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On-line Electrochemical Tagging of Free Cysteines in Peptides during Nanospray Ionisation Mass Spectrometry: An Overview

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Abstract: A recently developed nanospray interface for mass spectrometry (MS) was used to electro-generate benzoquinone from hydroquinone. When spraying biomolecules containing free cysteine residues in the presence of hydroquinone, the thiol cysteine moiety reacts *via* a 1,4-Michael addition with benzoquinone electrochemically generated at the electrode. The on-line labelling of cysteine was optimised using several substituted hydroquinones, and the kinetics of the addition was investigated by electrochemical methods such as cyclic voltammetry (CV). The mass spectrometric analysis of peptides containing up to three cysteines residues in the presence of selected probes allows the direct quantification of free cysteine residues. The on-line tagging method provides both the mass of the untagged peptides and that of the tagged peptides.

Keywords: Cyclic Voltammetry · Cysteine · Labelling · Mass Spectrometry · Quinone

1. Introduction

Characterisation and identification of proteins is a key feature in biological sciences. Proteomics refers to the determination of gene and cellular function directly at the protein level [1]. High-throughput methods are needed in this field and the use of mass spectrometry techniques has become the essential tool for rapid and sensitive screening of proteins. Usually, complex biological mixtures are first separated by two-dimensional gel electrophoresis and after excision of the spots, the proteins of interest are digested by enzymatic treatment. The resulting peptides are then analysed by MS and different techniques such as peptide mass fingerprinting [2], sequence tags [3][4] or MS/MS can be used to identify the selected proteins. The identification is then realized by using DNA and protein databases search software [5].

In order to enhance the identification of the protein and solve any ambiguity in a peptide mapping strategy, it can be useful to have supplementary information about the protein sequence. Knowing, for example, the number of a specific amino acid in the analysed peptides, the identification is improved as it eliminates many eventual possibilities of the same mass [6]. Moreover, as cysteine is present in a huge part of the proteome, the quantification of this amino acid is of great interest. The typical experiment consists in labelling the cysteine residues present in proteins or in peptides by a chemical reagent such as iodoacetamide [7]. The mass shift between the tagged and the untagged sample by MS yields the cysteine content of the studied biomolecule. As they require the preparation of two samples, these techniques can be called sequential. Besides, the labelling step is time consuming since the tagging reaction is often performed for several hours.

The recently developed on-line electrochemical tagging of cysteine using an electrospray ionisation (ESI) MS technique brings a possible answer to these drawbacks. The labelling reaction in the nanospray interface developed in the laboratory [8] is quasi-instantaneous and directly applied to the sample to be analysed. The nanospray chip device (see Fig. 1) is used to electrogenerate species likely to react with cysteine moieties. As the nanospray interface behaves like an electrolytic cell [9][10], in a positive ionisation mode, compounds such as benzoquinone can be generated at the electrode from hydroquinone and react *via* a 1,4-Michael addition with free cysteines [11]. It was shown that the geometrical and operational parameters of the MS-chip interface can be used to control the tagging degree [12]. The electrochemical mechanism occurring in the nanospray chip was elucidated and the rate constant pertaining to the addition of L-cysteine (**3**) onto benzoquinone were determined [13].

In order to enhance the tagging efficiency, several substituted hydroquinones presenting different reactivity toward the addition of **3** were employed. The kinetic constant for the addition on the different probes were determined by electrochemical studies. The final on-line tagging efficiency was investigated by nanoESI-MS experiments. For future application in protein identifications, this new method was then used for the quantification of cysteine residues contained in peptides.

