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Supporting Information

Ultrasensitive and simultaneous determination of RNA modified nucleotides by sheathless interfaced capillary electrophoresis-tandem mass spectrometry and its exploration on RNA modifications of HCT116 cells treated by nickel ion

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Experimental Section

Reagents

N^1-methyladenosine (m^1A) standard was purchased from Berry & Associates (Dexter, MI). N^6-methyladenosine (m^6A) standard was purchased from Macklin (Shanghai, China). Pseudouridine (ψ) standard was purchased from Toronto Research Chemicals (Ontario, Canada). Adenosine (A), cytidine (C) and 5-methylcytidine (m^5C) standards were purchased from J&K Scientific (Shanghai, China). 2'-O-methylguanosine (G_m) and 2'-O-methylcytidine (C_m) standards were purchased from Aladdin (Shanghai, China). 2'-O-methyladenosine (A_m), guanosine (G) and uridine (U) standards were purchased from Yuanye (Shanghai, China). 2'-O-methyluridine (U_m) standard was purchased from Meilunbio (Dalian, China). Acetic acid (CH_3COOH, 99.9985 %, metal basis) was purchased from Alfa Aesar (Ward Hill, MA, USA). Methanol was LC-MS Grade, purchased from J.T.Baker. EHNA hydrochloride (EHNA), deferoxamine mesylate salt powder (DFOM), Alkaline Phosphatase, Benzonase and Phosphodiesterase I were purchased from Sigma-Aldrich (Shanghai, China). Nickel sulfate ($NiSO_4$) and sodium chloride (NaCl) were purchased from Tongguang (Beijing, China). Magnesium chloride ($MgCl_2$) and hydrochloric acid (HCl) were purchased from Xilong Scientific (Guangdong, China). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Sinopharm Chemical Reagent (Shanghai, China). Formic acid (FA, LC-MS grade) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Ethylenediaminetetraacetic acid disodium salt (EDTA) was purchased from Xilong Scientific (Guangdong, China). Sodium dodecyl sulfate (SDS) was purchased from Bioruler (Beijing, China). DL-Dithiothreitol (DTT) was purchased from Genview (Beijing, China). Proteinase K was from Takara Biotechnology (Dalian, China). The water used throughout this work was ultrapure water purified using 0.45 μm MF-membrane filter (Merck Millpore, Germany).

CE-MS/MS

Sheathless CE-MS/MS experiments were carried out with a CESI 8000 plus capillary electrophoresis system from SCIEX coupled with a Triple Quad™ 5500 mass spectrometry (Brea, CA, USA) through the NanoSpray III source (Brea, CA, USA). This system was controlled by

Analyst software (SCIEX, CA, USA). Bare fused-silica capillaries (90 cm total length, 30 μm ID., 150 μm OD.) etched with a porous tip were obtained from SCIEX (Brea, CA, USA), which could be inserted into the nanospray interface. The capillary was flushed with methanol for 10 min at 100 psi, followed by water, 0.1 M sodium hydroxide, 0.1 M hydrochloric acid and water for 10 min each at 100 psi, and finally by the background electrolyte (BGE) of 10 % acetic acid (pH 2.2) for 10 min at 100 psi before first used. The BGE was also used as conductive liquid in the conductive liquid capillary. Before each run, the conductive liquid capillary was rinsed with BGE for 5 min at 100 psi. Samples for detection were stored at 4 °C in the CE system. Hydrodynamic injections were used in this study, and about 100 nL sample was injected into the separation system for each analysis. A voltage of +25 kV was applied during the separation and the current was between 3.0 to 3.2 μA. ESI was performed in positive ionization mode. The ionspray voltage was optimized to get the best nanospray stability and efficiency and +1750 eV was good enough for this study. The values for curtain gas, gas 1, gas 2, temperature, declustering potential, entrance potential and collision cell exit potential were 5, 0, 0, 0, 80, 10, 13, respectively. Detection was operated in the MRM mode for the quantitative analysis of nucleosides. Mass parameters for each analyte including precursor ion (Q1), product ion (Q3), quantitive ion and collision energy (CE) were summarized in Table S1.

Cell culture and Ni^{2+} treatment

Hela cell lines and HCT 116 cell lines were obtained from American Type Culture Collection (ATCC) cultured in DMEM medium (Sigma-Aldrich, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (Gbico,Waltham, MA USA) in a humidified atmosphere containing 5% CO_2 at 37 °C. For Ni^{2+} treatment, HCT 116 cells were treated with different concentrations of $NiSO_4$ including 0, 15, 40, 80, 160 and 640 μM for 48 h.

RNA extraction and enzymatic digestion

Total RNA was extracted using TransZol Up Plus RNA Kit (TransGen Biotech, Beijing, China) according to the manufacture's recommended procedure, and mRNA was extracted using GenElute™ mRNA Miniprep Kit (Sigma-Aldrich, Shanghai, China) according to the manufacture's recommended procedure. The concentrations of the purified RNA samples were determined by Nanodrop 2000 spectrophotometer from Thermo (Thermo, USA). Total RNA samples were diluted

to be 500 pg and mRNA samples were diluted to be 5 ng in 5 μL H_2O using ultrapure water. The enzymatic digestion protocol by digest mix for each biological sample was the same.

50 μL digest mix was composed by 0.1 μL Benzonase, 0.05 μL Alkaline Phosphatase, 5 μL Phosphodiesterase I (1 mU/μL), 0.13 μL 7.2 mg/mL EHNA, 0.16 μL 2.2 mM DFOM and 45 μL hydrolysis buffer (100 mM NaCl, 20 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.9). 3 μL digest mix was added into each biological sample (all in 5 μL H_2O) and the mixture was incubated at 37 °C for 2 hours. Then the mixture can be analyzed by CE-MS/MS system directly.

