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Quantifying the Cluster of Differentiation 4 Receptor Density on Human T Lymphocytes Using Multiple Reaction Monitoring Mass Spectrometry

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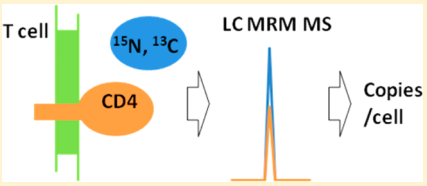
Supporting Information

ABSTRACT: Cluster of differentiation 4 (CD4) is an important glycoprotein containing four extracellular domains, a transmembrane portion and a short intracellular tail. It locates on the surface of various types of immune cells and performs a critical role in multiple cellular functions such as signal amplification and activation of T cells. It is well-known as a clinical cell surface protein marker for study of HIV progression and for defining the T helper cell population in immunological applications. Moreover, CD4 protein has been used as a biological calibrator for quantification of other surface and intracellular proteins. However, flow cytometry, the conventional method of quantification of the CD4 density on the T cell surface depends on antibodies and has suffered from variables such as antibody clones, the fluorophore and conjugation chemistries, the fixation conditions, and the flow cytometric quantification methods used. In this study, we report the development of a highly reproducible nano liquid chromatography−multiple reaction monitoring mass spectrometry-based quantitative method to quantify the CD4 receptor density in units of copy number per cell on human CD4+ T cells. The method utilizes stable isotope-labeled full-length standard CD4 as an internal standard to measure endogenous CD4 directly, without the use of antibodies. The development of the mass spectrometry-based approach of CD4 protein quantification is important as a complementary strategy to validate the analysis from the cytometry-based conventional method. It also provides new support for quantitative understanding and advanced characterization of CD4 on CD4+ T cells.



Cluster of differentiation 4 (CD4) is a glycoprotein that locates on the surface of immune cells such as T helper cells, monocytes, macrophages, and dendritic cells. As a coreceptor, CD4 amplifies the signal generated by the T cell receptor, which is essential for activation of many molecules involved in the signaling cascade of an activated T cell. In human T lymphocytes, CD4 receptor protein is encoded by the CD4 gene[1] and has four distinct extracellular domains (D1 to D4), a transmembrane portion, and a short intracellular tail.[2] The use of antihuman CD4 monoclonal antibodies generated against the four extracellular domains has been widely used to define T helper cells in immunophenotyping. Although the number of CD4+ T cells decreases in the progression of HIV-1 viral infection deriving from the gp120 viral protein binding to the CD4 receptor, Poncelet et al. reported that the surface CD4 density still remained constant on T helper cells of HIV-infected individuals.[3] Since then, multiyear research has supported the theory that CD4 expression/density can be used as a biological calibrator for quantification of other surface and intracellular proteins.[4−7]

Quantitative multicolor flow cytometry incorporating antibodies and a fluorescence detection method has played a critical role in clinical diagnostics and immunotherapies. Though the ultimate objective of quantitative flow cytometry is to measure the number of antigens or ligand binding sites associated with a cell, the task is carried out by measuring the number of antibodies bound per cell (ABC). It is critically important to produce biological cell reference materials that bear well-characterized protein markers such as CD4 for the transformation of a calibrated linear fluorescence intensity scale of a flow cytometer channel to a biologically meaningful ABC scale.[7] The quality of the cytometric measurements is affected by variables such as antibody clones, the fluorophore and conjugation chemistries, the fixation conditions, and the flow cytometric quantification methods used.[4,8−11] Hence, in addition to characterizing candidate reference cell preparations that use antibody-based cytometric methods,[12] it is necessary to develop a complementary approach to validate the absolute quantification of reference marker proteins such as CD4 without the use of antibodies.

