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REVIEW

Detection of nucleic acid modifications by chemical reagents

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ABSTRACT

Nucleic acids, especially RNA, naturally contain a diversity of chemically modified nucleosides. To understand the biological role of these modified nucleosides, nucleic acid scientists need tools to specifically label, detect and enrich modified nucleic acids. These tools comprise a diverse set of chemical reagents which have been established in the early years of nucleic acid research. Recent developments in high-throughput sequencing and mass spectrometry utilize these chemical labeling strategies to efficiently detect and localize modifications in nucleic acids. As a consequence the transcriptome-wide distribution of modified nucleosides, especially 5-methylcytosine and pseudouridine, in all domains of life could be analyzed. With the help of these techniques and the gained knowledge, it becomes possible to understand the functions of modifications and even study their connections to human health and disease. Here, the differential chemical reactivity of modified nucleosides and their canonical counterpart is reviewed and discussed.

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Introduction

In all domains of life, genetic information is stored by the sequence of the canonical nucleosides cytidine, guanosine, adenosine and thymidine in DNA and uridine in RNA, respectively. In addition, modified nucleosides in both DNA and RNA form a second layer of information, regulating major life processes like transcription and translation. To understand the biological role of these modified nucleosides, nucleic acid scientists need tools to specifically label, detect and enrich thus modified nucleic acids. These tools comprise a diverse set of chemical reagents which have been established in the early years of nucleic acid research. While their use has been nearly forgotten after the introduction of more sensitive techniques like mass spectrometry, some have been re-discovered in recent years. For example, the uridine isomer pseudouridine (Ψ, Fig. 1) is labeled with carbodiimides[1] (Table 1) or new carbodiimide derivatives[2] to enable the modification's detection by either mass spectrometry[3] or sequencing.[4-6] The return of this reagent has led to the discovery of Ψ in many mRNAs which resulted in renewed interest to study not only the distribution and function of Ψ, but also other modifications in mRNA and non-coding RNAs. Another example is bisulfite, which is now commonly used in RNA and DNA sequencing for detection and localization of 5-methylcytosine,[7] the so-called 5th base of DNA and a common RNA modification.

Here, chemical reagents used for the detection of modified nucleosides will be classified into (a) reagents used to covalently label modified nucleosides, (b) reagents converting nucleosides or inducing strand breaks and (c) reagents for enrichment of modified nucleic acids. This review summarizes these reagents and nucleoside targets which lead to the differential reactivity of modified nucleosides and highlight recent breakthroughs achieved by the combination of chemical labeling with modern sequencing techniques. Fig. 1 gives an overview of modified nucleosides detectable by chemical labeling approaches and the reactive groups of the modified nucleoside are indicated by arrows.

Covalent labeling of modified nucleosides

Chemical reagents, used for labeling of modified nucleotides, target certain functionalities in the modified residue. This target is, in the optimal case, only present in the modified nucleotide but not in the canonical, like the sulfur of 4-thiouridine (s^4U), which allows a high selectivity of the reagent. Structures of the reagents with arrows indicating their reactive center can be found in Table 1. The exact mechanisms for all reagents have been summarized by the Motorin lab.[8]

Thiols

In general, thiols are strong nucleophiles that attack all sorts of electrophiles like halomethylated fluorophores. So far an iodo- (9) and bromoacetamide.[10] have been used to label s^2U and s^4U (2-thio- and 4-thiouridine, respectively). In addition, a bromomethyl-coumarin.[11] was reported to react with s^4U, however the selectivity towards other thiolated nucleosides like s^2U or s^2C (2-thiocytidine) was not studied yet.