LC-MS/MS analysis of modified nucleosides in mRNA of Hela cells

Analysis of the nucleosides was performed on the LC-MS/MS system consisting of a Triple Quad™ 5500 mass spectrometer (Brea, CA, USA) with an ESI source (Turbo Ionspray) and a Thermo Scientific Dionex Ultimate 3000 HPLC (Thermo, USA). Data acquisition and processing were performed using AB SCIEX Analyst 1.5 Software (Applied Biosystems, Foster City, CA, USA). The HPLC separation was performed on a Zorbax Stablebond Analytical SB-C18 column (2.1 mm × 100 mm, 3.5 μm, Agilent Technologies, USA) at 35 °C. Water containing 0.0085% FA (v/v, solvent A) and MeOH containing 0.0085% FA (v/v, solvent B) were employed as the mobile phase. A gradient of 0-5% B for 6 min, 5-80% B for 0.5 min, 80% B for 5 min and 100% B for 5 min was used. The flow rate of the mobile phase was set at 0.3 mL min^{-1}. The mass spectrometry detection was performed under positive ESI mode. The nucleosides and labelled products were monitored using the multiple reaction monitoring (MRM) mode. The MRM parameters were shown in Table S1. The values for curtain gas, collision gas, ionspray voltage, ion source gas 1, ion source gas 2, interface heater temperature, declustering potential, entrance potential and collision cell exit potential were 20, 8, 5500, 55, 60, 600, 70, 10, 13, respectively. The mRNA samples were enzymatic digested using 5 μL digest mix at 37 °C for 2 h, and then add water to 80 μL. The digested mRNA was filtered by an ultrafiltration tube (10 kDa cutoff, Amicon, Millipore) to remove the enzymes, and the obtained solution was injected to LC-MS/MS system for analysis.

Method validation

To validate the sheathless CE-MS/MS method, the standard solutions of modified nucleosides standards / the corresponding normal nucleosides of varying molar ratio concentration were

prepared and analyzed using CE-MS/MS method described above. Each sample was analyzed at least three times. The quantification calibration curves of modified nucleosides were constructed by plotting the peak area ratios of modified nucleosides / the corresponding normal nucleosides (such as m^6A/A).

Determination of Hela cells mRNA sample

The resulting solutions of Hela cell mRNA sample were directly measured by CE-MS/MS. Each sample was analyzed for three times. The intra-day and inter-day precision of the CE-MS/MS method was investigated by quantification of mRNA sample per day for three consecutive days. The results were compared with data reported by others to validate the precision of our method.