Liquid chromatography coupled mass spectrometry has emerged as a versatile platform for quantitative protein/proteomics analysis due to its high specificity and sensitivity. Relative quantification of proteins can be achieved without the use of any internal standard for comparative analysis under the same analytical conditions. However, in many analyses such as clinical biomarker tests, absolute quantification of protein(s) in

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terms of molecule copy number per cell or per unit weight/volume of biological samples is required.[13−15] Absolute quantitative data enable valuable comparisons across different studies and conclusive interpretations of the disease states or treatment efficacy as well as the understanding of the whole body system biology probed from different angles in different studies. Multiple reaction monitoring mass spectrometry (MRM MS) combining proper separation and/or fractionation techniques has been proven to be an effective platform for protein quantification in biological samples.[16−18] In the present study, we report the development of an MRM MS-based approach that combines nanoscale liquid chromatography and a stable isotope-labeled full-length protein as the internal standard, enabling the quantification of the CD4 receptor density in units of copy number per cell on human CD4+ T cells without the use of antibodies.

EXPERIMENTAL SECTION

Materials. All chemicals and reagents, unless indicated specifically, were from Sigma-Aldrich Inc.

Determination of the Human CD4+ T Cell Count. Cryopreserved, negatively selected human CD4+ T cells with a purity of 98.5% were purchased from Astarte Biologics (Redmond, WA), confirmed internally, and used without further purification. The thawed cryopreserved CD4+ T cells were slowly added to 9 mL of RPMI-1640 containing 10% fetal bovine serum (FBS) in a 15 mL conical tube. After the tube was inverted three times, the cells were centrifuged at 400g_n for 10 min, and the supernatant was discarded. The resulting cells were washed once and resuspended in phosphate-buffered saline (PBS) with 1% FBS. The number of CD4+ T cells was counted by using both a hemocytometer and a flow cytometry with which TruCount beads from BD Bioscience (San Jose, CA) were used as the internal counting standard. Mouse antihuman CD4 fluorescein isothiocyanate (FITC; clone SK3, catalog number 340133, BD Biosciences) was used for cell staining, and CD4+ cells were counted using an Aria II flow sorter from BD Biosciences. Gating of CD4+ and TruCount beads was performed on a FITC histogram. The ratio of the respectively gated events of CD4+ cells and TruCount beads was used for obtaining the CD4+ cell number according to the manufacturer's procedure. The CD4+ cell numbers measured by the hemocytometer and flow cytometry were fairly consistent with a difference of no more than 6%, and therefore, the averaged cell count from both methods was used to derive the CD4 receptor density/copy number per cell.

Characterization of Isotope-Labeled Standard CD4. Isotope label (^{13}C and ^{15}N) was introduced on arginine and lysine residues in a standard CD4 protein from OriGene Technologies (Rockville, MD). The amino acid sequence of this standard CD4 protein is provided in the Supporting Information, Table S1. Since the isotope incorporation of the standard protein is not 100%, the percentage of isotope labeling was evaluated using MRM MS by comparing the chromatographic peak intensities of transitions from the isotope-labeled peptides to the peak intensities of the corresponding transitions from the unlabeled peptides. The concentration of the isotope-labeled internal standard CD4 was then determined using a recombinant CD4 protein (rCD4) (obtained from the NIH AIDS Research & Reference Reagent Program) with a known concentration. The rCD4 purity was determined to be above 96% using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and the concentration was calculated to be 31.84 μmol/L by amino acid analysis determined from averaging the concentrations of seven amino acid residuals, aspartic acid, glutamic acid, glycine, alanine, leucine, lysine, and arginine, using Standard Reference Material (SRM) 2389 of the National Institute of Standards and Technology (NIST) (amino acids in 0.1 mol/L HCl) as the amino acid calibration standard on an amino acid analyzer from Hitachi Instruments (Dallas, TX).

Sample Preparation for MRM MS. A preparation procedure of human CD4+ T cells for MRM MS measurements is illustrated in Figure 1. The isotope-labeled full-length

