Mass Spectrometry

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ESI Hydrogen/Deuterium Exchange Can Count Chemical Forms of Heteroatom-Bound Hydrogen**

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Heteroatom-bound (labile) hydrogen atoms in organic molecules undergo hydrogen/deuterium exchange (HDX) upon contact with D_2O, ND_3, D_2S, CH_3OD, CH_3COOD, or related compounds. Analytical applications of HDX can be classified into static and dynamic concepts. Typical static HDX experiments aim at complete exchange of all the labile hydrogen atoms in solution, for example, to improve peptide sequencing[1] or to support drug metabolite identification.[2] Dynamic experiments, for example, exploit the kinetics of in-solution HDX, which is dependent on chemical form, temperature, and pH value. In proteins, in-solution exchange rates of labile hydrogen atoms have been determined by NMR spectroscopy.[3] It was found that backbone amide hydrogen atoms have the slowest in-solution exchange rates. In addition, these rates are further reduced by participation in hydrogen bonds, which may increase their average life times to hours or days.[4] The individual exchange rates of chemically distinct hydrogen atoms are the basis for the use of in-solution HDX for the analysis of protein structure, dynamics, and interactions.[5] Gas-phase HDX depends on the gas-phase basicity difference between the reaction partners and on their structure. It is often performed in ion trap, ion mobility or FT-ICR mass analyzers and is typically characterized by slower exchange than in-solution HDX.[6] When the spatial arrangement of the reaction partners in gas-phase HDX favors a relay mechanism, the exchange reaction can be strongly accelerated.[7] This structural influence can lead to markedly different gas-phase exchange rates of chemically identical labile hydrogen atoms.[8] A typical application of gas-phase HDX is the study of the gas-phase structures of protein ions produced by electrospray ionization mass spectrometry (ESI-MS).[9] ESI-HDX is a separate, unique topic, since it exclusively proceeds in the time frame specified by the ESI process. Collisions between spray droplets and/or analyte ions with ambient water normally remain unnoticed, since only hydrogen–hydrogen exchange occurs. However, when an analyte is sprayed from normal water into a D_2O-containing atmosphere, deuterium in-exchange can be performed.[10] In the reverse situation, deuterium out-exchange (DHX) is observed.[11] Partial DHX is a general burden in mass spectrometric analysis of analytes labeled with deuterium at labile hydrogen positions.[12]

Herein, we address in-source ESI-HDX and -DHX in more detail, since we believe that an understanding of this technique will expand the analytical capabilities of ESI-MS. As model compounds, we selected small molecules, amino acids and modified amino acids. These classes of compounds exhibit a broad structural and chemical variability of labile hydrogen atoms. As experimental techniques, we used nano-ESI in combination with an open ion source, and ESI with a closed ion source design. Introduction of gaseous H_2O/D_2O into the source and other experimental details are described in the Supporting Information.

The nanoESI-HDX behavior of the standard amino acids was studied. Efficient HDX was observed only at carboxylic OH, aliphatic OH, and aromatic OH groups. This means that neutral and basic amino acids, as well as Asn, Gln, and Cys exchanged only one, whereas Ser, Thr, Tyr, Asp, and Glu exchanged two hydrogen atoms. Histidine was the only exception, since it efficiently exchanged four hydrogen atoms in nanoESI-HDX (see Figure S17). The standard amino acids were also subjected to ESI-HDX and -DHX. The results are summarized in Figure 1.

As shown in Figure 1, HDX and DHX experiments delivered mirrored results. The average degrees of deuteration of deuterium in- and out-exchange of all the experiments are close to 50%, so that no indication of a kinetic deuterium

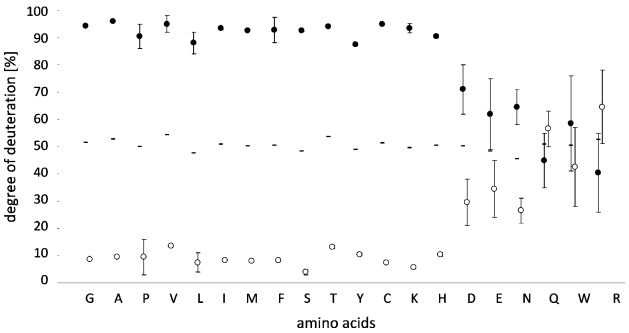

Figure 1. Efficiency of ESI-HDX and -DHX for the 20 standard amino acids. The degree of deuteration is normalized to the total number of exchangeable hydrogen atoms in each amino acid. (filled circles = ESI-HDX; open circles = ESI-DHX, error bars represent ±1 standard deviation for $n = 3$. Dashes represent the average between HDX and DHX experiments).