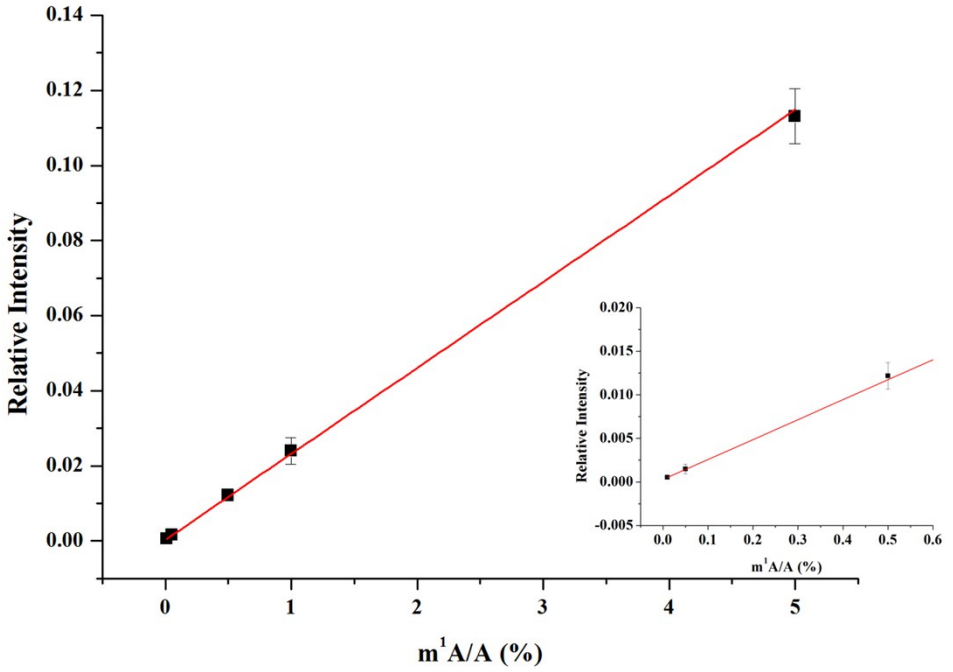
Determination of 100 Hela cells sample

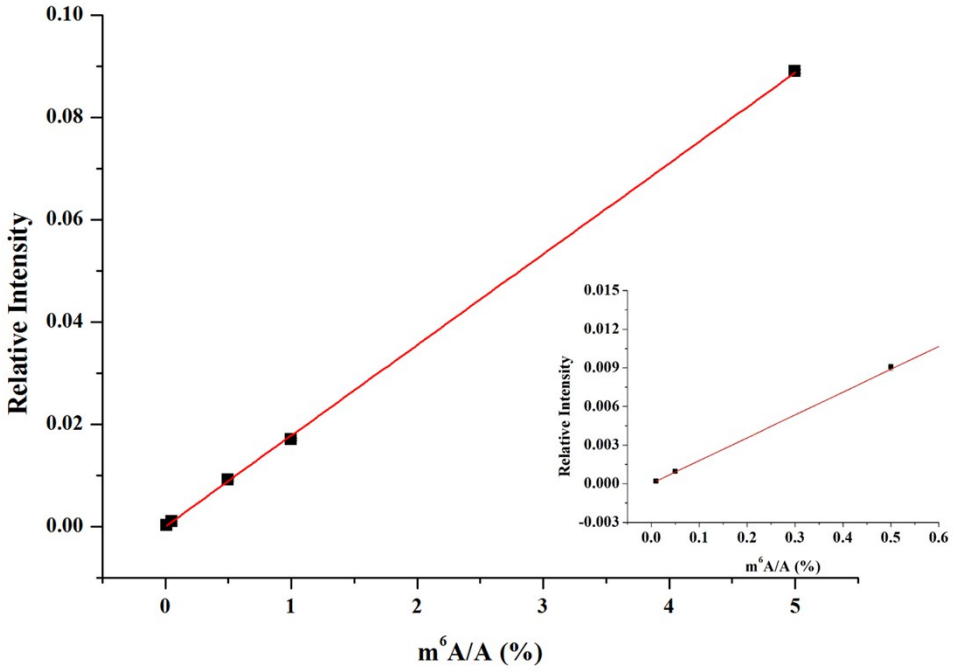
100 Hela cells were dissolved in 2 μL PBS buffer, and 2 μL lysis buffer was added to break the cells. The lysis buffer (pH 8.0) was composed by 10 mM Tris-HCl, 10 mM EDTA, 2% SDS and 80 mM DTT. Then 1 μL proteinase K was added followed with incubating at 56 °C for 1 h. The resulting sample was then incubated at 95 °C for 10 min to inactivate proteinase K. After adding 3 μL digest mix, the mixture (8 μL) was incubated at 37 °C for 2 h. The resulting solution was directly measured by CE-MS/MS.

Determination of RNA samples extracted from HCT 116 cells

The resulting solutions of RNA samples were directly measured by CE-MS/MS. Each sample was analyzed for three times.

Scheme S1 The schematic principle of RNA modified nucleotides determination by CE-MS/MS.


Relative Intensity
m1A/A (%)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
1
2
3
4
5
0.020
0.015
0.010
0.005
0.000
-0.005
0.0
0.1
0.2
0.3
0.4
0.5
0.6


Relative Intensity
m6A/A (%)
0.10
0.08
0.06
0.04
0.02
0.00
0
1
2
3
4
5
0.015
0.012
0.009
0.006
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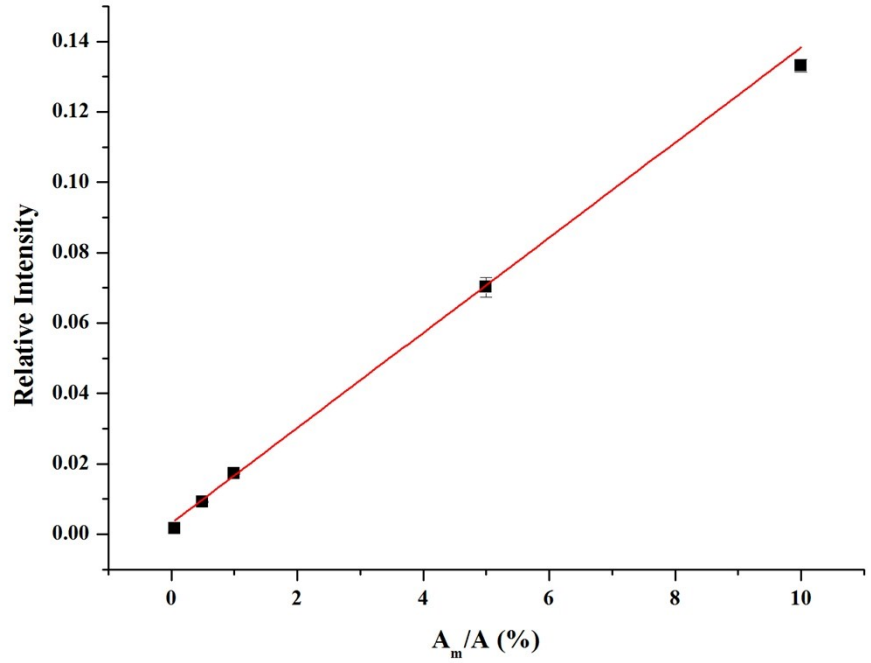
0.2
0.3
0.4
0.5
0.6