2. Results and Discussion

2.1. *On-line Tagging of L-Cysteine (3)*

The electrochemically induced tagging of L-cysteine (**3**) has been previously stud-

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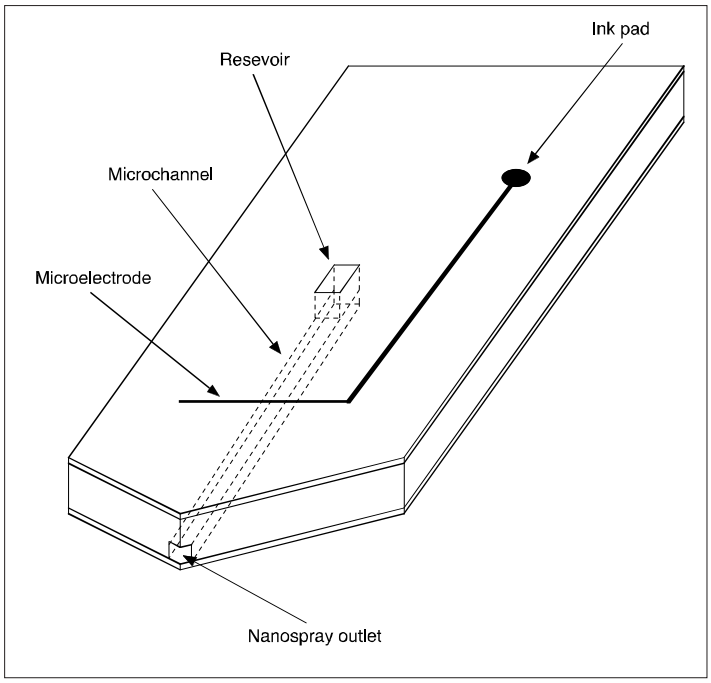
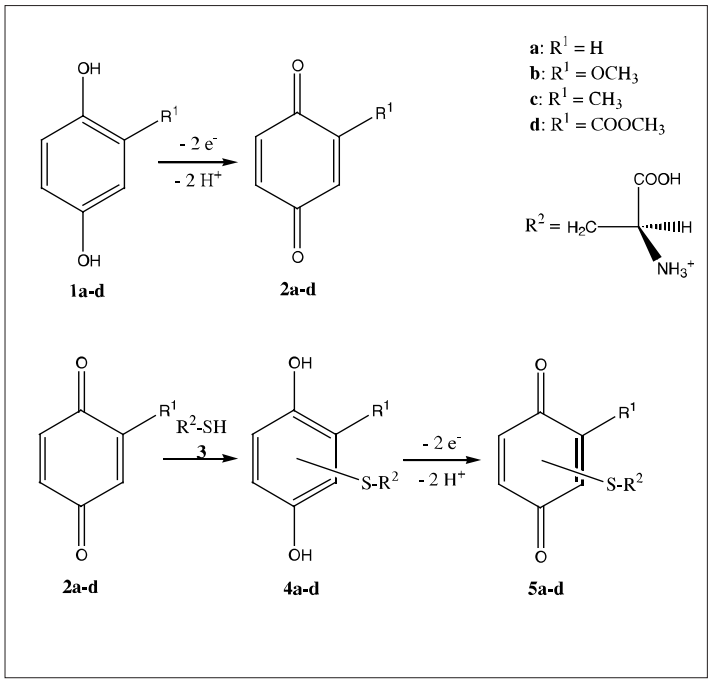


Fig. 1. Representation of the nanospray interface used for electrogeneration of tags



Scheme. Mechanism corresponding to the reaction between electrochemically generated benzoquinone and L-cysteine

