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Short communication

# Monitoring the redox cycle of low-molecular peptides using a modified target plate in MALDI-MS

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ABSTRACT

A new method is being proposed for preparing MALDI target plates with a hydrophobic polymer coating and hydrophilic anchors. The particles of the MALDI matrix were pre-mixed with a poly[4,5-difluoro-2,2-bis(trifluoromethyl)-1,3-dioxole-*co*-tetrafluoroethylene] solution prior to their placement on a mass-spectrometric sample support. This technique led to the formation of matrix microspots with a diameter of less than 1 mm inside the polymer. The polymer and matrix concentration as well as the amount of suspension placed on the target plate influenced the size and quality of microspots to a great extent. The sensitivity of the mass-spectrometric analysis was confirmed by obtaining the mass spectra of fmole concentrations of an apomyoglobin tryptic digest. The potential proteomic application of this type of MALDI surface preparation was demonstrated by performing the redox cycle using glutathione and its analogue. All reactions were carried out directly on a MALDI plate, which accommodates low volumes of reagents and prevents sample loss.



## 1. Introduction

Obvious advantages such as high accuracy, high throughput and low cost have led to the extensive use of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) as a powerful analytical tool in proteomics. A typical application of the technique is the analysis of large and medium-sized molecules such as proteins and peptides [1–3]. The detection of peptides with a mass smaller than 1000 Da is usually limited by the abundance of interfering matrix ions in the low-mass range of spectra [4]. Moreover, the main reason for problems encountered with the sensitivity and reproducibility of the method is the non-homogeneous distribution of the analyte when using conventional target plates, particularly with low-volume and diluted samples. This MALDI-MS problem can now be overcome by developing new methods for the formation of various hydrophobic supports that lead to the elimination of sample dispersion on a target plate.

Various types of materials have been applied to modify the surface of the target plate, and have been successfully employed with excellent reproducibility and low limits of detection to rapidly analyze mixtures of proteomic and pharmaceutical analytes. In 1998, Hung et al. [5] used paraffin wax film (Parafilm™) as a sample support coating for the analysis of DNA fragments. Compared with the commonly used metal surface, the new coating improved MALDI performance in DNA analysis and remarkably increased detection sensitivity. Later, the same research group tested polytetrafluoroethylene (Teflon™) for the mass-spectrometric analysis of DNA and proteins. It was found that the polymer coating enhanced sensitivity and salt tolerance, and could be used as a transfer membrane in protein analysis [6]. Schuerenberg et al. achieved further results in this area by introducing a new concept of MALDI-MS sample preparation utilizing pre-structured sample supports [7]. Target plates were coated with a Teflon layer that carried an array of 200 μm gold spots, which provided hydrophilic sample anchors. The water-repellent property of the Teflon layer allowed sample concentration and localization within the anchor area. This technology is now used in the preparation of commercial target plates.

Various technologies have been used for the preparation of specific MALDI surfaces to obtain better accuracy, high resolution and sensitivity. van Kampen et al. recently demonstrated that a strongly hydrophobic fluoropolymer coating on a MALDI target plate appreciably improved the means of relative error, precision and lower limits of quantitation [8]. Other methods include DropStop™ hydrophobic foil [9], an AnchorChip pre-structured surface for peptide analysis [10] and a removable coating based on 3M Scotch Gard™ [11], as well as various other materials and technologies [12–18].

This research investigates the possibility of introducing suitable MALDI matrixes into a Teflon-like solution (polyfluorodioxole) before a polymer is placed onto the target plate. During solvent evaporation, the matrix concentrates in the polymer and

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forms a hydrophilic anchor with a diameter of less than 1 mm. This strategy improves sample preparation and mass spectrum quality without a loss of sensitivity. The technique demonstrates the potential for minimal sample handling and eliminating sample loss by on-plate oxidation of both glutathione (GSH) and its analogue (UPF1, 4-methoxy-L-tyrosinyl-γL-glutamyl-L-cysteinyl-glycine) and reducing their dimers by using an enzymatic reaction in conjunction with direct MALDI-MS analysis.

The tripeptide GSH (L-γ-glutamyl-L-cysteinyl-glycine) is the most abundant nonprotein thiol in mammalian cells. Reduced glutathione is a central intracellular nonenzymatic water-soluble antioxidant that has several other functions in the human body [19–21]. In the presence of GSH as a substrate, the endogenously produced radicals are effectively reduced by the glutathione peroxidase (GPx). During this reaction, which is known as a redox cycle, GSH is converted to dimer GSSG, which is recycled back to two molecules of GSH by the glutathione reductase (GR). GSH is also converted to GSSG by the action of the hydroxyl radical and $H_2O_2$.