Relative Intensity
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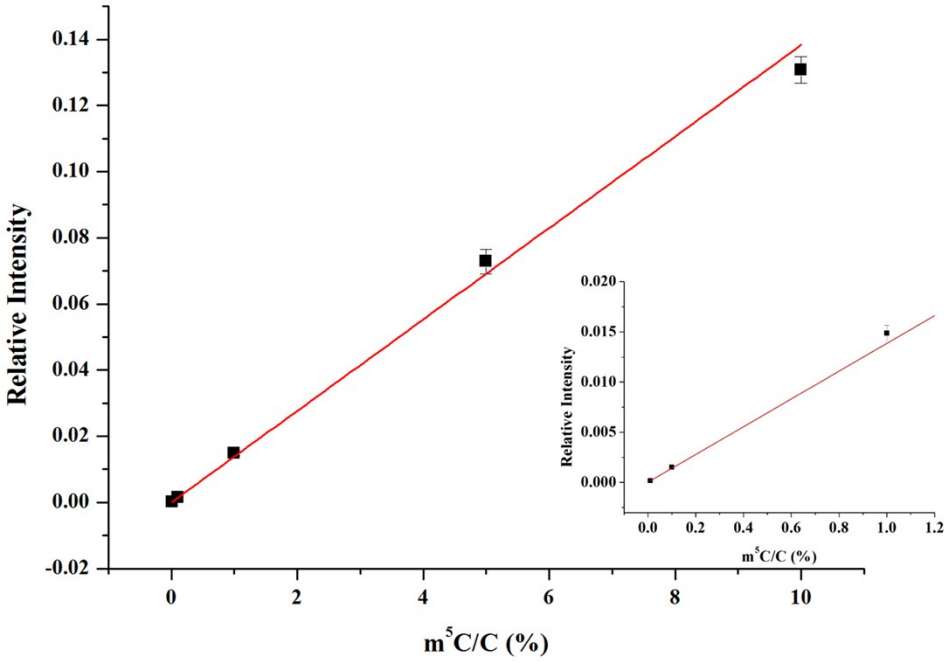
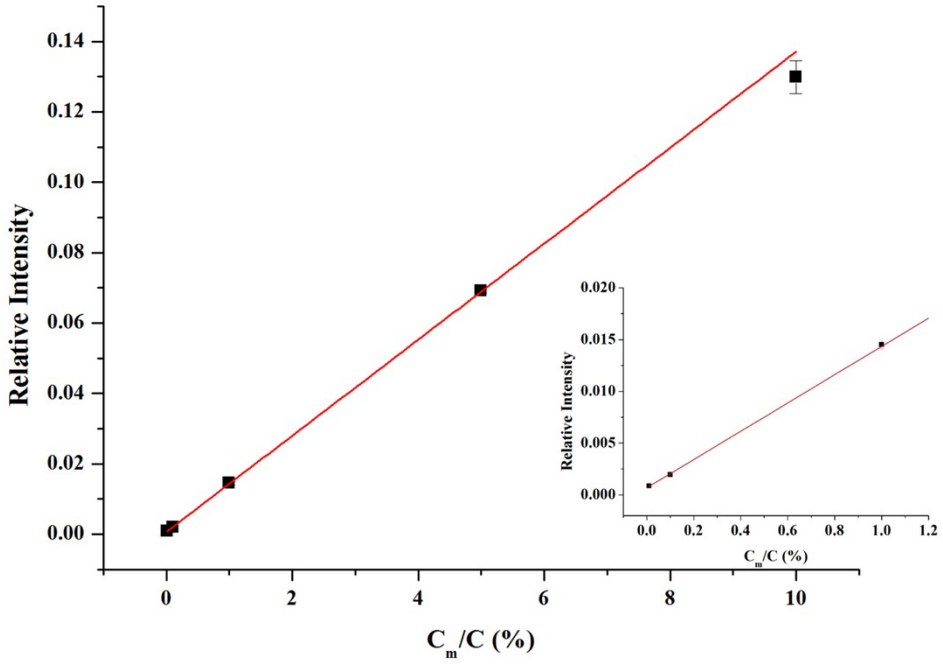
0.10
0.08
0.06
0.04
0.02
0.00
0
2
4
6
8
10
A_m/A (%)


Relative Intensity
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
0
2
4
6
8
10
m5C/C (%)
Relative Intensity
0.020
0.015
0.010
0.005
0.000
0.0
0.2
0.4
0.6
0.8
1.0
1.2
m5C/C (%)


Relative Intensity
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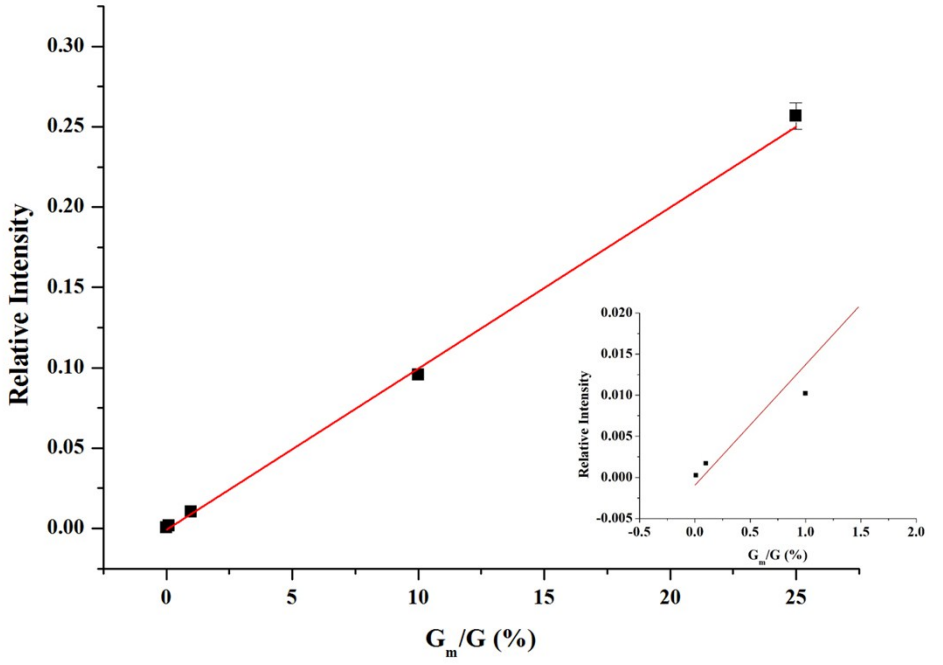
0.10
0.08
0.06
0.04
0.02
0.00
0
2
4
6
8
10
C_m/C (%)
Relative Intensity
0.020
0.015
0.010
0.005
0.000
0.0
0.2
0.4
0.6
0.8
1.0
1.2
C_m/C (%)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0.10
0.05
0.00
0
5
10
15
20
25
G_m/G (%)
Relative Intensity
0.020
0.015
0.010
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-0.5
0.0
0.5
1.0
1.5
2.0
G_m/G (%)