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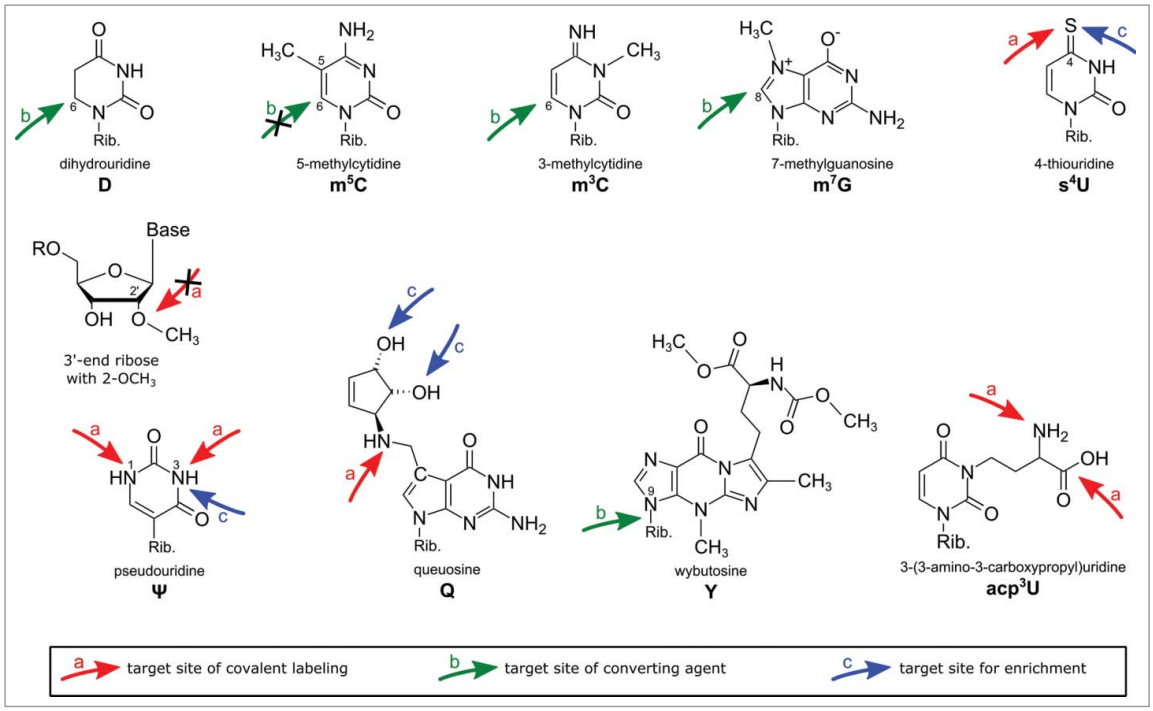


Figure 1. Overview of modified nucleosides. The arrows indicate the target sites in the modified nucleosides responsible for their reactivity with the described reagents. Red arrows: covalent reaction, green arrows: converting reaction, blue arrows: physicochemical interaction.

5-methyl-ribopyrimidine

Deoxythymidine and 5-methyldeoxycytidine (m^5dC or just mC) in DNA can be specifically labeled with OsO_4 or osmate complexes, which react with the diastereotopic 5–6 double bond.[12] of the nucleobase. Only recently, this approach was presented in context of RNA where the osmium tetroxide-bipyridine complex reacted with both m^5C and m^5U on the nucleoside level. Interestingly, a change in diastereoselectivity leads to an almost complete loss of selectivity towards 5-methylcytosine (m^5C) in a 5-mer ribonucleotide context, while 5-methyluridine (m^5U) remained about 8 times more reactive than the canonical pyrimidines.[13]

Carboxylic acids

Although common in other macromolecules, free carboxy groups are only found in modified nucleosides conjugated with amino acids like 3-(3-amino-3-carboxypropyl)uridine (acp^3U), N6-threonylcarbamoyladenosine (t^6A) or N6-methyl-N6-threonylcarbamoyladenosine (m^6t^6A). It was shown that these nucleosides react selectively with primary amines in e.g. aniline or ethylendiamine in soluble carbodiimide.[14] Many more carboxyl functionalized nucleosides (e.g. N6-glycinylcarbamoyladenosine (g^6A), uridine 5-oxyacetic acid (cmo^5U), 5-carboxymethylaminomethyluridine ($cmnm^5U$), etc.) have been discovered, since this report was published, and it is very likely that these might also be labeled under the same conditions.