We have previously investigated a glutathione analogue, which exhibited remarkably better hydroxyl radical scavenging properties than glutathione itself [22,23]. In previous research [24] we demonstrated that UPF1 affected the activity of the GSH-bound enzyme, GPx. One of the aims of this research was to investigate how the oxidized form of UPF1 and mixed dimers (GSH/UPF1) affected the activity of GR. Based on the results of the study, novel GSH analogues to be used in oxidative stress treatment will be developed.

## 2. Experimental

### 2.1. Reagents

Poly[4,5-difluoro-2,2-bis(trifluoromethyl)-1,3-dioxole-*co*-tetrafluoroethylene] (polyfluorodioxole) (CAS No. 37626-13-4), a 35% hydrogen peroxide solution, L-glutathione, glutathione reductase, β-nicotinamide adenine dinucleotide phosphate (NADPH) as well as 2,5-dihydroxybenzoic acid (DHB) were obtained from Sigma–Aldrich (Steinheim, Germany). The solvent AB103516 for dissolving polymers came from ABCR GmbH & Co (Denmark). α-Cyano-4-hydroxycinnamic (CHCA), and sinapic (SA) acids were acquired from Fluka (Steinheim, Germany). The glutathione analogue UPF1 (MW 483.5 Da) was synthesized at the Institute of Biochemistry, University of Tartu (Estonia) as described elsewhere [25]. All of the solvents were MS-grade and purchased from Sigma–Aldrich (Steinheim, Germany). The deionized water was from a Milli-Q water system (Millipore, France). The tryptic digest of horse apomyoglobin was prepared as follows: 60 nmol of horse apomyoglobin was reconstituted in 6 mL of 10 mM ammonium bicarbonate and 20 μg of Promega modified trypsin was added subsequently. The mixture remained at ambient temperature for 15–18 h to obtain a protein digest with a final concentration of 10 pmol/μL.

### 2.2. Preparation of the hydrophobic surface and MALDI-MS instrumentation

The stainless steel MALDI plate was sonicated in ethanol for 20 min and then dried under a stream of air. Powdered polyfluorodioxole was mixed with the solvent to obtain 0.25, 0.5 and 1% solutions and was left for 24 h for swelling. 0.2–0.4 mg of the MALDI matrix was then added to the polymer solution and sonicated for 5 min until a suspension formed. Finally, immediately after sonication, 1.2 μL of the mixture was placed on the MALDI plate and dried at room temperature for a minimum of 30 min. The polyfluorodioxole/matrix mixture was freshly prepared every day. For the MS analysis, an aliquot of the sample (0.5–1 μL) was placed on top of the concentrated matrix and dried at room temperature. All the MALDI-TOF MS analyses employed the delayed extraction (20–100 ns) positive-ion reflector mode on a Bruker Autoflex II mass spectrometer (Bruker Daltonics, Denmark) equipped with a nitrogen laser (337 nm). The laser power was set to obtain a good resolution and signal/noise ratio (approx. 50%). The mass range of $m/z$ 200–1200 ($m/z$ 200–2000 for the digest of apomyoglobin) was scanned and 50 shots were averaged for each spectrum.

### 2.3. Sample preparation for the on-plate redox cycle reaction

Fresh solutions of 25 mM and 25 μM ammonium bicarbonate (pH 7.8) were prepared prior to the experiments. The glutathione reductase was diluted by one thousand times in 25 mM $NH_4HCO_3$ and a 3.8 μM NADPH solution was prepared using 25 μM $NH_4HCO_3$. The individual oxidation of GSH and UPF1 was accomplished using a diluted solution of hydrogen peroxide as follows: 0.5 μL of the freshly made 0.24 mM solution of UPF1 or GSH was placed on top of the 0.5 mm concentrated microspot of matrix. As the sample droplet evaporated, the appearance of the monomer peak was confirmed by registering the MS-spectra. 0.5 μL of a 0.3% solution of hydrogen peroxide was then added to the same spot and, after drying, MS analysis was performed repeatedly to monitor the peaks of GSH and UPF1 dimers. Finally, 0.5 μL of the GR solution was dropped onto the microspot and, after 2 min, the same aliquot of NADPH was added to the droplet of GR. After the reaction time (RT), which was equal to the time it took for the droplet to dry, the completeness of the redox cycle was monitored by MALDI-MS.

