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Mechanistic aspects of nucleic-acid oxidation studied with electrochemistry-mass spectrometry

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ABSTRACT

Oxidation reactions play a major role in the modification of nucleobases in DNA and RNA. Enzymatic oxidation reactions are involved in the control of epigenetic signaling as part of DNA-demethylation pathways. Oxidative stress gives rise to non-enzymatic oxidation. Many different oxidative DNA modifications have been identified. The cellular responses to such oxidative damage involve several processes, such as DNA repair, cell-cycle arrest and apoptosis. Persistent DNA damage may result in genomic instability, which is considered to play a role in the development of cardiovascular and neurological diseases, aging and cancer. Due to the involvement of nucleic-acid oxidation in many biological processes, understanding the underlying mechanisms is of the utmost importance. Herein, we demonstrate the vast potential of electrochemistry coupled to liquid chromatography-mass spectrometry as a tool for studying the oxidative stability of nucleic-acid species and identifying important oxidation products

Contents

1. Introduction

In the early days of nucleic-acids research, naturally occurring nucleic-acid polymers (DNA and RNA) were thought to contain only four conical nucleosides: adenosine (A), cytidine (C), guanosine (G), and uridine (U) or thymidine (dT). However, over the past 100 years, a large number of nucleoside derivatives were identified in DNA and RNA of living organisms, viruses, mitochondria and chloroplasts. The variety of modification reactions is enormous; often, oxidation reactions are involved as part of enzymatic or non-enzymatic processes.

The most important enzymatic modification in genomic DNA is methylation of the C5 atom of cytosine [1,2]. Cytosine methylation

Abbreviations: m^5dC, 5-methyldeoxycytidine; G + O, 8-hydroxyguanosine; A, Adenosine; dG, Deoxyguanosine; DNA, Deoxyribonucleic acid; C, Cytidine; EC, Electrochemistry; ESI, Electrospray ionization; GC, Gas chromatography; g, Guanine; G, Guanosine; $E_{1/2}$, Half-wave potential; ·OH, Hydroxyl radical; I.D., Inner diameter; LC, Liquid chromatography; MS, Mass spectrometry; O.D., Outer diameter; ROS, Reactive oxygen species; RNA, Ribonucleic acid; U, Uridine; dT, Thymidine.

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is accomplished by S-AdoMet-dependent methyltransferases. The extent of post-replicative modifications of deoxycytidine (dC) into 5-methyldeoxycytidine (m^5dC) for bacterial, archaeal and eukaryal genomic DNA is generally 1–8% of the total dC, except for mammalian and plant DNAs where m^5dC can reach 30% of total dC.

DNA methylation has functional consequences for the cell, most notably in the control of gene expression. There is a clear correlation between gene silencing and methylation of dCpdG islands [3]. One hallmark of epigenetic signaling is that it is heritable, but flexible; this implies that pathways for the removal of epigenetic marks are required.

Recently, two oxidation products of m^5dC (5-(hydroxymethyl) deoxycytidine and 5-formyldeoxycytidine) were identified [4–8] and are considered to constitute part of the pathways that lead to active DNA demethylation [9]. Given that these epigenetic changes are of vital importance to development biology and numerous areas of human diseases, including acquired autoimmune, cardiovascular, and neurological diseases, aging and cancer, it is of great importance to elucidate the underlying mechanisms of formation and removal.

The most widespread enzymatic RNA modifications are base and ribose methylations and isomerization of U into pseudouridine [1]. The extent of total modifications is especially high in tRNA molecules, being 2–15% in bacteria and reaching up to 25% in mammals and plants. The modifications have various functions, including roles in tRNA aminoacylation, modulation of translational efficiency, gene regulation and central or intermediary metabolism.

Non-enzymatic reactions are another source of nucleic-acid modifications [10,11]. The vast majority of these reactions falls into just two general categories [12]:

1 alkylation of a DNA/RNA nucleophile by an electrophile; or,
2 the reaction of a pi bond or C-H bond in DNA/RNA with a radical intermediate.

Species interacting with nucleic acids are of endogenous or exogenous origin. They can be intrinsically reactive; usually, however, they are activated by oxidation reactions [13]. Potential endogenous sources of oxidative stress include oxidative phosphorylation, cytochrome P450 metabolism, peroxisomes, and inflammatory-cell activation [14]. Diet, lifestyle and other environmental conditions and factors (e.g. ultraviolet light, ionizing radiation, toxins, pollutants, and antioxidants) can alter the amount of DNA damage that an organism undergoes.