Aliphatic amines

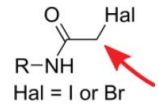
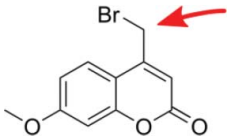
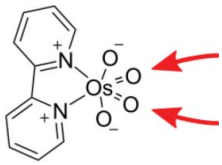
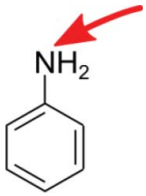
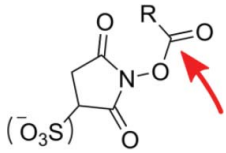
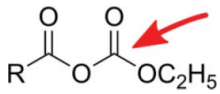
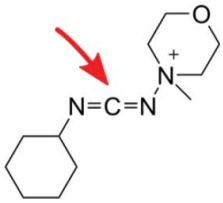
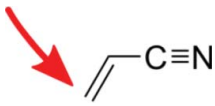
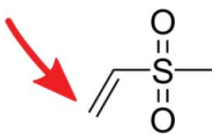
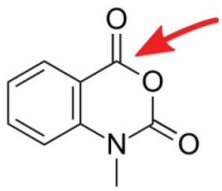
Similar to carboxyl groups, aliphatic amines are only found in some modified nucleosides like acp^3U, 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) and queuosine (Q). Fluorescein-isothiocyanate (FITC) was reported to react with both Q[15] and acp^3U,[16] while the latter also reacts with N-hydroxysuccinimides.[10,17,18] The aliphatic amino group of an uncommon bacterial wobble modification mnm^5s^2U was found to react with a spin-labeled anhydride[19] and in addition showed reactivity with a carbodiimide derivative.[10]

Aromatic amines

Fig. 2 shows the N-acylation of usually aromatic amines by the carbodiimde CMCT (N-cyclohexyl-N'-β-(4-methylmorpholinium)ethylcarbodiimide p-tosylate) which leads to products of guanosine, uridine, inosine (I), 2-methlythio-6-isopentenyladenosine (ms^2i^6A) and pseudouridine (Ψ). In a second step, the acyl-moieties can be removed from guanosine, uridine and inosine[20] residues by alkaline treatment, leaving only N3-acylated pseudouridines[21] and an unknown derivative of 2-methylthio-N6-isopentenyladenosine (ms^2i^6A).[3] The acylation product is relatively bulky, which allows detection not only by mass spectrometry but additionally by reverse transcription (RT).[1,3] This reagent was recently used for sequencing which allowed a transcriptome wide mapping of Ψ in yeast,[6] human cells[4] and dyskeratosis congenital patient samples.[5] In addition, an azide modified CMC derivative was developed which allows enrichment of pseudouridylated RNA prior to sequencing by azide-alkyne cycloaddition and subsequent Biotin pulldown (encircled in Fig. 2).[2]

CMC labeling is a multi-step process with precisely defined conditions necessary to achieve the desired pseudouridine selectivity. Therefore special attention is required at all steps of the labeling procedure to avoid false positive results. In addition to

Table 1. Overview of all reagents used for covalent labeling and detection of modified nucleosides. Red arrows indicate the active groups responsible for the reaction with the target modification. The term "target modification" lists the modified nucleosides known to react with the reagent from the literature. "Potential side reactions" lists all nucleosides that are chemically able to react with the reagent but were not described in the literature.

