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RESEARCH ARTICLE

Graphite Supported Preparation (GSP) of α-Cyano-4-Hydroxycinnamic Acid (CHCA) for Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) for Peptides and Proteins

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Abstract

Graphite as MALDI matrix or in combination with other substances has been reported in recent years. Here, we demonstrate that graphite can be used as target coating supporting the crystallization of the α-cyano-4-hydroxycinnamic acid matrix. A conventional dried-droplet preparation of matrix and analyte solution on a graphite-coated metal target leads to a thin, uniform layer of cubic crystals with about 1 μm edge length. Commercially available graphite powder of 1–2 μm particle size is gently wiped over the target using a cotton Q-tip, leading to an ultra-thin, not-visible film. This surface modification considerably improves analysis of peptides and proteins for MALDI MS using conventional dried-droplet preparation. Compared with untreated targets, the signal intensities of standard peptides are up to eight times higher when using the graphite supported crystallization. The relative standard deviation in peak area of angiotensin II for sample amounts between 1 and 50 fmol is reduced to about 15 % compared with 45 % for untreated sample holders. For a quantification of 1 fmol of the peptide using an internal standard the coefficient of variation is reduced to 3.5 % from 8 %. The new graphite supported preparation (GSP) protocol is very simple and does not require any technical nor manual skills. All standard solvents for peptides and proteins can be used.

Key words: MALDI-MS, Sample preparation, Graphite supported crystallization, α-Cyano-4-hydroxycinnamic acid (CHCA)

Introduction

MALDI–MS is often used for identifying proteins via peptide mass fingerprinting and for the analysis of intact proteins. Since its introduction [1] great efforts have been undertaken to find new and better matrices and to improve sample preparation protocols [2–6]. Nevertheless, only very few matrices and preparation protocols are used today. While sDHB (a 9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) [7] and sinapic acid (SA) [8] are mostly used for analysis of intact proteins CHCA is the first choice for peptide analysis and proteomics. Due to the homogeneous distribution of small crystals this matrix is particularly suited for automatic measurements and high throughput proteome analysis. Sample preparation techniques are either dried droplet preparation (DDP) or thin layer preparation (TLP). While the DDP comprises simple application of one droplet of matrix and one of analyte solution or one droplet of a mixture of both on the MALDI target there are various techniques for TLP [3, 5,

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6, 9]. There are several reports on the use of graphite for MALDI mass spectrometry. Solid graphite plates were used for matrix-free laser desorption/ionization of low molecular weight nonpolar compounds [10], polymers [11], or fatty acids [12]. A simple way to produce a graphite layer on a metal target is to use a pencil. This has been used for direct (matrix-free) analysis of actinides [13] and small organic molecules [14]. Glycerol was added to enhance ionization of polymers under laser irradiation in the visible [15], matrix was added to analyze folates [16] and cocaine and its metabolites in hair [9]. Graphite powder mixed with glycerol [17] or other liquid matrices [18] was applied to analyze peptides, proteins, oligosaccharides, and polymers. Strong cationization is observed in all cases and graphite particles promote the risk of instrument contamination. Improved results for peptide mapping were reported by Li et al. [19] on a silicone/graphite layer using a standard matrix. They fabricated a small device for coating of metal targets with the silicone/graphite mixture which has to be removed by a silicone cleaner after measurement. Here we describe a simple procedure for coating MALDI targets with a thin-layer of graphite that improves analysis of peptides and proteins using the conventional dried-droplet method with CHCA as matrix.