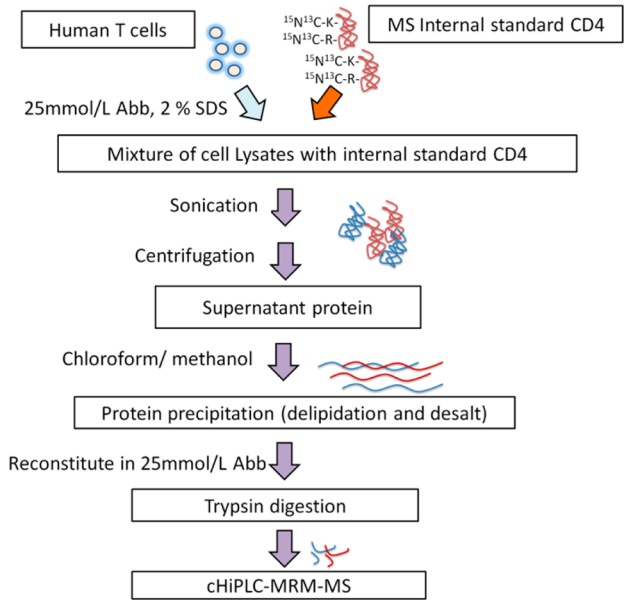

Figure 1. Flowchart of sample preparation for nano-LC−MRM MS analysis.

standard CD4 of known concentration was mixed with a known number of human T cells in 150 μL of 25 mmol/L ammonium bicarbonate buffer (Abb), pH 7.9, with 2% SDS. The cell and protein mixture was lysed by sonication on ice at 20 W using three 10 s continuous cycles (Sonicator 3000 from Misonix, Farmingdale, NY). The mixture was treated with 20 mmol/L DTT and incubated at room temperature for 60 min to allow reduction of cysteines and was then treated with 50 mmol/L iodoacetamide for another 60 min for alkylation. The cell lysate was centrifuged at 175000g_n for 30 min to remove insoluble fragments. Proteins in the supernatant were precipitated using chloroform/methanol[19,20] to remove salts and lipids. Briefly, 1 volume of sample solution was combined with 4 volumes of methanol, 1 volume of chloroform, and 3 volumes of water. The solution was mixed by vortexing and centrifuged at 20000g_n at room temperature for 10 min. The upper phase was removed carefully, and 4 volumes of methanol was added to the lower phase and interphase, which contained precipitated proteins. The mixed solution was centrifuged again at 20000g_n for 10 min to pellet the protein. The precipitated protein mixture was then reconstituted in 100 μL of 25 mmol/L Abb followed by protease digestion using trypsin (sequence grade modified, Promega, 1:50 w/w trypsin/protein) overnight at 37 °C. After enzymatic digestion, the sample was treated with 0.5% trifluoroacetic acid and centrifuged at 175000g_n for 30 min. The supernatant, which contained soluble peptides, was transferred to a fresh microcentrifuge tube and dried by vacuum

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centrifugation (Eppendorf AG, Hamburg, Germany) for subsequent mass spectrometry analysis.

Nano-LC−MRM MS Analysis. The digested peptides were reconstituted in Milli-Q H_2O with 3% acetonitrile (ACN) containing 0.1% formic acid followed by nano-LC−MRM MS analysis. Peptide separation and mass spectrometry analysis were performed on a hybrid triple-quadrupole/linear ion trap mass spectrometer (4000 QTRAP, ABI/MDS-SCIEX) coupled to an Eksigent nanoLC-2D system (Dublin, CA). Peptides were separated and eluted at a flow rate of 300 nL/min over 30 min with a gradient of acetonitrile from 15% to 35% in H_2O containing 0.1% formic acid using an Eksigent cHiPLC-nanoflex system equipped with a nano cHiPLC column, 15 cm × 75 μm, packed with ReproSil-Pur C18-AQ, 3 μm (Dr. Maisch, Germany). The eluted peptides were directed into the mass spectrometer with a nanospray source. The subsequent MRM detection of CD4 signature peptides was performed in the positive ion mode with the following key parameters: ion spray voltage of 2200 V, curtain gas pressure of 15 psi, source gas pressure of 20 psi, interface heating temperature of 170 °C, declustering potentials of 76 V for +2 precursor ions and 65 V for +3 precursor ions, collision cell exit potentials of 16 V for +2 precursor ions and 13 V for +3 precursor ions, and dwell time of 40 ms for each transition pair. The collision energy of each target transition was optimized empirically using peptides from unlabeled rCD4 and isotope-labeled standard CD4. The peptides detected and optimized collision energy (CE) values are listed in the Supporting Information, Table S2. The mass spectrometer was operated using Analyst 1.5.1 (AB SCIEN). Since the detectable ions of different peptides from a single protein can be different in different mass spectrometers, we selected and optimized the target CD4 peptides from human T cells and working MS parameters using our MRM analysis for the standard proteins based on favorable transition peak intensities, stable retention times, and minimum biological matrix effects. Considering the complexity of the cell lysate, the similarity of the intensity ratios of multiple transitions from the selected peptides from standard CD4 and the counterpart in the cell lysate confirmed minimal interference from the biological matrix. Each selected peptide was further confirmed as a unique CD4 peptide by sequence blast against the human nonredundant genome database (NCBI).