Reagent	Structure=active site	Target modification	Potential side reactions
Halo-acetamides		thiolated nucleosides, *e.g.* s^2U,[10] s^4U[9]	s^2C
Bromomethyl-coumarin		thiolated nucleosides,[11] pseudouridine[25]	uridine, thymidine[26]
Osmate complex		5-methyl-ribopyrimidines, e.g. m^5U [13] and dm^5C[12]	m^5C, thymidine[12]
Activated amines, *e.g.* aniline and ethylenediamine		Carboxyl functions, *e.g.* acp^3U, t^6A[14]	Other modified nucleosides with carboxyl groups like g^6A, $cmnm^5U$, etc.
Isothiocyanate		Aliphatic amines, *e.g.* Q[15] and acp^3U[16]	Other aliphatic amines in *e.g.* wobble uridines
N-hydroxy-succinimides		Primary aliphatic amines, acp^3U[18]	
anhydride		Aliphatic amines, mnm^5s^2U[10]	s^4U
Carbodiimide		Amines, *e.g.* pseudouridine	inosine, guanosine and uridine[1] (avoidable), ms^2i^6A,[3] mnm^5s^2U[10]
acryolnitrile		Amines, *e.g.* pseudouridine	I,[24] m^5C, m^5U
methylvinylsulfone		Amines, *e.g.* pseudouridine	I, m^5C, m^5U[23]
N-methylisatoic anhydride (NMIA)		blocked by 2′O-methylation	