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isotope effect was obtained. In general, ESI-HDX was more effective than nanoESI-HDX, because with ESI-HDX SH, α-NH, and ε-NH hydrogen atoms were also exchanged. Fourteen out of 20 standard amino acids exhibited almost complete (over 90%) HDX and DHX efficiency. Six amino acids grouped on the right side of Figure 1 had both a reduced exchange efficiency and an enhanced variability of results. Figure 2 shows this situation with tryptophan as an example.

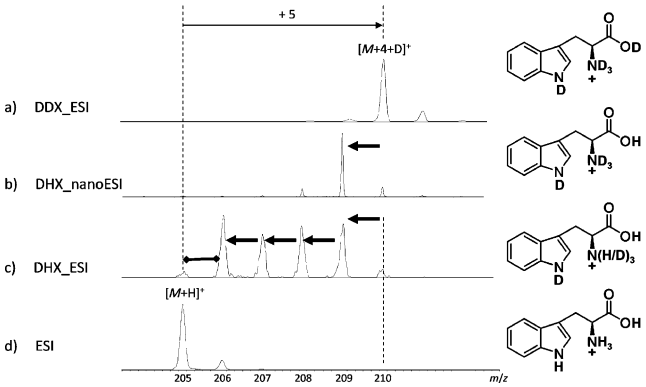

Figure 2. Differentiation of three classes of labile hydrogen atoms in tryptophan; a) complete HDX using ESI-MS with D_2O as solvent and D_2O-saturated nitrogen as sheath/sweep gas; b) differential DHX of carboxy OH by nanoESI with D_2O as solvent and ambient air in the ion source; c) differential DHX of carboxy OH plus three α-amino hydrogen atoms using ESI with D_2O as solvent and H_2O-saturated nitrogen as sheath/sweep gas; d) normal positive ion ESI analysis.

The reduced exchange efficiency can be ascribed to the presence of labile hydrogen atoms resistant to ESI-HDX (amide, indole, and guanidino NH). This phenomenon becomes visible by a gap in the isotope pattern between the species with the lowest number of deuterium atoms and the natural monoisotopic peak (Figure 2c and Supporting Information). The increased exchange variability in Asp, Glu, Asn, Gln, and Trp is correlated with the α-amino hydrogen atoms which show an exchange pattern containing all the isotopologues of the $-NH_3^+$ group, so that the presence of three equivalently exchangeable hydrogen atoms is clearly visible (Figure 2c and Supporting Information).

Combined nanoESI- and ESI-HDX/DHX measurements as displayed in Figure 1 were performed for all the standard amino acids, a set of modified amino acids, and selected small molecules (see Supporting Information). Exchangeable hydrogen atoms can be classed as either, 1) efficiently exchanging, 2) moderately exchanging, or 3) not exchanging (Table 1).

To explain this ranking, we compared the site-specific in-solution exchange constants of the labile hydrogen atoms in proteins as determined by NMR spectroscopy.[3] This comparison revealed that the class 1 hydrogen atoms, which are selectively exchanged during nanoESI-HDX exhibit very short average life times in the submillisecond range. Class 2 hydrogen atoms which additionally exchange during ESI-HDX exhibit longer lifetimes of 1–10 milliseconds. Class 3 hydrogen atoms, which are resistant to both nanoESI- and ESI-HDX, have lifetimes in excess of 50 ms. The lifetimes correlate closely to the lifetimes of the corresponding electrospray droplets.

Table 1: Ranking of chemically different forms of labile hydrogen atoms according to their exchange efficiency by nanoESI and ESI.