In order to reduce the dimer mixture, UPF1 and GSH were first dissolved in the bicarbonate buffer at an equimolar ratio and left for 2 days at room temperature to permit oxidation. The reduction process of the peptide mixture was then carried out similarly to that described above.

## 3. Results and discussion

### 3.1. Formation of the matrix microspot in hydrophobic polymer solution

The property of matrix particles to form densely concentrated microspots in a viscous solution of polymers underlies a new method of sample preparation in the MALDI-MS technique. Fig. 1 compares the formation of matrix spots by conventional (upper row) and proposed (lower row) approaches using three prevalent MALDI matrices. As shown in the figure, all these matrices are able to form inside the hydrophobic polymer well-concentrated microspots.

Preliminary experiments indicated that analyte ionization was achieved to a considerable degree. Concerns that the polymer layer covering the matrix microspot would totally absorb the laser energy were not substantiated. After the addition of the sample droplet to the microspot, the polymer was probably partially dissolved and subsequently acted as a matrix ion suppressor, eliminating the formation of matrix clusters. The quality of the concentrated matrix microspots depends on several parameters such as polymer and matrix particle concentration, matrix particle size and the volume of the colloidal solution on the target plate.

In this research, three different concentrations of polyfluorodioxole – 0.25, 0.5, 1 and 2% – were examined for the formation of matrix microspots. The results showed that at all concentrations the polymer formed a stable hydrophobic surface with an internal hydrophilic anchor. The water droplet contact angle on this surface was 100–112°, as measured by a microphotocamera. However, the polymer solvent in lower-concentration samples evaporated

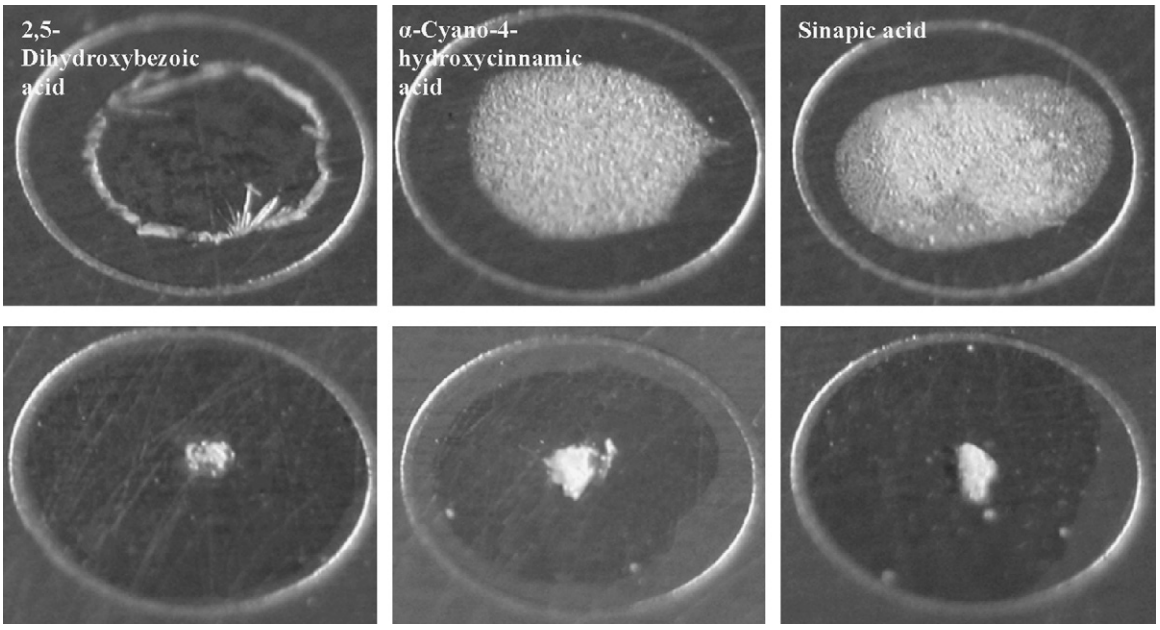


**Fig. 1.** Preparation of matrix supports using conventional (upper row) and proposed (lower raw) target deposition methods.

quickly, which prevented the matrix particles from grouping in one microspot. At a concentration of 2%, the polymer layer formed above the matrix microspot was too thick. Analyte ionization was still possible, but the sensitivity of the method decreased remarkably.