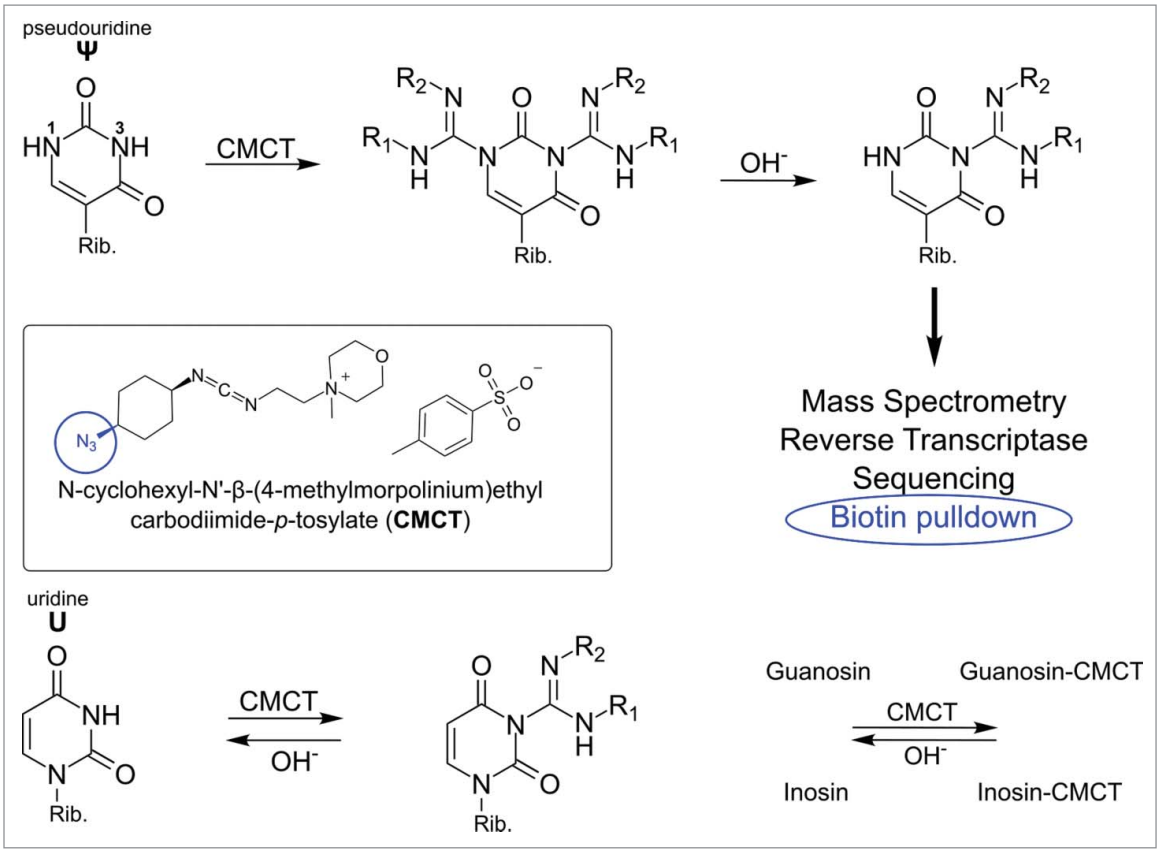


Figure 2. Reaction of carbodiimides with pseudouridine and uridine. Top: acylation of pseudouridine (Ψ) with the carbodiimide CMCT (full name in text). The carbodiimide group reacts with both N1 and N3, but after alkaline treatment the N1-CMCT is cleaved. The remaining CMC at N3 enables detection by mass spectrometry, reverse transcription, sequencing or even biotin pulldown in case of the CMC-azide (encircled, blue). Bottom panel: Like pseudouridine, uridine gets labeled at the N3 position, which is removed after alkaline treatment. The same was found to be true for guanosine and inosine. Insert: Structure of CMCT.

pseudouridine, it was shown that carbodiimides react with aliphatic amines e.g. in mnm^{5}s^{2}U[10] and ms^{2}i^{6}A.

Similar site reactivity (here I, m^{5}C and m^{5}U) was observed for Ψ labeling with the N-alkylating reagents acrylonitrile and methylvinylsulfone which are mostly applied in mass-spectrometric studies[22,23] since the reverse transcriptase is not stopped by these labels. For mass spectrometry these reagents are greatly beneficial since Ψ as a uridine isomer has the same m/z as the canonical nucleoside. By labeling of Ψ it can be detected as the conjugate and the thereby increased m/z. The side reactivity of inosine with acrylonitrile has been exploited for inosine mapping in the human transcriptome.[24]

In 1974, the fluorescent reagent 7-methoxy-4-bromomethylcoumarin was reported to react selectively with pseudouridine under specific reaction conditions.[25] The development of an azide functionalized coumarin derivative did not exert the same selectivity towards Ψ, but can be used to functionalize any native RNA or DNA instead.[26]

Blocking target sites

All techniques mentioned above exploit the higher reactivity of functional groups introduced by the natural nucleoside modification. However, it is also possible that a modification e.g. a methyl group, blocks a functional group of a canonical nucleoside. N-methylisatoic anhydride (NMIA) is a reagent used for RNA structural analysis which reacts with free unrestrained ribose 2′OH.[27] In theory, methylation of the 2′ OH will omit the labeling with NMIA. Using this reagent, mapping of 2′O-methylated nucleosides should be possible as the label leads to a stop of reverse transcriptase.

Conversion of modified nucleosides

The second class of chemical reagents used for the detection of modified nucleosides comprises all reactions of modified nucleosides leading to their conversion or subsequent strand-breaks. In comparison to the first class of reagents, no bulky labels are introduced which allow direct detection of the modification by e.g. primer extension, sequencing or fluorescence. Therefore the techniques described in the following section are usually combined with primer extension assays or sequencing approaches.

Bisulfite conversion

In both RNA and DNA, the C6 of cytosine reacts readily with bilsulfite which leads to its deamination into uracil (see Fig. 3a). In contrast, methylation of cytosine C5 increases the electron density in the pyrimidine ring, which makes it less prone to reaction with bisulfite.[28] The comparison of bisulfite and mock treated samples after high throughput sequencing is used to locate all 5-methylated cytosines since they still pair with guanosine, while the