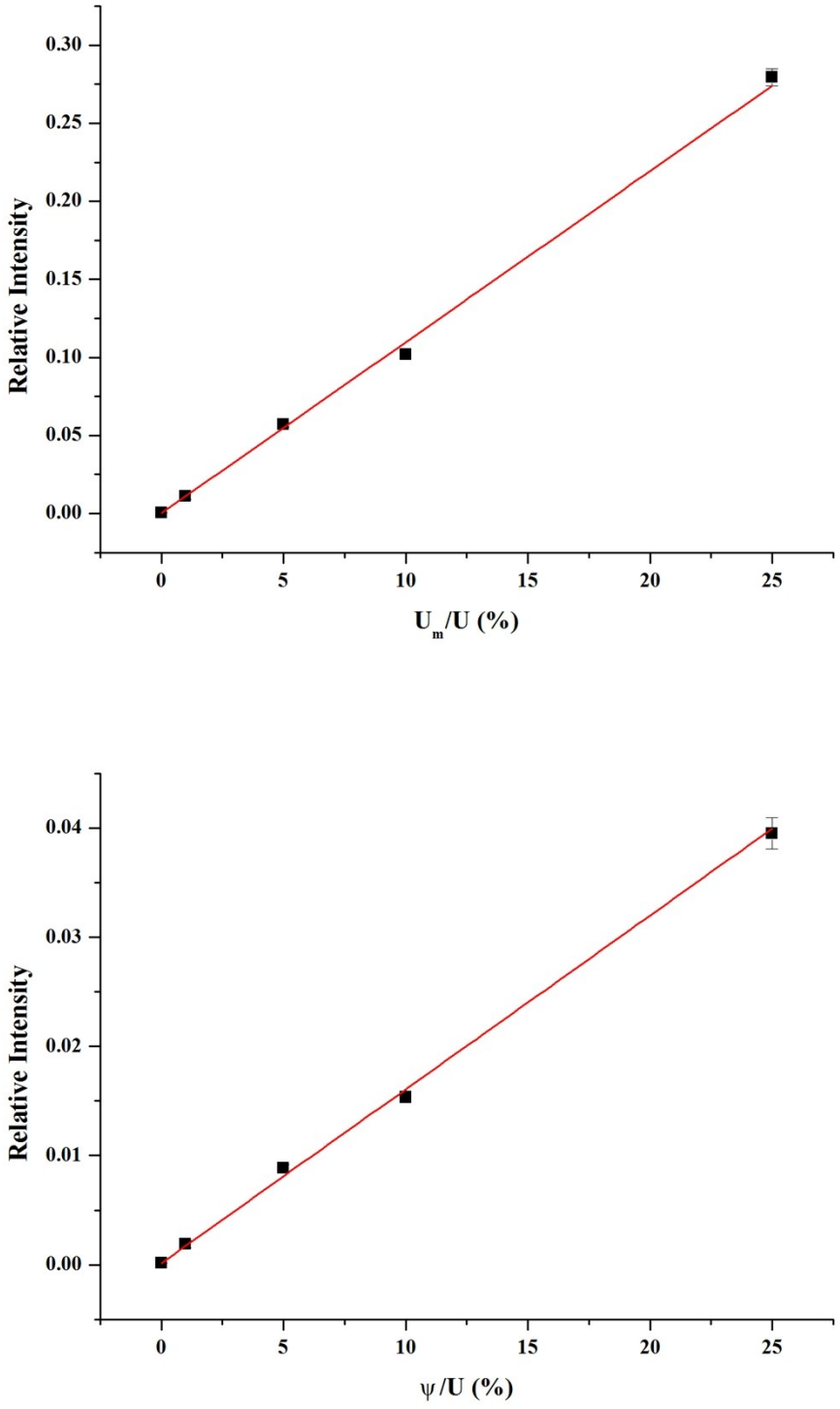

Fig. S1 Calibration curves of eight modified nucleosides. Error bars represent SD of three independent analysis. RSD < 10%.