ied using non-substituted hydroquinone [13] under different experimental conditions [14]. The overall mechanism can be described as in the Scheme for the experimental conditions used in the present work. The compounds under study are hydroquinone (**1a**), methoxyhydroquinone (**1b**), methylhydroquinone (**1c**), and carboxymethylhydroquinone (**1d**). It is expected that an electron pair attracting group will increase the electrophilicity of the electrogenerated substituted benzoquinone (compared to the non-substituted benzoquinone) whereas an electron pair donating group will decrease it, leading to a difference of reactivity for the Michael addition. The CVs displayed in Fig. 2 describe the electrochemical behaviour of the different hydroquinones **1a**-**d** in the presence (bold line) or in the absence (thin line) of **3**. When there is no **3**, the CVs show a first peak (intensity Ip^0), situated around 0.4 to 0.8 V/SCE, corresponding to the oxidation of hydroquinones into benzoquinones and a second peak (–0.2 to 0.2 V/SCE) corresponding to the reduction of benzoquinones into hydroquinones. When **3** is added to the electrochemical cell (bold line), the CVs shapes change, exhibiting the decrease or the total vanishing of the benzoquinone signal and the increase of the hydroquinone oxidation peak intensity (*Ip*). The disappearance of the benzoquinone signal is attributed to its reaction with **3** and the current increase (Ip/Ip^0) is explained by the oxidation of the addition products **4a**-**d**, which occurs at a similar potential to **1a**-**d**. The evolution of the Ip/Ip^0 ratio *versus* the CV scan rate can be use to determine the homogenous rate constant corresponding to the addition of **3** onto electrogenerated benzoquinone. Some digital simulations of the CVs at different scan rate were used to extract the addition kinetic constant for each hydroquinone [13][15]. The kinetic constants for **1a**, **1b**, **1c** and **1d** were found to be 210, 50, 50 and 5000 $M^{-1}.s^{-1}$ respectively.

In ESI, a high voltage is applied between the spray source and the MS. Recently a new polymeric nanospray interface incorporating a microelectrode was developed and micromachined by laser photoablation [8]. When a miniaturised nanospray interface (*i.e.* with a high electrode surface to channel volume ratio) is employed, the high positive voltage may be used to electrogenerate compounds such as benzoquinones. These oxidative products can react with nucleophiles and the formed adducts can subsequently be detected by MS. A similar procedure was used in the current investigation and it was found that all the substrates were able to electro-tag **3** as all adducts can be observed in the ESI-MS spectra (Fig. 3). However, when the relative abundances of **3** ([MH^+]: m/z = 122) and adducts are compared, it is evident that at least the apparent conversion yields are not similar. The average values of the apparent tagging yield (recorded for 70 min) indicates that the reactivity increases in the order: **1c** < **1a** < **1b** < **1d**. Based on the electrochemical studies the apparent tagging yields are surprising as the rate constant corresponding to the addition of **3** to the benzoquinones follows the electronic effect of the substituent (*i.e.* **1d** > **1a** > **1c** ≈ **1b**). However, an important factor to consider is the ionisation efficiency of the products **4a**-**d** in ESI-MS. The spectrum obtained by infusing an equimolar mixture of the chemically generated adducts **4a**-**d** shows that the ionisation efficiency increases as: **4a** ([MH^+]: m/z = 230)< **4c** ([MH^+]: m/z = 244)< **4b** ([MH^+]: m/z = 260)< **4d** ([MH^+]: m/z = 288). It may thus be concluded that without accurate knowledge of the actual ionisation efficiency, the real tagging yield cannot be determined on the basis of the mass spectra alone. However, based on both kinetics and MS, **1d** reveals itself as the best electrotagging probe.

The selectivity of the studied hydroquinones **1a**-**d** towards **3** in such on-line tagging experiments was studied using several peptides as substrates, which do not contain any cysteine residues but other nucleophilic amino acids. For instance, using bradykinin, Leu-enkephalin and Met-enkephalin-Arg-Phe, no tag reaction was observed. As well, myoglobin from horse heart, which contains all amino acids except cysteine, infused in the presence of the different hydroquinones did not shown any tag product.

2.2. *On-line Tagging of Peptides*

On the basis of the previous studies, carboxymethylhydroquinone (**1d**) was chosen to investigate the on-line electrochemical