The concentration density of matrix particles can be controlled by the volume of matrix/polymer mixture applied to the stainless steel plate. The optimal volume of mixture was found to be 1.2 μL per 3.5 mm target plate circle. At higher volumes, the mixture of polymer and matrix tended to spread outside the labeled borders. At lower volumes, the layer of suspension was too thin, leading to the immediate evaporation of the solvent.

The quantity of matrix in the polymer solution influenced the final size of the matrix microspot. Thus, a polymer matrix concentration of 0.2–0.3 mg/100 μL produced microspots with a diameter of less than 0.3 mm; a concentration of 0.4–0.6 mg/100 μL resulted in microspots with a diameter of approximately 0.5 mm. It is predicted that the matrix-to-analyte ratio plays an important role in sample ionization in MALDI-MS analysis. Thus, depending on the analyte concentration, different sizes of matrix microspots should be used to achieve better ionization.

If needed, the polymeric cover on the target plate can easily be removed without leaving any polymer traces on the stainless steel surface, by washing with ethanol.

### 3.2. Evaluation of the sensitivity of the method

The model apomyoglobin digest was used to evaluate the sensitivity of the method. The peptide mixture was dissolved in 0.1% TFA to create a series of different concentrations—200, 100, 50, 25, 10 and 5 fmol/μL. 0.3 mm microspots of the DHB matrix applying a 1% polymer solution were used in the MALDI-MS analysis. 0.5 μL apomyoglobin digest aliquots were deposited in the center of the matrix microspot and allowed to dry. Fig. 2 shows the MALDI-MS spectrum obtained from the tryptic digest diluted to 25 fmol (per spot). Compared with the digest analysis using a stainless steel plate, the signal-to-noise ratio was improved by more than two times. Moreover, the mean of RSD ($n = 3$) for $m/z$ 1606, for example, was decreased from 15.7% for a stainless steel plate to 5.1% for a modified target plate. Mass-spectrometric measurement of 5 fmol/μL showed an increase of the mean RSD by up to 15%; therefore, the concentration of 10 fmol/μL was determined to be

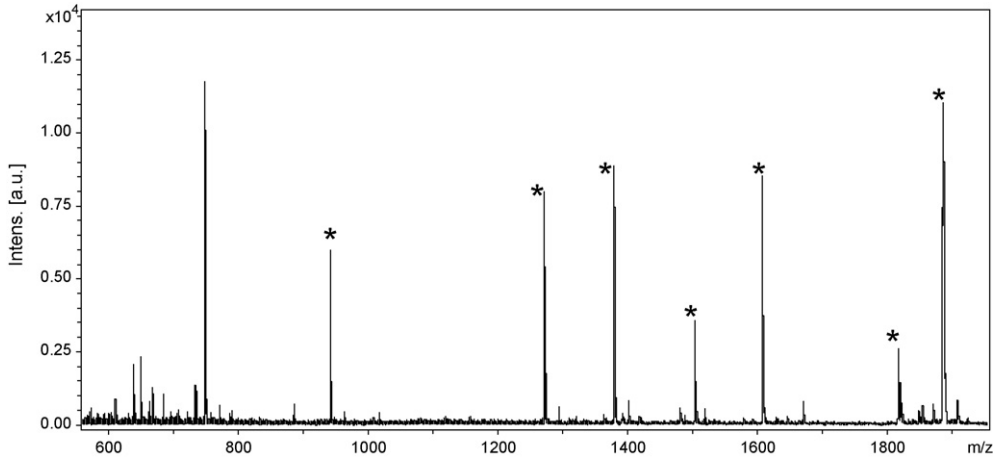


**Fig. 2.** MALDI mass spectra of a tryptic digest of apomyoglobin obtained using polyfluorodioxole as a hydrophobic coating and a DHB matrix as a hydrophilic anchor. The matched peptides are indicated by an asterisk.