Data Analysis. Calibration curves showed linearity and low scatter over the range of 0.1−5 pmol/μL tested for the internal standard. The concentration of the stable isotope-labeled standard CD4, N_{iso}, was calculated according to the following equation:

$$N_{iso} = \frac{I_{iso}}{I_r} N_r \quad \text{where} \quad I_r = I_{n\text{-}iso} - I_{iso} \times 0.23 \tag{1}$$

I_{iso} and I_r refer to the intensity of the isotope-labeled peptide peak and intensity of the rCD4 peptide peak, respectively. $I_{n\text{-}iso}$ corresponds to the intensity of the total non-isotope-labeled peptide peak detected, and the constant 0.23 is the ratio of the nonlabeled to the labeled peptide obtained from the internal standard CD4. N_r is the concentration (mol/L) of rCD4 derived from the amino acid analysis. The endogenous CD4 protein concentration, N_{end}, was derived in the same fashion from the ratio of the nonlabeled and labeled MRM transition peak intensities multiplied by the known amount of standard spiked into the sample on the basis of the following equation:

$$N_{end} = \frac{I_{end}}{I_{iso}} N_{iso} \quad \text{where} \quad I_{end} = I_{n\text{-}iso} - I_{iso} \times 0.23 \tag{2}$$

I_{end} stands for the intensity of the endogenous CD4 peptide peak.

The identities of the selected peptides were confirmed on the basis of the two parameters of the internal standard run under the same conditions, the retention time of the given peptide, and the proportional ratio among the MRM transitions. Each pair of transitions from a given peptide was treated as an independent measure for the peptide, resulting in a concentration value expressed as the copy number of the quantified peptide per cell. Analysis of each selected signature peptide was based on the mean value of multiple transitions from the peptide. Three signature peptides were employed to evaluate the endogenous CD4 concentration. Each sample was measured in triplicate, and a total of three cell lysate replicates were prepared and measured independently.

RESULTS AND DISCUSSION

Concentration and Isotope Incorporation Efficiency of Stable Isotope-Labeled Standard CD4. With the nano-LC−MRM MS approach, we applied the stable isotope-labeled internal standard CD4 for quantification of the endogenous CD4 receptor protein from human CD4+ T cells. Therefore, the isotope incorporation and the concentration of the internal standard CD4 protein are key factors for accurate quantification of endogenous CD4 on T cells in the MRM MS-based quantification scheme and were carefully investigated in the present study using mass spectrometry.

We measured the isotope incorporation efficiency in the standard CD4 using MRM MS based on the intensity ratio of the natural isotope abundance peptide to the stable isotope-labeled peptide within the standard protein sample. The selected peptides and the isotope incorporation percentage are listed in Table 1. Each individual peptide was analyzed by at least three transitions, and the isotope incorporation percentage of each peptide was the average value of multiple transitions. The average isotope incorporation of the standard CD4 is 81.6 ± 0.7% based on four peptides per multiple-replicate experiment. The ratio of nonlabeled to labeled protein was calculated to be 0.23 and used for calculations of the endogenous CD4 density.

Table 1. Isotope Incorporation (%) in the Internal Standard CD4[a]

peptide	rep1	rep2	rep3	mean
SLWDQGNFPLIIK	80.65	81.30	81.30	81.08
ILGNQGSFLTK	81.97	80.65	81.30	81.30
SWITFDLK	82.64			82.64
ASSIVYK	81.30			81.30
mean				81.58
std dev				0.72
coefficient of variation				0.88
nonlabeled/labeled				0.23

[a]The isotope incorporation percentage in the standard CD4 was determined using four selected peptides. The individual peptide was analyzed on the basis of at least three transitions. Two of the four peptides were repeatedly tested in multiple experiments. The mean incorporation percentage of each peptide, the standard deviation, and the coefficient of variation are indicated in the table.