Materials and Methods

Materials

All standard proteins and peptides, Angiotensin-II (Ang-II), [Val] Angiotensin-II ([Val] Ang-II), human Neurotensin, bovine insulin β-chain, bovine serum albumin (BSA), and chemical reagents, such as trifluoroacetic acid (TFA), acetonitrile (ACN), methanol (MeOH), acetone, as well as the graphite powder (synthetic, 1–2 µm) were obtained from Sigma Aldrich (Munich, Germany). Water was prepared in house using a Purelab Ultra Genetic water purification system (ELGA, Celle, Germany). The MALDI matrix α-cyano-4-hydroxycinnamic acid (CHCA) was obtained from Bruker Daltonics (Bremen, Germany). MALDI targets used were either a stainless steel Opti-TOF 384 well insert or a 100 well Voyager STR stainless steel target without well inserts (AB SCIEX, Darmstadt, Germany). Wenol metal polish was from Reckitt Benckiser (Mannheim, Germany). CHCA was prepared as a 3 mg/mL solution in 70:30 ACN/0.1 % aqueous TFA. BSA and a mixture of all four peptides

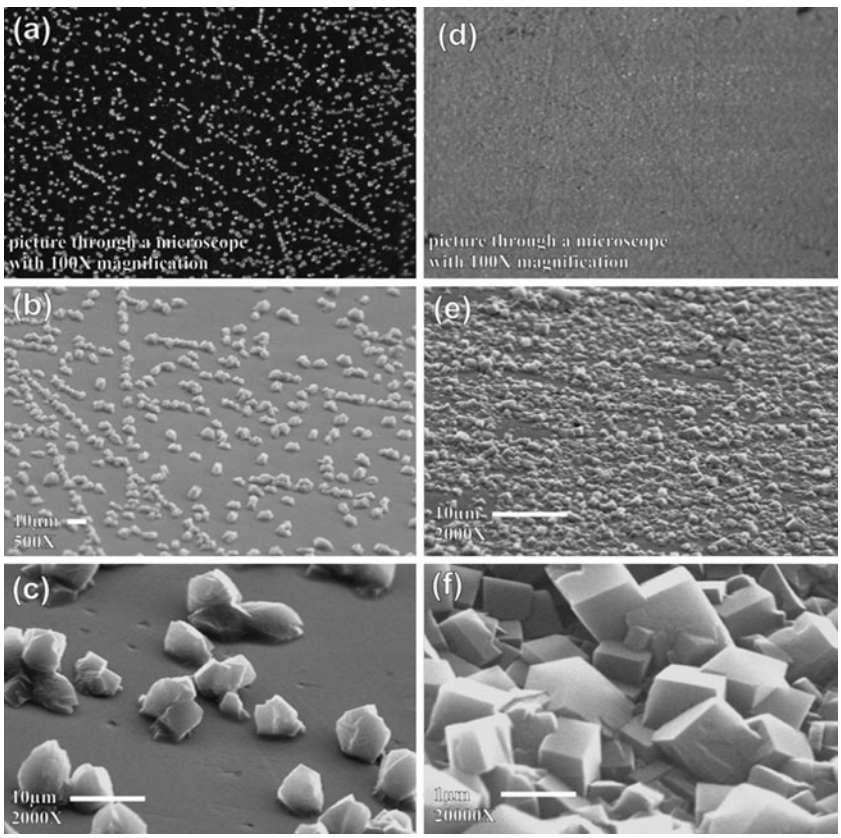

Figure 1. Optical microscope and SEM pictures of CHCA preparations. (**a**)–(**c**) Show dried droplet preparation on an unmodified target. Photographic image through an optical microscope with a magnification of 100× (**a**), SEM pictures of the crystals with a magnification of 500× (**b**), and 2000× (**c**). (**d**)–(**f**) Show graphite supported preparation. Optical microscope picture with a magnification of 100× (**d**), SEM pictures with a magnification of 2000× (**e**), and 20,000× (**f**)

were freshly prepared in H_2O with different concentrations (as indicated in the text or the figure captions).