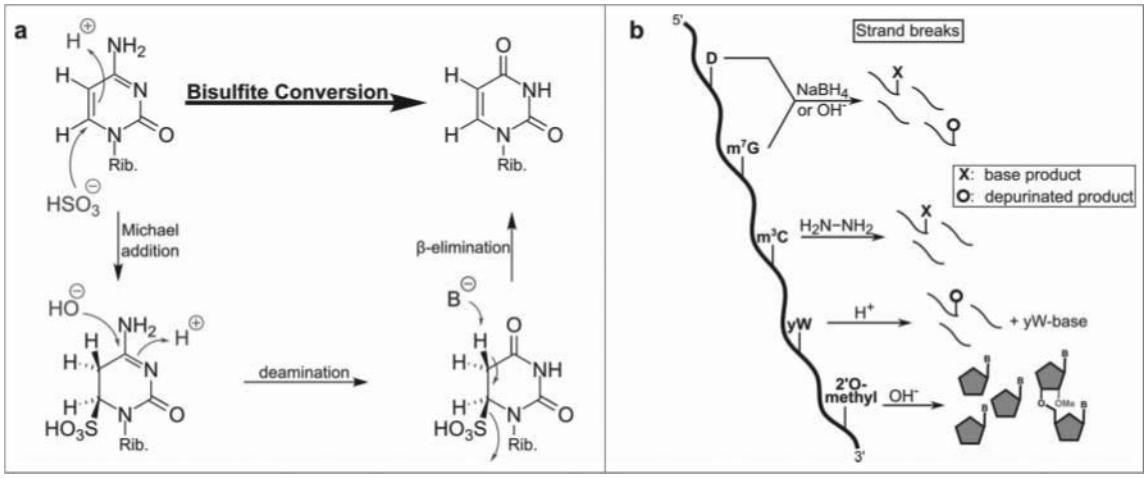


Figure 3. Conversion of different modified nucleobases and reagents. (a) Mechanism of cytosine to uracil conversion by bisulfite treatment. 5-methylated cytosine (m^5C) does not react with bisulfite, due to its lower electrophilicity, and thus is not converted to uracil. (b) Strand break analysis reveals the presence of modified nucleosides after treatment with various reagents. These reactions with modified bases lead to *e.g.* depurination which results in strand breaks after anilin treatment. In contrast, 2′O-methylated ribose is protected from alkaline induced cleavage which allows detection of the methylated site.

converted cytosines pair with adenosine. The technique of bisulfite sequencing was first developed for DNA and adjusted for RNA just a few years ago.[7] Since then it has been used for mapping of m^5C in all domains of life and various RNA species.[29-32] A more detailed description of the sequencing method is reviewed by the Motorin lab in the same issue.

Variations of bisulfite reaction

With the discovery of the oxidized derivatives of m^5C in DNA and RNA, namely 5-hydroxymethylcytosine (hm^5C), 5-formylcytosine (f^5C) and 5-carboxycytosine (ca^5C), the reactivity of bisulfite has been re-analyzed. It was found that both f^5C and ca^5C undergo the bisulfite induced conversion into uracil, while hm^5C is sulfonated but not deaminated. This finding showed that regular bisulfite sequencing is not sufficient to distinguish m^5C from hm^5C or even detect f^5C and ca^5C. Therefore chemical and enzymatic pre-treatments have been developed. After catalytic oxidation to f^5C,[33] hm^5C converts into uracil after bisulfite treatment while m^5C stays unaffected. By comparison of non-oxidized and oxidized samples, the localization of hm^5C ca be assigned. Although this technique has been developed for DNA, it is likely that it can be applied to hm^5C detection in RNA.

Similarly f^5C can be reduced by $NaBH_4$ into hm^5C in DNA[34] or RNA[35,36] before bisulfite treatment. The comparison of reduced and untreated sample can be used to locate f^5C in the nucleic acid of interest (see Table 2).

Table 2. Overview of conversion products after bisulfite reaction of cytidine and its modified derivatives. Without pretreatment (2nd column from left) C, f^5C and ca^5C convert into uracil after bisulfite treatment. Catalytic oxidation of hm^5C into f^5C (3rd column from the left) leads to its conversion into uracil after bisulfite treatment while all cytidines are protected from conversion after enzymatic methylation (first column from the right). Thus f^5C and ca^5C become detectable.

		Pretreatment		
	bisulfite only	catalytic oxidation	$NaBH_4$ reduction	enzymatic methylation
Cytidine **C**	U	U	U	–
5-methylcytosine **m^5C**	–	–	–	–
5-hydroxymethylcytosine **hm^5C**	–	U	–	–
5-formylcytosine **f^5C**	U	U	–	U
5-carboxycytosine **ca^5C**	U	U	U	U

Enzymatic demethylation

An enzymatic approach is used for detection of f^5C and ca^5C in DNA by bisulfite sequencing. Here, the sample is methylated by a CpG methyltransferase M.Sssl in vitro. After this step all cytidines are methylated and no longer convert into uridine, while f^5C and ca^5C still undergo conversion after bisulfite treatment.[37]