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By comparing the peak intensity ratio of the targeted peptides of the stable isotope-labeled standard CD4 and rCD4 with a known concentration, the concentration of the heavily labeled standard CD4 is calculated to be 0.22 ± 0.03 μmol/L according to eq 1 (Table 2). Six peptides and at least three transitions per peptide were employed to determine the concentration of the isotope-labeled standard protein. These 6 peptides contain 61 amino acids and cover 13.3% of the full-length CD4, ranging across the extracellular portion of the protein (Supporting Information, Table S1). This experiment was repeated three times. The mean value of all the peptides measured was taken as the concentration of the isotope-labeled internal standard CD4.

Table 2. Concentration (μmol/L) of Isotope-Labeled Internal Standard CD4[a]

peptide	av ± std dev
SLWDQGNFPLIIK	0.21 ± 0.04
ILGNQGSFLTK	0.23 ± 0.04
EGEQVEFSFPLAFTVEK	0.18 ± 0.03
SWITFDLK	0.24 ± 0.01
ATQLQK	0.23 ± 0.02
VTQDPK	0.25 ± 0.03
mean	0.22 ± 0.03

[a]The concentration of heavy isotope-labeled standard CD4 was averaged from multiple peptides, quantified by the MRM peak intensity ratio of the standard CD4 peptide and rCD4 peptides with a known concentration.

Quantification of Endogenous CD4 Receptor on the Surface of Human T Cells. The target peptides employed for CD4 quantification were selected on the basis of favorable transition intensities and minimum matrix effects from our empirical data. Representative ion chromatograms of selected transitions from the signature peptides are shown in Figure 2. Each peptide was evaluated using no less than three pairs of transitions. The comparable intensity ratios of the transition pairs from the different peptides indicated that the unique target CD4 protein was measured. The CD4 quantification in our study was performed with a total of three replications from different cell lysates for statistical purposes. The protein density per copy number on the surface of the CD4+ T cell was derived from the mean values of all selected signature peptides according to eq 2. The results of endogenous CD4 quantification are summarized in Table 3. On the basis of our data, the copy number of CD4 protein receptors on a human CD4+ T cell varies from 1.43×10^5 to 1.50×10^5 with a mean of 1.46×10^5. The results are larger than those obtained using the conventional flow-cytometry-based method (~(0.90–1.10) × 10^5 measured), which relies on the affinity binding between CD4 receptors and anti-CD4 antibodies.[12]

In normal resting human helper T cells, CD4 glycoproteins controlled by the encoding gene are exclusively distributed on the cell surface.[1,2] Down-modulated CD4 cell surface expression and subcellular localization[21] and depletion of the surface CD4 protein[22] have been reported in the literature in the case of HIV infection. For the present study, purified CD4+ helper T cells were obtained from a normal blood donor tested for blood-borne pathogens HIV-1 and -2, hepatitis B, hepatitis C, and HTLV-1. Hence, endoplasmic CD4 proteins are expected to be negligible. With the method described, we avoided using an antibody-based affinity assay as it is used in the conventional cytometry approach. Thus, the variations resulting from the antibody clone and binding specificity and fluorescent label specific issues do not interfere with our CD4 measurement. Moreover, the antibody-based approach only measures protein quantity through recognition of a single protein epitope. The association of CD4 receptor with lipid rafts[23] could affect the affinity binding by the anti-CD4 antibody, resulting in a lower detectable CD4 density. The quantification by the MRM MS approach was based on multiple unique peptides of CD4, providing more quantitative information on the full length of the protein. It would be of particular interest for CD4 analysis in cells in different biological conditions since various protein functions are usually associated with unknown cleavages and modifications. We did not detect any membrane-associated and cytoplasmic CD4 peptides in this study due to the limitation of the peptide length