MALDI Target Preparation

After cleaning with household cleaners (dishwashing detergent for example, Bref power) and 10 min ultrasonication in a 1:1:1 water/MeOH/acetone mixture, the MALDI target was polished using metal polish on a lint-free paper followed by a final acetone wash. Although this cleaning procedure is nonessential for the presented graphite supported preparation (GSP), it provides very clean MALDI plates and avoids memory effects and spreading of the droplets; both effects are particularly significant for analysis of peptides in the low concentration range. Afterwards, the stainless steel MALDI target was modified by wiping graphite powder over the surface, using a commercial cotton Q-Tip. An excess of loose graphite powder on the target was gently wiped off with lint-free paper. The change in the surface was neither visible to the naked eye nor by microscopic or even electron microscopic imaging. To provide an

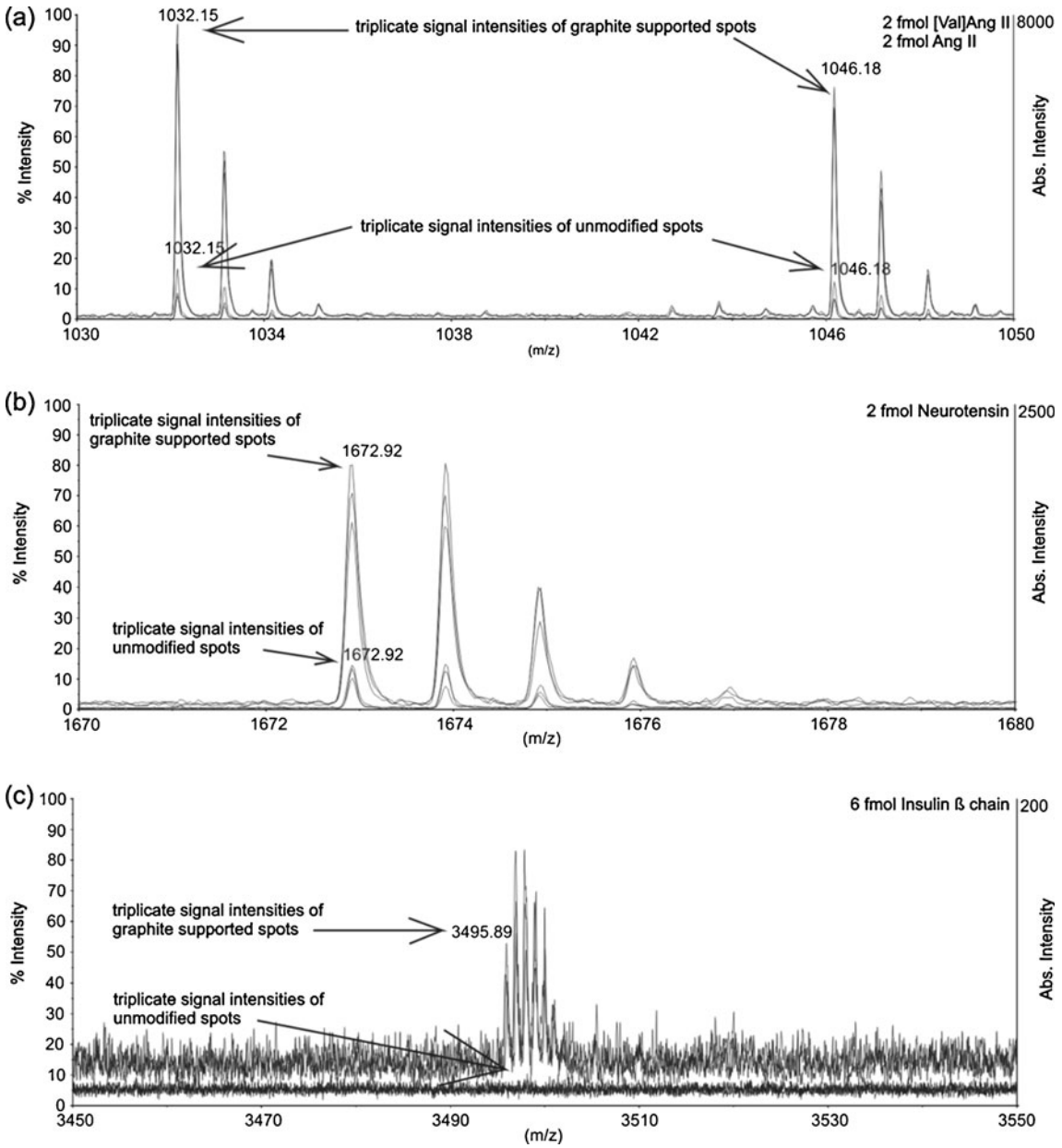

Figure 2. Extracted spectra from graphite supported and unmodified spots of the peptide mixture. The spectra are obtained from 2 fmol of each peptide of [Val]Ang II, Ang II (**a**) and neurotensin (**b**) and from 6 fmol of insulin β chain (**c**). In each case, three spectra are displayed in stacking mode. All spectra are taken under the same conditions from the same target in automated run on the AB SCIEX 4800 TOF-TOF in the positive-ion reflector mode. The signals in each extract are normalized to the same absolute intensity