Class 1 exchangeable by nanoESI-HDX	Class 2 additionally exchangeable by ESI-HDX	Class 3 resistant to both ESI-HDX and nanoESI-HDX
carboxylic OH	α-amino NH	amide NH
aliphatic OH	other amino NH	indole NH
aromatic OH	sulfhydryl SH	guanidino NH
phosphoryl OH		

NanoESI droplets (diameter under 200 nm) are estimated to have a submillisecond lifetime, whereas the much larger ESI droplets (diameter 1–10 μm) have lifetimes in the range of several milliseconds.[13] A common view on ESI droplet lifetimes and in-solution lifetimes of different classes of labile hydrogen atoms is given in Figure 3. The correlation displayed can explain the observed characteristics of ESI-HDX on a kinetic basis. In this explanation, forms of hydrogen with average lifetimes near the droplet lifetimes are of major importance. For instance, in nanoESI-HDX Tyr-OH is exchanged, whereas Cys-OH and α-NH_3^+ are stable. As evident from Figure 3, the mean lifetime of Tyr-OH hydrogen is still within the nanoESI droplet lifetime, whereas the hydrogen lifetimes of Cys-OH and α/ε-NH_3^+ exceed this time frame. The same trend can be observed for ESI-HDX: The mean hydrogen lifetimes of Cys-SH and α/ε-NH_3^+ are within the ESI droplet lifetime, whereas all other, exchange-resistant hydrogen atoms have longer average lifetimes. The partially incomplete HDX at the $-NH_3^+$ groups of the six amino acids at the right part of Figure 1 probably is caused by an increased average lifetime of the amino hydrogen atoms, which in turn is caused by their intramolecular interaction with their polar side chain. The proposed explanation implies that the observed exchange is mainly a kinetically controlled in-solution processes and not, or only to a minor extent, a gas-phase event. This interpretation implies that in the ESI process, besides solvent evaporation, recondensation of water

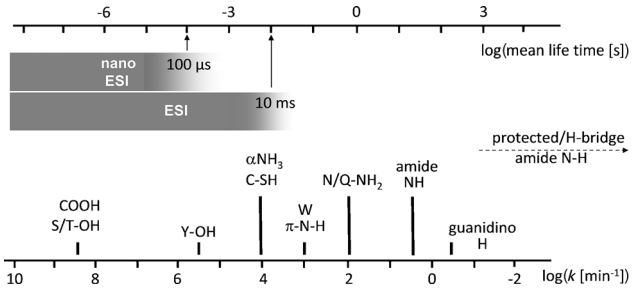

Figure 3. Display of in-solution exchange rates of different classes of exchangeable hydrogen atoms, their corresponding mean lifetimes, and the lifetimes of nanoESI and ESI droplets.

from the in-source atmosphere plays an important role. As a result, prior to ion emission, a droplet, or at least its surface, has to acquire the hydrogen isotopic composition of ambient atmospheric water. The excess charge at the droplet surface probably favors water recondensation, since charge centers tend to attract molecules for their solvation.[14] Water recondensation at the electrospray droplet has been used to explain the ion intensity increase, observed when a dry nitrogen ESI sheath gas was spiked with moderate amounts of water.[15] The data of nanoESI- and ESI-HDX experiments can be ordered in terms of the chemical nature of the labile hydrogen atoms. This supports the conclusions that in-solution HDX is the main active exchange process. If gas-phase HDX would be the prevailing process, a consistent classification by chemical parameters probably would not be possible because of the dominating influence of structural effects. For instance, gas-phase HDX for lysine and ornithine has a strikingly different outcome,[16] whereas these homologous compounds behave similarly during ESI-HDX (see Supporting Information).

The combined use of nanoESI-HDX and ESI-HDX expands the analytical capabilities of electrospray ionization MS towards differentiation of three classes of heteroatom-bound hydrogen atoms with typical in-solution lifetimes under 1 ms, 1–10 ms, and above 10 ms. The characteristics of nanoESI-HDX and ESI-HDX as an in-solution process allows the behavior of a labile hydrogen to be predicted from its chemical environment. This allows the distribution of the three kinetically distinct classes of labile hydrogen atoms in a molecule to be determined and offers the possibility to localize ionizing protons. The unexpected recognition of in-source HDX as a kinetically controlled in-solution process opens a way to study fast HDX processes. In addition, it makes the combined use of nanoESI-HDX and ESI-HDX an attractive novel tool for studies of the electrospray ionization process.

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