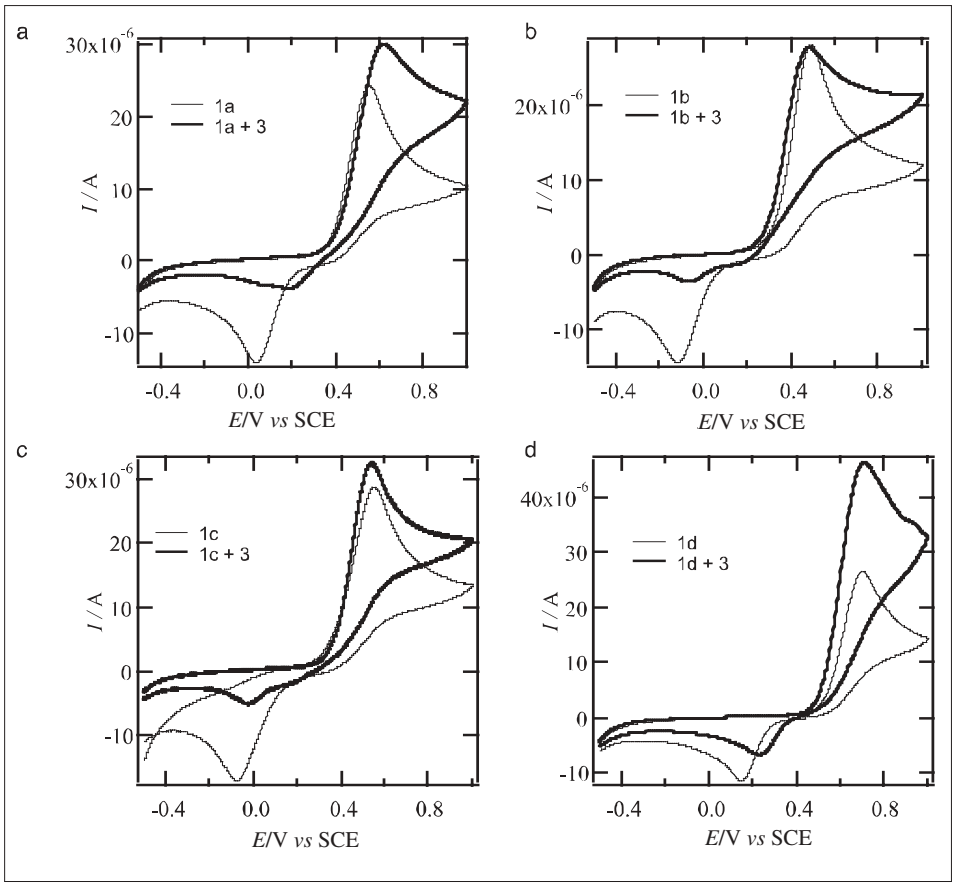


Fig. 2. Cyclic voltammograms recorded at 25 mV.s^{-1} of 2 mM of different hydroquinones 1a–d (a to d respectively) without **3** or in the presence of 2 mM of **3**

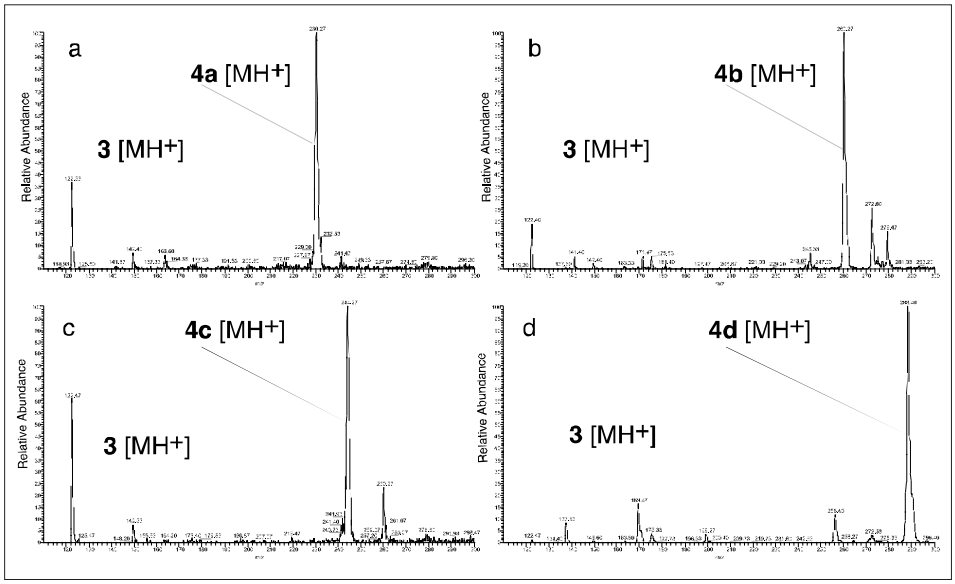


Fig. 3. Mass spectra obtained by infusing **3** in the presence of hydroquinone **1a–d** (a to d respectively)

MS tagging on peptides. The experiments were performed with the non-substituted hydroquinone (**1a**) too, in order to show the relevance of this choice.