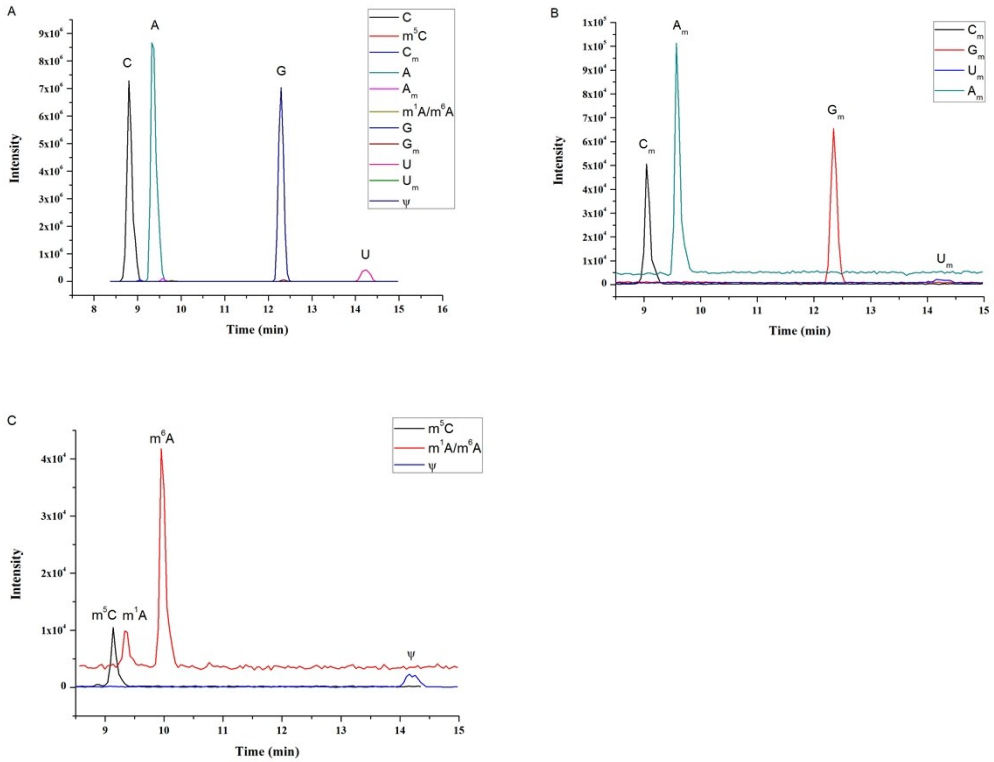

Fig. S2 (A) CE-MS/MS chromatograms of twelve nucleosides for Hela cell mRNA sample. (B) CE-MS/MS chromatograms of C_m, G_m, A_m and U_m. (C) CE-MS/MS chromatograms of m^5C, m^1A, m^6A and ψ.

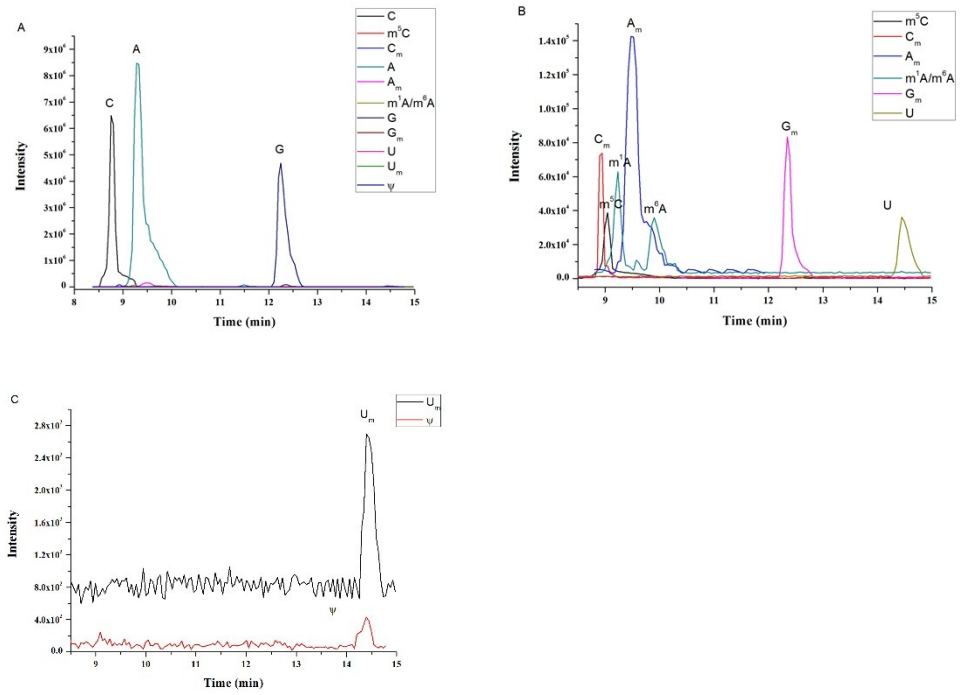

Fig. S3 (A) CE-MS/MS chromatograms of twelve nucleosides in 100 Hela cells. (B) CE-MS/MS chromatograms of m^5C, m^1A, m^6A, C_m, G_m, A_m and U. (C) CE-MS/MS chromatograms of U_m and ψ.

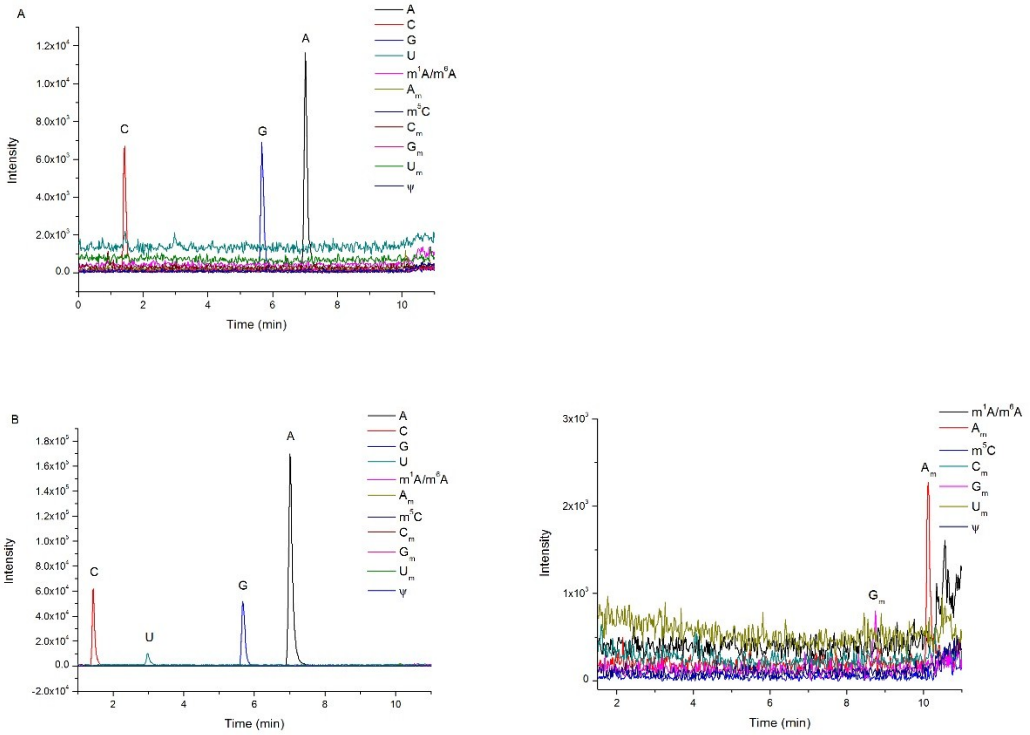

Fig. S4 MRM chromatograms of (A) 500 pg total RNA and (B) 5 ng mRNA using LC-MS/MS. Only A, C and G can be detected in 500 pg total RNA and A, C, G, U, A_m and G_m can be detected in 5 ng mRNA.