optimal comparison, only one half of the MALDI target was modified so that both preparation techniques could be prepared on the same target.

MALDI-MS Measurement

All peptide spectra were acquired on an AB SCIEX 4800 MALDI TOF-TOF (AB SCIEX, Darmstadt, Germany) in the positive-ion reflector mode. All measurements were replicated six times on separate spots under otherwise identical conditions. One thousand shots per spectrum were accumulated. All spectra were recalibrated after recording to provide a direct comparison in the overlaid mode of the spectra as extracted from the Data Explorer (AB SCIEX, Darmstadt, Germany). Mass spectra of the intact protein BSA were acquired on a Voyager STR from AB SCIEX (Darmstadt, Germany) in the positive linear ion mode. The instrument settings on the Voyager STR were set as followed: acceleration voltage 20 kV, grid voltage 90 %, delayed extraction 1200 ns, mass range 4000 to 75,000 Da, low mass gate 4000 Da and accumulation of 1000 shots per spectrum. Analyte samples were mixed 1:1 with the MALDI matrix and vortexed prior spotting. The total volume per spot was 1 µL of the premixed sample-matrix solution, regardless whether a normal polished stainless steel target or graphite treated polished stainless steel target was used. The droplets were allowed to dry at room temperature or in a cool stream of air.

Optical Microscope and SEM Pictures

The SEM pictures are taken with a 1920 ECO-SEM from AMIKA/AMRAY equipped with a OXFORD EDS [ATW Si(Li) detector]. The accelerating voltage was set to 20 kV with a working distance of 15 mm and a tilt angle of 20°. The magnification is mentioned in every picture displayed in Figure 1. The optical microscope pictures were taken with an Canon EOS 400D (Canon, Krefeld, Germany) connected to an Olympus CH30 (Olympus, Hamburg, Germany) by an LM-Scope from Micro Tech Lab (Graz, Austria).

Results and Discussion

Matrix Crystallization

While crystallization of CHCA on an untreated target takes place over the entire period of solvent evaporation and, thus, leads to a slow crystal growth over minutes, crystallization on the graphite supported target surface is quite fast and occurs within some seconds after droplet deposition. On the first glance, the spots resemble those prepared with the thin layer preparation technique by Vorm et al. [6]. The crystals on the graphite treated MALDI target in comparison with the untreated target were inspected under an optical microscope and by scanning electron microscopy. The pictures obtained