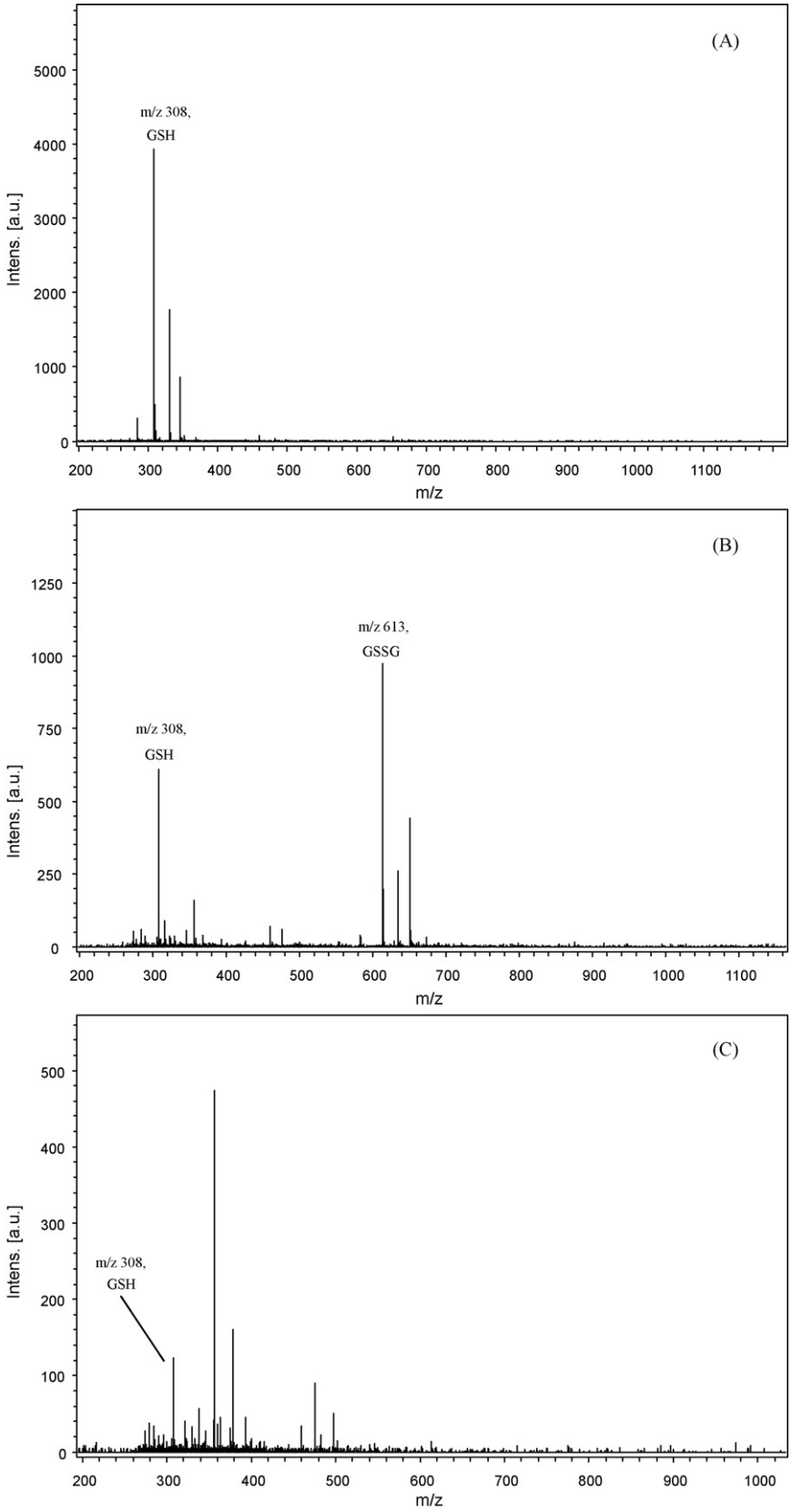


**Fig. 3.** The redox cycle of glutathione. (A) Mass spectra of 0.25 mM GSH, (B) dimerization of glutathione with a 0.3% of hydrogen peroxide, and (C) the reduction of GSSG by enzymatic reaction.

