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.

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 carbodiimides1 (Table 1) or new carbodiimide derivatives2 to enable the modification’s detection by either mass spectrometry3 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 (s4U), 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 s2U and s4U (2-thio- and 4-thiouridine, respectively). In addition, a bromomethyl-coumarin,11 was reported to react with s4U, however the selectivity towards other thiolated nucleosides like s3U or s2C (2-thiocytidine) was not studied yet.

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5-methyl-ribopyrimidine

Deoxythymidine and 5-methyldeoxycytidine (m5dC or just mC) in DNA can be specifically labeled with OsO4 or osmate complexes, which react with the diastereotopic 5–6 double bond.12 Only recently, this approach was presented in context of RNA where the osmium tetroxide-bipyridine complex reacted with both m5C and m5U on the nucleoside level. Interestingly, a change in diastereoselectivity leads to an almost complete loss of selectivity towards 5-methylcytosine (m5C) in a 5-mer ribonucleotide context, while 5-methyluridine (m5U) 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 (acp3U), N6-threonylcarbamoyladenosine (t6A) or N6-methyl-N6-threonylcarbamoyladenosine (m6t6A). It was shown that these nucleosides react selectively with primary amines in e.g. aniline or ethylenediamine in soluble carbodiimide.14 Many more carboxyl functionalized nucleosides (e.g. N6-glycinylcarbamoyladenosine (g6A), uridine 5-oxyacetic acid (cmo5U), 5-carboxymethylaminomethyluridine (cmnm5U), 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 carboxy groups, aliphatic amines are only found in some modified nucleosides like acp3U, 5-methylaminomethyl-2-thiouridine (mnm5s2U) and queuosine (Q). Fluorescein-isothiocyanate (FITC) was reported to react with both Q15 and acp3U16 while the latter also reacts with N-hydroxysuccinimides.10,17,18 The aliphatic amino group of an uncommon bacterial wobble modification mnm5s2U was found to react with a spin-labeled anhydride19 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′-(4-methylmorpholinium)ethylcarbodiimide p-tosylate) which leads to products of guanosine, uridine, inosine (I), 2-methylthio-6-isopentenyladenosine (ms2i6A) and pseudouridine (Ψ). In a second step, the acyl-moieties can be removed from guanosine, uridine and inosine residues by alkaline treatment, leaving only N3-acylated pseudouridines and an unknown derivative of 2-methylthio-N6-isopentenyladenosine (ms2i6A).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 yeast6 human cells4 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

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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.

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**Figure 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-methylthio-6-isopentenyladenosine (ms2i6A) and pseudouridine (Ψ). In a second step, the acyl-moieties can be removed from guanosine, uridine and inosine residues by alkaline treatment, leaving only N3-acylated pseudouridines and an unknown derivative of 2-methylthio-N6-isopentenyladenosine (ms2i6A). The acylation product is relatively bulky, which allows detection not only by mass spectrometry but additionally by reverse transcription (RT). This reagent was recently used for sequencing which allowed a transcriptome wide mapping of Ψ in yeast, human cells and dyskeratosis congenital patient samples. 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). 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. s²U,¹⁰                      | s⁷C                                       |
|                            |                       | s⁴U                                                     |                                            |
| Bromomethyl-coumarin        |                       | thiolated nucleosides,¹¹ pseudouridine²⁵               | uridine, thymidine²⁶                      |
|                            |                       | pseudouridine²⁵                                         |                                            |
| Osmate complex              |                       | S-methyl-riboymidines, e.g. m⁵U¹¹ and dm⁵C¹²            | m⁶C, thymidine¹²                          |
| Activated amines, e.g. aniline and ethylenediamine |                       | Carboxyl functions, e.g. acp⁵U, t⁷A¹⁴ | Other modified nucleosides with carboxyl groups like g⁷A, cmm³⁵U, etc. |
| Isothiocyanate              |                       | Aliphatic amines, e.g. Q¹⁵ and acp⁵U¹⁶                  | Other aliphatic amines in e.g. wobble uridines |
| N-hydroxy-succinimides      |                       | Primary aliphatic amines, acp⁵U¹⁶                      |                                            |
| anhydride                   |                       | Aliphatic amines, mmm⁵-s²U¹⁰                          | s⁴U                                      |
|                            |                       | s²U                                                     |                                            |
| Carbodiimide                |                       | Amines, e.g. pseudouridine                              | inosine, guanosine and uridine¹ (avoidable), m₅C, m₅U¹⁰ |
|                            |                       | Amines, e.g. pseudouridine                              | i,²⁴ m⁵C, m⁵U                            |
| acrylonitrile               |                       | Amines, e.g. pseudouridine                              | i,²⁴ m⁵C, m⁵U                            |
| methylvinylsulfone          |                       | Amines, e.g. pseudouridine                              | i, m⁵C, m³⁵U                            |
| N-methylisatoic anhydride (NMIA) |                     | blocked by 2’O-methylation                              |                                            |
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 C-labeling with the N-alkylating reagents acrylonitrile and methylvinylsulfone which are mostly applied in mass-spectrometric studies since the reverse transcriptase is not stopped by these labels. For mass spectrometry these reagents are greatly beneficial since C as a uridine isomer has the same m/z as the canonical nucleoside. By labeling of C 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.