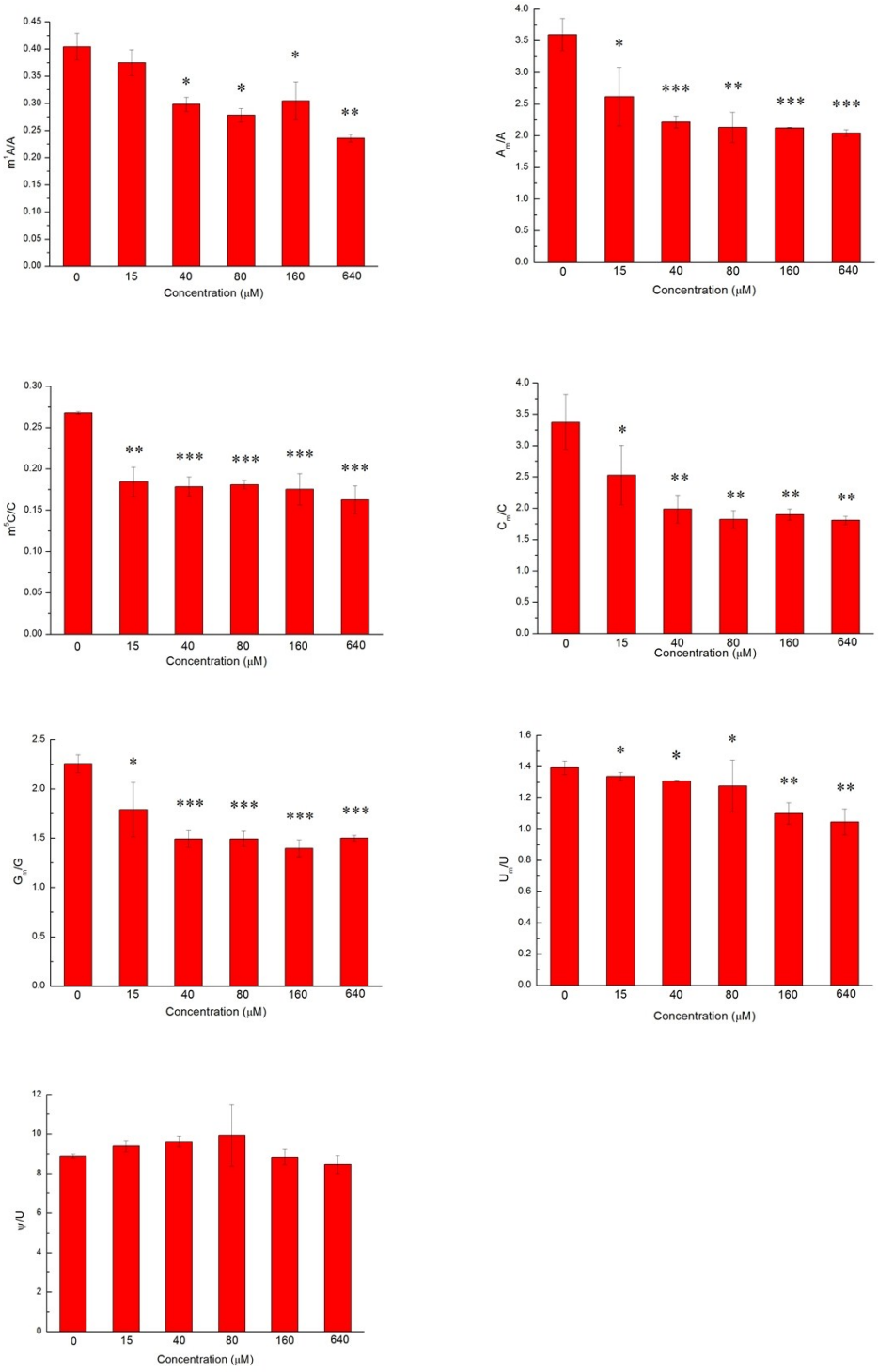

Fig. S5 The trends of modified nucleotides in total RNA with the increases of nickel concentration. The error bars represent SD of three independent analysis. *P < 0.1; **p < 0.05; ***p < 0.01.