Using the bacterial enzyme AlkB, tRNA modifications like 1-methyladenosine, 3-methylcytidine or 1-methylguanosine are demethylated and become detectable by comparative sequencing.[38,39]

Chemical conversion

For 1-methyladenosine (m^1A), it has long been known, that dimroth rearrangement in alkaline conditions leads to formation of 6-methyladenosine (m^6A).[40] This chemistry was recently rediscovered to identify the number of false-positive m^1A signals during m^1A-seq of eukaryotic mRNA.[41]

RNA cleavage/depurination

While bisulfite is the most prominent nucleoside detection reagent, other chemicals were established to detect modifications in RNA. After disruption of the nucleobase's aromaticity by chemical reagents or after depurination, strand break analysis after aniline induced strand cleavage is a useful tool for the detection of modified nucleosides. Some modifications decrease the electron density in the nucleobase leaving the base more electrophilic and therefore prone to e.g. nucleophilic reactions or reduction (see Fig. 3b). 7-methylguanosine (m^7G).[42] and dihydrouridine (D)[43] can be detected by reduction with $NaBH_4$

or under mild alkaline conditions. In case of m^7G these treatments lead to depurination, which makes the RNA prone to aniline induced strand breaks. The reaction of dihydrouridine leads to a ring opening of the nucleobase and subsequent strand breaks. Strand breaks are detectable by primer extension assays, where a radiolabeled primer is extended by a polymerase until it reaches the strand break and stops.[44] The length of the extended primer is compared on a gel to an alkaline RNA digest which assigns the location of the modification in the original sample.

Methylation of cytidine at position 3 decreases the electron density in the pyrimidine ring. Therefore, m^3C reacts more readily with hydrazine than e.g. cytidine which leads to aniline induced strand breaks.[45]

As mentioned above, alkaline treatment of RNA leads to strand breaks. Ribose 2′OH methylation is a common modification of RNA which prevents alkaline RNA cleavage which allows detection of 2′OH methylated nucleotides by primer extension. Only recently, the alkaline stability of 2′OH methylation was exploited by combining the treatment with high throughput sequencing.[46,47] The described method detects all ribosomal RNA methylations with high accuracy. Surprisingly, the alkaline treatment provided information on other modifications like pseudouridine or base methylated nucleosides as mentioned here.[48]

The hypermodified nucleoside Wybutosin (yW) is prone to depurination after mild acidic treatment[49] which makes it easily detectable by aniline induced strand-break analysis.

Detection and enrichment by physicochemical properties

Most modified nucleosides are low abundant compared with the canonical nucleosides, which makes it difficult to study their distribution and function among RNA species. To study these modifications it can be quite useful to enrich the modified RNA species before analysis. As described above, pseudouridinylated RNA can be enriched after covalent labeling with an azide functionalized CMCT derivative.[2] While this enrichment is based on covalent reaction with the modification, it is also possible by non-covalent interactions between the modification and a reagent. Affinity purification of e.g. thiolated RNA or cis-diols in RNA is commonly used and simply exploits the features introduced by a modification.

Mercury forms a coordinate covalent bond with sulfur as a ligand, retaining thiolated nucleic acids and reducing the migration of thus modified RNAs. Since 1977 organomercury functionalized agarose and 1978 mercury containing cellulose[50] has been used for the purification of synthetically thiolated RNA[51] and later natural thiol and selenium containing RNAs.[52,53] Here the strong interaction of sulfur and selenium with mercury is used to retain the modified RNA on the agarose beads. After stringent washing and removal of non-modified RNA, the RNA is eluted by addition of organic sulfur compounds like dithiothreitol.