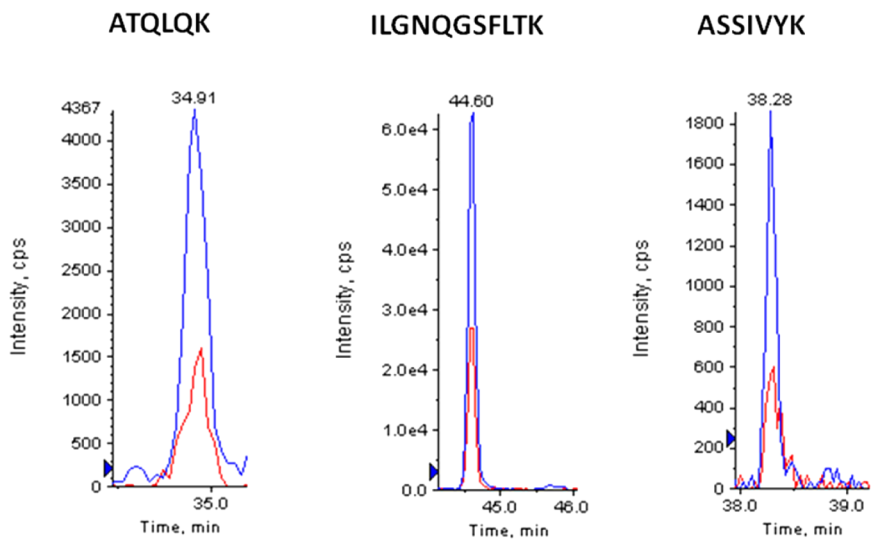

Figure 2. Representative extracted ion chromatograms of selected transitions from three signature peptides of human CD4 protein showing comparable heavy/light ratios and comparable quantification: red peaks, heavy isotope-labeled peptides; blue peaks, light (nonlabeled endogenous) peptides.

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Table 3. Copy Number of CD4 Receptors ($\times 10^5$) on a Human CD4+ T Cell[a]

	rep1 [15.0 million CD4+ T cells (CV < 6%)]	rep2 [10.1 million CD4+ T cells (CV < 6%)]	rep3 [10.1 million CD4+ T cells (CV < 6%)]	mean ± std dev
ILGNQGSFLTK	1.32 ± 0.05	1.43 ± 0.10	1.55 ± 0.16	
ASSIVYK	1.61 ± 0.10	1.13 ± 0.22	1.38 ± 0.44	
ATQLQK	1.54 ± 0.07	1.88 ± 0.17	1.34 ± 0.20	
mean ± std dev	1.49 ± 0.15	1.48 ± 0.38	1.42 ± 0.12	1.46 ± 0.03

[a]Quantification of CD4 on human CD4+ T cells in units of copy number per cell was based on three peptides and three independent sample preparations/measurements. The peptide level evaluated by multiple transitions was employed to determine the averaged protein CD4 level. Quantification of CD4 was replicated by a total of three sets of cell lysates. In each biological replicate, the number of CD4+ T cells was counted multiple times with variations of no more than 6%.

and detection sensitivity. Additional effort will be taken to resolve both the intracellular portion and membrane-associated portion of CD4 in a future study.

CONCLUSIONS

We reported the development of a nano-LC–MRM MS-based quantitative method to quantify the CD4 density on a human CD4+ T cell. The full-length stable isotope-labeled CD4 served as the internal standard for the quantification of the CD4 receptor density on a human CD4+ T cell based on the MRM transition intensity ratio of selected peptides. Application of isotope-labeled full-length proteins as internal standards overcomes potential quantitative errors from protein hydrolysis and variations associated with complex biological sample processing such as sample fractionation.

This MRM MS-based method is relatively simple to implement with less variation compared to other available approaches. The quantification method in our study showed great reproducibility with low standard deviations. The method can be applied for quantification of other cell marker proteins. It would be of great interest to examine the limit of detection of this method on proteomic biomarkers with diverse expression levels. As demonstrated in this study, MRM MS is a powerful tool for biomolecule quantification and can potentially assist the biomolecular analysis in clinical laboratories.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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