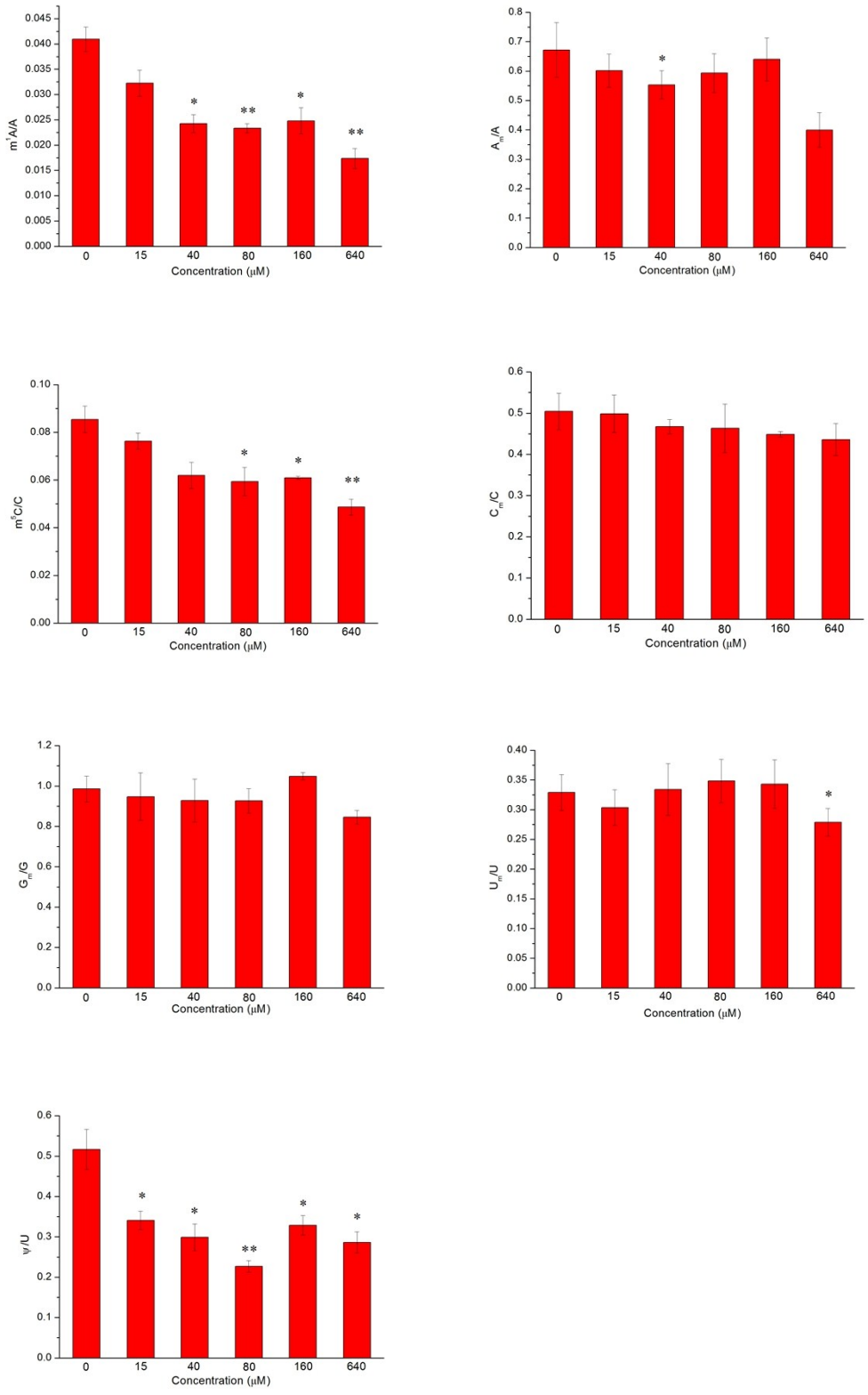

Fig. S6 The trends of modified nucleotides in mRNA with the increases of nickel concentration. The error bars represent SD of three independent analysis. *P < 0.1; **p < 0.05; ***p < 0.01.

Table S1 Optimized MS parameters and the retention times of the target analytes.

Analyte	Q1	Q3	CE (eV)	Retention time (min)
C	244.1	112.1	24.00	8.80
C_m	258.0	112.0	20.00	8.99
m^5C	258.0	126.1	27.16	9.09
m^1A	282.1	150.1	26.00	9.23
A	268.2	136.1	26.00	9.38
A_m	282.0	136.1	23.00	9.57
m^6A	282.0	150.1	24.00	9.71
G	284.1	152.2	23.33	12.39
G_m	298.1	152.1	21.00	12.58
ψ	245.1	179.1	14.72	14.64
U	245.1	113.0	19.00	14.73
U_m	258.9	113.1	20.00	14.73

Table S2 Comparison of the LODs of m^6A and m^5C obtained in different analytical methods

Analytical method	LODs	
	m^6A	m^5C
LC-MS/MS	4.0 fmol[1]	4.2 fmol[2]
HPLC-MS/MS with derivatization		60 amol[2]
CE-MS/MS (this work)	5 amol	2.5 amol

Table S3 The comparison of contents of modified nucleosides in Hela cells obtained by CE-MS/MS and LC-MS/MS.

	CE-MS/MS	LC-MS/MS
m^1A/A	0.053 ± 0.003	0.052 ± 0.003
m^6A/A	0.544 ± 0.029	0.564 ± 0.013
A_m/A	0.897 ± 0.071	0.867 ± 0.046
U_m/U	0.320 ± 0.014	0.306 ± 0.017
G_m/G	0.893 ± 0.055	0.805 ± 0.040
C_m/C	0.509 ± 0.033	0.472 ± 0.026
m^5C/C	0.080 ± 0.003	0.072 ± 0.004
ψ/U	0.243 ± 0.006	0.222 ± 0.010

Table S4 Intra-day RSD and inter-day RSD of modified nucleosides by CE-MS/MS

	Intra-day RSD	Inter-day RSD
m^1A/A	5.9	11.3
m^6A/A	5.4	6.1
A_m/A	8.0	6.9
U_m/U	4.5	11.1
G_m/G	6.2	8.0
C_m/C	6.5	2.7
m^5C/C	4.4	6.9
ψ/U	2.3	2.2

1. B. F. Yuan, *Methods Mol Biol*, 2017, **1562**, 33-42.
2. W. Huang, M. D. Lan, C. B. Qi, S. J. Zheng, S. Z. Wei, B. F. Yuan and Y. Q. Feng, *Chem Sci*, 2016, **7**, 5495-5502.