Later Igloi and co-workers developed an organomercury variant that could be co-polymerized into polyacrylamide gels, thus allowing separation of thiolated and non-thiolated RNA.[54] Furthermore, the authors showed that the degree of retardation depends on the type of RNA modification with 4-thiouridine being more retarded than 2-thiouridine or 5-methylaminomethyl-2-thiouridine (mnm^5s^2U). For purification of all thiol containing RNAs regardless of their affinity, a method involving 3-layered polyacrylamide gels in which only the middle layer contains a high amount of the organomercurial compound was developed (see Fig. 4).[55] Organomercury gels are now commonly used to study the position 34 thiolated uridines in tRNAs.[56,57]

A non-mercury containing approach was presented for the enrichment of 4-thiouridine modified RNAs using activated disulfide reagents, which allow reductive release after enrichment.[58] Here, methylthiosulfonate-activated biotin (MTS-biotin) was used to efficiently react with s^4U (after biosynthetic introduction into miRNA in HEK cells) and enrich the modified RNA.

The ribose of free nucleosides has vicinal OH groups on the 2′ and 3′ position which form a complex with boronic acids. RNAs with a non-phosphorylated 3′ end can be retained by boronic

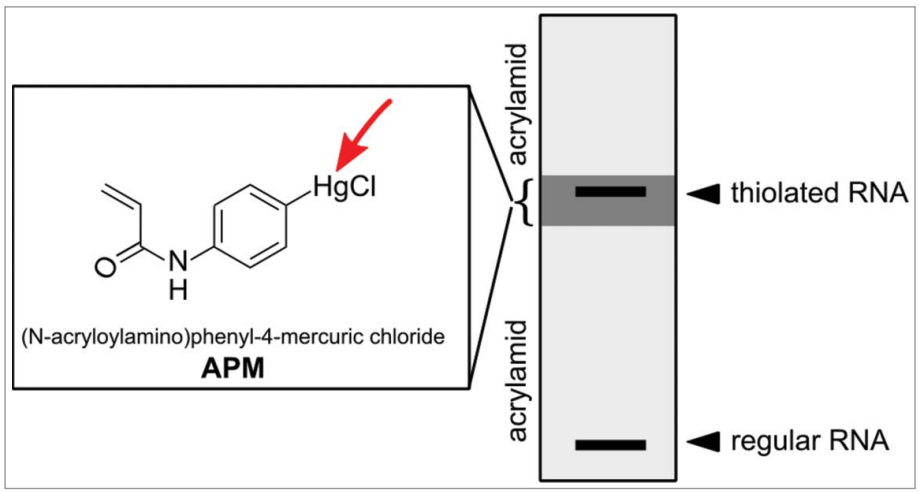


Figure 4. Enrichment of thiolated RNA on a 3 layered acrylamide gel. The upper and lower gel layer allow normal migration of RNA, while the 2nd layer contains APM (structure shown in the box) which retains all thiolated RNA.

acid. tRNAs of Bacteria and Eukarya contain queosine (Q), a hypermodified 7-deazaguanosine nucleoside with a cis diol on an additional cyclopenten. Queosine containing RNA is more strongly retained on boronate polyacrylamide gels compared with unmodified RNA,[59] which was recently used to study the degree of Q scavenging and incorporation into RNA as an effect of nutrient availability.[60] This study revealed a strikingly direct mechanism by which recoding of entire genomes results from changes in utilization of the nutrient queosine. The second exception is capped RNA, where the 5′ cap-structure is connected with its 5′OH to the 5′ end of the RNA chain, leaving the 3′ and 2′-OH free. In 2015, the bacterial NAD cap was discovered, which was the first report of bacterial epitranscriptomics.[61] The connection of NAD to the RNA via a pyrophosphate bridge leaves a free 3′OH on the cap structure next to the 2′OH which interacts with boronate. Only recently, this technique (APB copolymerization with acrylamide) was utilized to purify bacterial cofactor-capped RNAs, e.g. NAD-RNA and study the demodification kinetics by NudC and which could potentially be used to study bacterial cofactor modification kinetics in general.[62]

In this review we have shown small chemical reagents for RNA modification labeling, chemicals for conversion and enrichment of modified RNA. Modern techniques like RNA sequencing and mass spectrometry utilize the presented strategies to deepen our understanding of nucleic acid modifications.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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