Several synthetic peptides containing up to three cysteines were tested in on-line electrochemical tagging. **1d** allows all cysteines present in the peptide to be quantified whereas hydroquinone **1a** does not go further than two cysteines (moreover, the two-tagged peptide presents a very low intensity peak). As the tagging reaction is not quantitative, it is possible to extract from a single sample the peptide mass as well as its cysteine content (at least up to three moieties). Another experiment on a peptide having three cysteine residues, two of which are consecutives, confirms that it is possible to access its cysteine content (Fig. 4).

3. Conclusion

On-line electrochemical tagging of cysteine was optimised exploring several hydroquinone probes. The best tagging efficiency of L-cysteine (**3**) was obtained with carboxymethylhydroquinone (**1d**). The withdrawing group borne by this compound increases the electrophilicity toward nucleophilic addition of thiol moieties. Cyclic voltammetry and digital simulation allowed the determination of the addition kinetics for the addition and confirms the statement.

Peptides containing up to three cysteine residues were successfully tagged by the selected compound. These last results show that the method is compatible with the on-line labelling of peptides resulting from a protein digestion. The method can be employed in order to emphasize the certainty of protein identification [6].

4. Experimental

4.1. *Chemicals*

Myoglobin from horse heart (>90%) and 1,4-hydroquinone (>98%), lithium trifluoromethanesulfonate (purum) were purchased from Fluka, 2-methylhydroquinone (99%), 2-methoxyhydroquinone (99%) from Acros, 2-carboxymethylhydroquinone (methyl 2.5-dihydroxy benzoate) (99%) from Aldrich and bradykinin (99%), Met-enkephalin-Arg-Phe (98%) from Sigma. Leu-enkephalin (>99%) and Lys-Cys-Thr-Cys-Cys-Ala (70%) were bought from Bachem. Methanol (>99.8%, Riedel-de Haën), acetic acid (>99.5%, Fluka) were used without any further purification. Deionised water (18.5 MΩ) was prepared using a Milli-Q system from Millipore.

4.2. *Electrochemical Set-up*

Analytical experiments were performed in a three compartments cell fitted with a saturated calomel reference electrode (SCE), glassy carbon working electrode (diameter 3 mm) and a platinum wire counter electrode, using as electrochemical solvent a mixture of ($MeOH/H_2O/AcOH$ 50:49:1) with 0.1 M of lithium trifluoromethanesulfonate. The solvent was degassed under nitrogen. The CVs were recorded on a Metrohm Autolab PGSTAT 30.

4.3. *Microchip Device*

For the fabrication of the nanospray device, a polyethylene terephthalate (PET) substrate (100 μm thick Melinex® sheet from