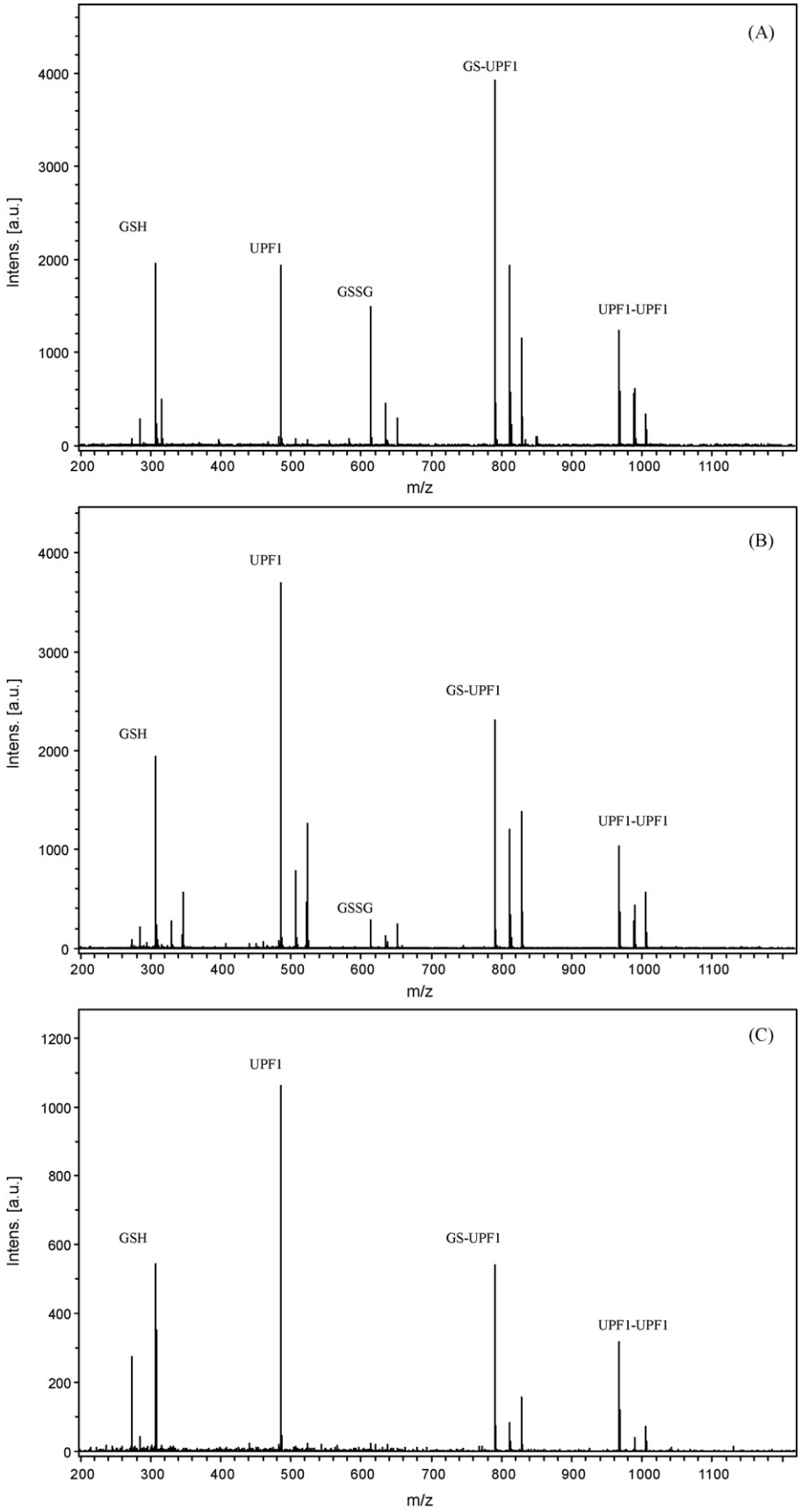


**Fig. 4.** The reduction of peptide dimers by glutathione reductase. MALDI mass spectrum of (A) the initial peptide mixture (RT = 0), (B) after the addition of 1 μL of GR/NADPH (RT = 15 min), and (C) after the addition of 2 μL of GR/NADPH (RT = 30 min).

the limit of detection for the proposed method. In our opinion, the main factor limiting detection sensitivity was the possible incorporation of the dissolved polymer into the sample droplet, leading to the suppression of ionization of the analyte.

### 3.3. The on-plate redox cycle of GSH

Unlike UPF1, GSH is more stable at room temperature in different solutions [21]. The mass spectrum of a freshly prepared UPF1 solution (in 25 mM ammonium bicarbonate buffer, pH 7.8) already contained a peak of the dimer. For this reason, the glutathione monomer was selected to perform redox cycling reactions. During these reactions, it was expected that GSH would be converted by hydrogen peroxide to the glutathione dimer, GSSG, and then recycled back to the monomer by enzymatic reaction.