An important reactive oxygen species (ROS) is the hydroxyl radical (·OH), which reacts with nucleobases by addition to double bonds of nucleobases and by abstraction of an H atom from the methyl group of dT [15]. Many oxidative DNA modifications have been identified in the mammalian genome and very important alterations seem to be 8-hydroxyguanine, 8-hydroxyadenine, 5,6-dihydroxy-5,6-dihydrocytosine, 5,6-dihydroxy-5,6-dihydrothymine, 5-hydroxymethyluracil, and 5-formyluracil [16–18]. The majority of these lesions are premutagenic and/or lethal. The cellular responses to oxidative damage involve several processes, such as DNA repair, cell-cycle arrest and apoptosis [19]. Persistent DNA damage can result in arrest or induction of transcription, replication errors, and genomic instability. These processes are considered to play a role in the development of cardiovascular and neurological diseases, aging and cancer [20].

Oxidative damage is not exclusively observed in DNA. RNA molecules also are oxidatively modified, and there is increasing evidence that this oxidation, and the consecutive loss of integrity of RNA, is a mechanism contributing to disease development [21].

Oxidation reactions are involved in the production of important DNA/RNA modifications. Understanding the underlying mechanisms and their biological consequences is of the utmost importance for disease prevention and treatment. Accordingly, there is a lot of interest in the chemical characterization of lesions. A number of assays are available for qualitative and quantitative analysis of modified nucleic-acid species formed. Due to the sensitivity, the accuracy and the specificity of liquid chromatography (LC) coupled to mass spectrometry (MS), there is a clear trend towards its use for that purpose [22–24]. A challenge in detection and structure identification of modified nucleic acids *in vivo* is that these species are formed in complex mixtures and at exceedingly low levels [25].

DNA-adduct levels as low as one damaged base in 10^6–10^{12} are common. Accordingly, there is a strong need for *in-vitro* assays that allow controlled and reproducible production of lesions at concentrations sufficiently high for LC/MS characterization. Controlled one-electron oxidation of nucleic-acid species is accomplished by treatment with sulfate, carbonate or Br_2 radical anions, and by single-photon or two-photon ionization [17,26]. Alternatively, electrochemistry (EC) can be applied.

The electroactivity of nucleic acids was discovered in the 1960s [27]. Oxidation mainly relies on the intrinsic electrochemical activity of the nucleobases [28,29], even though the oxidation of the sugar has also been demonstrated with copper electrodes [30]. The first attempts were made to identify important oxidation products of purines in the 1970s [31], when structure elucidation was accomplished off-line from EC with, e.g., UV/VIS spectroscopy and gas chromatography (GC)-MS after derivatization into volatile products [32–35].

On-line coupling of EC to MS for studying purine oxidation was first realized by Brajter-Toth and co-workers [36,37]. More recently, in the EC/MS set-up used to study nucleic-acid oxidation, thermospray was substituted by electrospray ionization (ESI) [38,39]. Furthermore, LC and capillary electrophoresis (CE) were integrated to enable the separation of the oxidation products formed [40–42].

Principally, EC/LC/MS is a versatile tool for studying oxidation reactions involving different kinds of nucleic-acid species (Fig. 1). However, in the past, it has mainly been used to study the oxidation of guanine (g) at the nucleobase level and inside the nucleoside and nucleotides. Herein, we summarize current knowledge on the electrochemical oxidation of these important nucleic-acid constituents. Furthermore, we provide new insights into the reaction mechanisms involved and demonstrate that g oxidation can also be studied in small oligomers. Finally, we show the applicability of EC to study the oxidation of pyrimidines, including cytidine (C), 5-methylcytidine (m5C), uridine (U), and thymidine (dT), and demonstrate the tremendous impact of C5 methylation on oxidation chemistry.

EC/LC/MS is a young, exciting tool for nucleic-acids research. As the body of knowledge available in publications was found to be insufficient to describe the capabilities of this technique fully, we decided to extend the reviewing with unpublished data from our laboratory. The Electronic Supplementary Material contains details on the experimental conditions applied.

2. Experimental set-up typically applied for EC/LC/MS of nucleic acids

EC/LC/MS integrates electrochemical oxidation with chromatographic separation and MS detection. There are different ways of linking EC to LC/MS, and these were reviewed recently [43]. The simplest set-up is the off-line approach, where LC/MS is used to analyze samples manually transferred from an electrochemical-reactor cell to the injection system. Alternatively, the electrochemical cell can be integrated into the LC/MS system. Set-ups using the electrochemical cell as a post-column or pre-column reactor were developed.