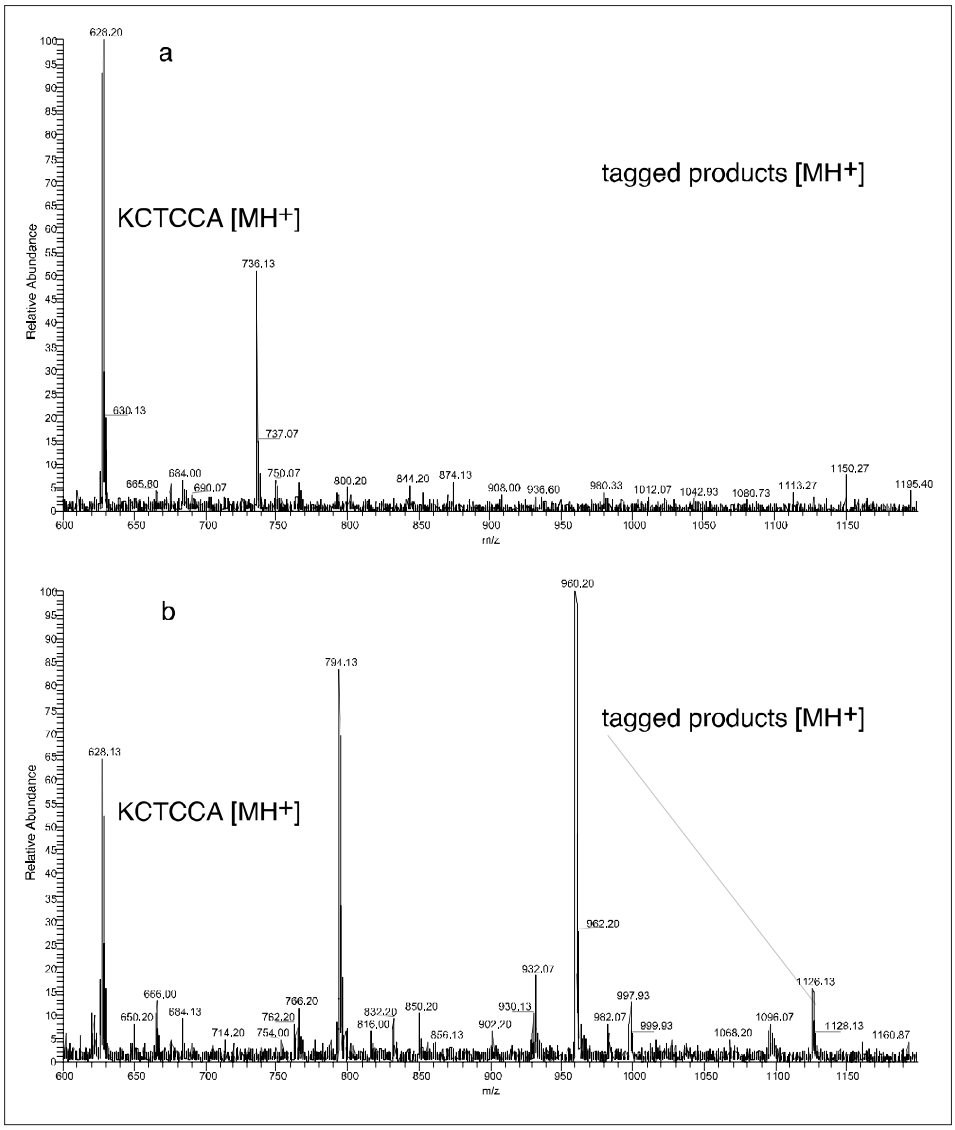


Fig. 4. Mass spectra of KCTCCA peptide infused in the presence of (a) hydroquinone (**1a**) and (b) carboxymethylhydroquinone (**1d**). In the lower spectrum, KCTCCA peptide ([MH+]: m/z = 628) is visible as well as the three tagged products ([MH+]: m/z = 794, [MH+]: m/z = 960 and [MH+]: m/z = 1126)

Dupont) was photoablated with a UV excimer laser (ArF 193 nm from Lambda Physics). The fabrication of the nanospray interface has been previously described [8]. The electrode was 70×25 μm^2, the channel had dimensions of 35×30 μm^2, and the distance from the electrode to the outlet was 1.8–2 cm.

4.4. *MS Set-up*

An LCQ DUO ion trap mass spectrometer Finnigan was used. With the ESI interface removed, the microchip holder was mounted on the probe slide adapter of the mass spectrometer. The device was coupled to a syringe pump (kdScientific) to introduce the solution. The flow rate was set to 250 $nl.min^{-1}$ and the voltage applied was 3.5–4 kV. The distance from the outlet of the microchip to the entrance of the spectrometer varied between 1 and 2 cm in order to optimise the signal and the trap injection time. L-cysteine 0.2 μM and peptides 50 μM were infused in the presence of hydroquinones 20 mM in MeOH/H_2O/AcOH 50:49:1. Proteins 5 μM were infused in the presence of hydroquinones 5 mM.

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