It can be seen in Fig. 3A that the 0.25 mM of freshly prepared GSH solution generated only one peak at $m/z$ 308 that corresponded to the protonated glutathione monomer. The peaks at $m/z$ 330 and 346 belonged to the $Na^+$ and $K^+$ adducts of the monomer. 15 min (the drying time of the droplet) after the addition of 0.5 μL of a 0.3% solution of hydrogen peroxide to the spot containing only the monomer, a peak of GSSG at 613 $m/z$ appeared, and those of its adducts were visible at 635 and 651 $m/z$ (Fig. 3B). The presence of a peak of the monomer is indicative of incomplete oxidation. Reduction of the oxidized glutathione took place via a multi-step reaction involving reduction of the glutathione reductase by means of an NADPH and disulfide interchange. The mass spectrum in Fig. 3C clearly demonstrates by the total disappearance of the peak of GSSG that the reduction process was complete. (See Section 2 for a description of the reduction). The remarkable loss in peak height can be explained by the contamination of the mass range by the formation of some unknown peaks due to repeated additions of reagent droplets and the partial degradation of the matrix microspot.

### 3.4. The redox cycle of the GSH and UPF1 mixture

It is well known that glutathione and related peptides are prone to oxidation in solution. We used the ammonium bicarbonate buffer (pH 7.8) to investigate the oxidation of the GSH/UPF1 mixture. The oxidized forms of UPF1 ($m/z$ 967) and GSH ($m/z$ 613) as well as the heterodimer GS-UPF1 ($m/z$ 790) were detected by MALDI-MS analysis after a 2-day incubation at room temperature (Fig. 4A). The exact time of the appearance of dimers was not established, because this was not the aim of the study. This procedure was performed to avoid forced oxidation, to carry out the first step of the redox cycle reaction via a spontaneous dimerization of the GSH/UPF1 mixture in a neutral buffer, and to investigate the activity of GR by using three peptide dimers.

Fig. 4B shows the MALDI mass spectrum after depositing equal aliquots (0.5 μL) of GR and NADPH onto the matrix microspot containing the peptide mixture. The time of evaporation of the GR/NADPH droplet, which is equal to the reaction time, was 15 min. It can be seen that the intensity of the GSSG peak decreased significantly at these amounts of glutathione reductase and NADPH. The addition of the repeatable portion of GR/NADPH (the total reaction time was 30 min) resulted in the disappearance of the GSSG signal (Fig. 4C). At the same time, the intensity of the UPF1-UPF1 peak did not decrease, the heterodimer GS-UPF1 was partially recycled back to GSH and UPF1 monomers, and the intensity of the respective peak was decreased.

Based on the results of these experiments, we can conclude that the monodimer GSSG and heterodimer GS-UPF1 both affected the activity of the glutathione reductase, but the monodimer UPF1 did not affect the activity of GR.

The traditional approach to measuring the glutathione redox cycle is a spectrophotometric method based on the Bioxytech GR-340 Assay (Bioxytech; OXIS International, Inc., Portland, USA). Proposed MALDI-MS analysis could considerably reduce the concentrations of reagents involved in on-plate reactions. For instance, 240 nmol of GSSG sufficed for one spectrophotometric measurement. Furthermore, 60 pmol of GSSG is enough to obtain the products of the reaction for the on-plate method. Therefore, due to the high sensitivity of the method, the consumption of some very expensive reaction reagents was minimized.

## 4. Conclusions

This study describes a simple new method for fabricating MALDI matrix hydrophilic microspots coated with a hydrophobic polymer. The evaluation of target surface modifications led to the conclusion that the quality of mass spectra in MALDI-MS directly depends on the concentration of the matrix and polymer, as well as on the amount of suspension on the MALDI target plate. The application of low volumes of the mixture and on-plate enrichment resulted in highly reproducible MALDI spectra of 25 fmol of the model tryptic digest. Moreover, the possibility of treating samples directly on the MALDI plate was demonstrated by performing redox cycle reactions of glutathione and its analogue and their mixtures. Further optimization of the "lab-on-a-plate" mass-spectrometric method is underway. One of the prospective benefits of the proposed method, when matrix spots already exist in the hydrophobic environment of the MALDI target plate, is its combination with off-line capillary electrophoresis. The high throughput of the method contributes to the rapid analysis of dozens of fractions collected during electrophoretic separations.

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