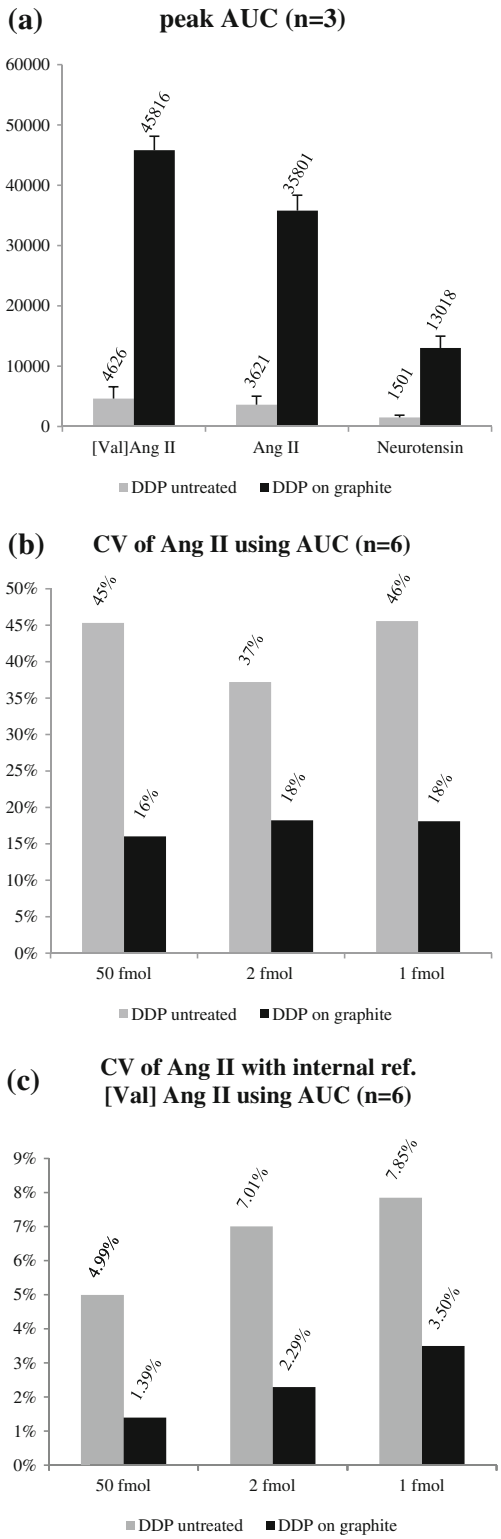

Figure 3. Comparison of results obtained for peptides on the modified and unmodified target in bar charts. The measurements were made as triplicates for (**a**) with error bars indicating the standard deviation. The CVs are calculated from six measurements from six different spots. (**a**) Peak areas given in AUC, (**b**) CV of the peak areas, (**c**) CV of the peak areas of Ang II relative to [Val]Ang II as internal standard

are shown in Figure 1. With an optical microscope with 100× magnification, differences are already distinct and visible. Small crystals homogeneously distributed all over the sample/matrix spot are observable on the untreated target (Figure 1a), while a thin layer of the matrix/sample mixture is formed on the treated target (Figure 1d). For the dried droplet preparation, the electron microscopic pictures show irregularly formed isolated crystals, which are equally distributed over the spot and have diameters of about 10 µm (Figure 1b, c). A dense layer of interconnected prismatic crystals with a maximum edge length of 1 µm are formed for the GSP preparation as displayed in Figure 1e, f. Most likely, the 1–2 µm graphite particles serve as nucleation centers for crystal growth as already postulated by Li et al. [19]. We also investigated the behavior of the 4-chloro-alpha-cyanocinnamic acid [2] using the GSP protocol but only marginal effects were observed (data not shown).

Spectra Derived from Standard Peptides

The mass spectra acquired from the peptide mixture on the modified and unmodified target were compared after automated measurement under identical conditions. In Figure 2a and b, the *m/z*-range including the peaks from [Val] Ang-II (1032.15), Ang-II (*m/z* 1046.18), and Neurotensin (*m/z* 1672.92) from a total amount of 2 fmol per peptide on-target are displayed. In each case, signals derived from unmodified and graphite modified spots are shown in overlaid mode. In all cases, the signal intensities from the GSP are by a factor of about 5–10 (peak area and height) higher compared with the normal DDP for [Val] Ang-II (1032.15), Ang-II (*m/z* 1046.18), and Neurotensin (*m/z* 1672.92). This applies also for sample amounts of 50 and 1 fmol. The exact values are given in Figure 3a as bar graphs. Signals from insulin B chain with a molecular mass of 3495 were only detected from a 6 fmol/µL-solution using the GSP (Figure 2c), whereas this sample amount was not sufficient to detect signals from the unmodified target. Beside signal intensity, the reproducibility is enhanced due to the more uniform crystallization. This is illustrated in more detail in Figure 3b for six replicates of Ang II. While the relative standard deviation for the DDP is about 40 %, it is reduced to about 15 % with the GSP. This is independent of the concentration in the tested range from 50–1 fmol/µL. Similar values were obtained for the other peptides tested in this study. The enhanced reproducibility is especially important for quantitation of low amounts of peptides. Typically, an internal standard is used for quantification in mass spectrometry and the signal intensity of the analyte is determined relative to the standard. This is simulated for Ang II with [Val]Ang II as internal standard. The relative standard deviation of the peak area for Ang II with this method on the modified target is below 1.5 % for sample amounts of 50 fmol, it increases to only 3.5 % for 1 fmol samples (see Figure 3c). Also in this respect the dried droplet preparation on the unmodified target is clearly inferior. While at a sample amount of 50 fmol the reproducibility is about 5 %, it increases to only 7 %–9 % for 2 fmol or 1 fmol, respectively.