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

**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. 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 bisulfite 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. 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
converted cytosines pair with adenosine. The technique of bisulfite sequencing was first developed for DNA and adjusted for RNA just a few years ago. Since then it has been used for mapping of m5C in all domains of life and various RNA species. 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 m5C in DNA and RNA, namely 5-hydroxymethylcytosine (hm5C), 5-formylcytosine (f5C) and 5-carboxycytosine (ca5C), the reactivity of bisulfite has been re-analyzed. It was found that both f5C and ca5C undergo the bisulfite induced conversion into uracil, while hm5C is sulfonated but not deaminated. This finding showed that regular bisulfite sequencing is not sufficient to distinguish m5C from hm5C or even detect f5C and ca5C. Therefore chemical and enzymatic pre-treatments have been developed. After catalytic oxidation to f5C, hm5C converts into uracil after bisulfite treatment while m5C stays unaffected. By comparison of non-oxidized and oxidized samples, the localization of hm5C can be assigned. Although this technique has been developed for DNA, it is likely that it can be applied to hm5C detection in RNA.

Similarly f5C can be reduced by NaBH4 into hm5C in DNA or RNA before bisulfite treatment. The comparison of reduced and untreated sample can be used to locate f5C in the nucleic acid of interest (see Table 2).

Enzymatic demethylation

An enzymatic approach is used for detection of f5C and ca5C 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 f5C and ca5C still undergo conversion after bisulfite treatment. Using the bacterial enzyme AlkB, RNA modifications like 1-methyladenosine, 3-methylcytidine or 1-methylguanosine are demethylated and become detectable by comparative sequencing.

Chemical conversion

For 1-methyladenosine (m1A), it has long been known, that dimroth rearrangement in alkaline conditions leads to formation of 6-methyladenosine (m6A). This chemistry was recently rediscovered to identify the number of false-positive m1A signals during m1A-seq of eukaryotic mRNA.

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 (m7G) and dihydrouridine (D) can be detected by reduction with NaBH4.

Table 2. Overview of conversion products after bisulfite reaction of cytidine and its modified derivatives. Without pretreatment (2nd column from left) C, f5C and ca5C convert into uracil after bisulfite treatment. Catalytic oxidation of hm5C into f5C (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 f5C and ca5C become detectable.
or under mild alkaline conditions. In case of m7G 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. 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, m3C reacts more readily with hydrazine than e.g. cytidine which leads to aniline induced strand breaks.

As mentioned above, alkaline treatment of RNA leads to strand breaks. Ribose 2'-0H methylation is a common modification of RNA which prevents alkaline RNA cleavage which allows detection of 2'-0H methylated nucleotides by primer extension. Only recently, the alkaline stability of 2'-0H methylation was exploited by combining the treatment with high throughput sequencing. 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-methylamino-methyl-2-thiouridine (mnm5s2U). 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).

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. Here, methylthiosulfonate-activated biotin (MTS-biotin) was used to efficiently react with s4U (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...
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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