecules. Furthermore, there is a natural turnover of cellular RNA, i.e. the gene expression and degradation of RNA are continuous processes within the cells. Owing to these reasons, the effects of RNA adduction have often been considered to be less important than DNA adduction. Nevertheless, RNA adduction has been associated with different diseases which include cancer. The same methods that have been used to detect DNA adducts can also be applied to the detection and quantification of RNA adducts. To ensure higher specificity is achieved, it is important to remove any genomic DNA from cellular RNA samples prior to the RNA adduct analysis. As mentioned earlier in this chapter, the most effective approach to remove genomic DNA is by carrying out the DNase digestion, and the intact cellular RNA that remains in the sample can be easily purified by using one of the standard RNA purification methods. After that, cellular RNA is ready to be digested into nucleotides, unless the information on the location of RNA adducts is desired. To determine the location of adducts within an RNA fragment, the PCR-based methods can be used. For instance, reverse transcriptase would prematurely stop the cDNA synthesis at the adduct location and produce a template which is unique to the adduct location for the subsequent PCR reaction. In general, the PCR-based methods have lower specificity than those methods that have been developed to analyze a mixture of nucleotides including RNA adducts.

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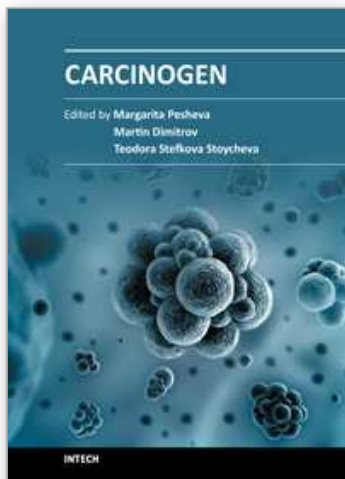
Carcinogen

Edited by Dr. Margarita Pesheva

During the last decades, cancer diseases have increased all over the world. The low quality of food and strong pollution of environment are the main prerequisites for carcinogenesis. The main problem for scientists is to find strategy for prevention of cancer diseases. Therefore, the information about the models for studying carcinogenesis and mutagens which appear during cooking, environmental pollutants, and tests for specific detection of carcinogens is particularly important. The book "Carcinogen" is intended for biologists, researchers, students in medical sciences and professionals interested in associated areas.

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