Protein Mass Spectra

BSA, a protein with a mass of 66432 Da, was chosen to test the influence of the GSP on protein mass spectra. Unfortunately, sDHB, which is the preferentially used matrix for proteins above 25 kDa [7], does not behave like CHCA and does not form a uniform crystal deposit. CHCA is a harder matrix and shows a certain degree of prompt fragmentation which affects the peak width of the measured protein ion signal. Nevertheless, it is sometimes used for analysis of proteins as it has the advantage to produce higher charge states [20]. The spectrum in Figure 4 shows a broad distribution with charge states ranging from 1 to 8 for 75 fmol BSA. Normal dried droplet preparations show only a maximum charge of $[M + 6H]^{6+}$ for 75 fmol BSA near the detection limit. The limit of detection on graphite is about 15 fmol for BSA. The necessary laser irradiance for BSA on the GSP spots is lower than for the unmodified spots. With respect to MALDI of intact proteins the presented GSP method well compares to the "ultrathin-layer technique" [5, 21], but uses a much less laborious protocol**.** For the third MALDI protein matrix, sinapic acid, also a more homogenous crystal layer is formed on the graphite-modified surface; however, the crystal size as inspected microscopically is still large compared with CHCA, which agrees with the fact that no benefit is achieved for this matrix over normal preparation.

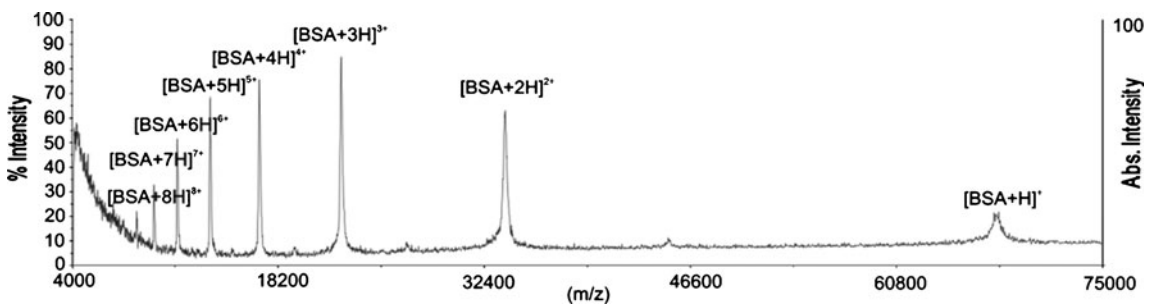

Figure 4. MALDI MS spectra of BSA using graphite modified spots with a total amount of 75 fmol

Conclusions

The graphite modification of the metal MALDI target presented here changes the crystal morphology of CHCA; 2 μm graphite particles wiped over the target surface provide a basis for the formation of a thin, uniform layer of tiny crystals when a droplet of CHCA/analyte solution is applied. Compared with the normal dried droplet preparation on an unmodified target, the signal intensities are enhanced by a factor of up to eight for low peptides at low concentration. The limit of detection for bovine serum albumin is reduced by a factor of five. The homogeneous preparation provides a high reproducibility of the signal intensities. This makes the method well suited for quantification of low amounts of peptides. For the reproducibility of Ang II signals from 1 fmol on target related to an internal standard, a value of 3.5 % compared with 8 % was determined. There are no limitations in the sample solvent so that automatic spotting of separated peptides after gradient elution with HPLC is possible. The described procedure is quick, easy to use, and inexpensive.

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