



Nanokit coupled electrospray ionization mass spectrometry for analysis of angiotensin converting enzyme 2 activity in single living cell



Xinqi Chang^a, Nina Wang^a, Depeng Jiang^{b,*}, Hong-Yuan Chen^a, Dechen Jiang^{a,*}

^a State Key Laboratory of Analytical Chemistry for Life Science, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, China

^b Department of Respiratory Medicine, The Second Affiliated Hospital, Chongqing Medical University, Chongqing 400010, China

ARTICLE INFO

Article history:

Received 12 January 2022

Revised 20 April 2022

Accepted 12 May 2022

Available online 16 May 2022

Keywords:

Nanokit

Electrospray ionization mass spectrometry

Single cell analysis

The enzymatic activity

ACE2

ABSTRACT

Angiotensin-converting enzyme 2 (ACE2) is not only an enzyme but also a functional receptor on cell membrane for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Here, the activity of ACE2 in single living cell is firstly determined using a nanokit coupled electrospray ionization mass spectrometry (nanokit-ESI-MS). Upon the insertion of a micro-capillary into the living hACE2-CHO cell and the electrochemical sorting of the cytosol, the target ACE2 enzyme hydrolyses angiotensin II inside the capillary to generate angiotensin 1–7. After the electrospray of the mixture at the tip of the capillary, the product is differentiated from the substrate in molecular weight to achieve the detection of ACE2 activity in single cells. The further measurement illustrates that the inflammatory state of cells does not lead to the significant change of ACE2 catalytic activity, which elucidates the relationship between intracellular ACE2 activity and inflammation at single cell level. The established strategy will provide a specific analytical method for further studying the role of ACE2 in the process of virus infection, and extend the application of nanokit based single cell analysis.

© 2023 Published by Elsevier B.V. on behalf of Chinese Chemical Society and Institute of Materia Medica, Chinese Academy of Medical Sciences.

Angiotensin-converting enzyme-2 (ACE2) is a type I integral membrane protein that functions as a carboxypeptidase to hydrolyse angiotensin II into angiotensin. It is known that ACE2 plays an important role in hypertension, cardiac function, heart function and diabetes. More specially, the epidemic of Coronavirus disease 2019 (COVID-19) became a global pandemic in the past two years, which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Current studies have shown that ACE2 is a specific receptor of SARS-CoV-2, mediating the entry of virus into host cells [1,2]. Accordingly, the complete investigation of ACE2, including its catalytic activity, is important for the fully understanding of its physiological role. Many methods have been developed for the determination of ACE2 activity from a population of cells [3,4], however, the measurement at single cell level is still challenging. Considering the importance of the cellular heterogeneity in the knowledge of ACE2 role [5–9], the establishment of a robust strategy to measure its activity in single living cell is needed.

Currently, probe based optical method is popular for the analysis of the enzymatic activity in single living cell [10–17]. Although

this strategy is robust, the design and synthesis of the specific probe to recognize the enzyme is complex and time-consuming. Previously, our group reported nanokit based strategy for the measurement of the enzymatic activity in single living cells [18,19]. In this strategy, a nanopipette carrying the kit components was inserted into a living cell. The components was electrochemically pumped into the cell, which reacted with the target enzyme to generate the product and byproduct of hydrogen peroxide. By the electrochemical detection of hydrogen peroxide using the ring electrode at the orifice of the nanopipette, the activity of the target enzyme in one cell was determined. This method can be adapted for the measurement of many enzymes by choosing the proper kits, however, the byproduct of hydrogen peroxide is needed that restricts the application of this nanokit based analysis.

Single-cell mass spectroscopic analysis is the emerging technology with its advantages of non-label measurement, high sensitivity and selectivity [20]. Many ionization modes, including electrospray ionization (ESI), secondary ion ionization, matrix-assisted laser desorption ionization and inductively coupled plasma ionization, have been developed to record the molecular profiles at single cells [21–25]. Among them, ESI is a soft ionization method that utilizes a microcapillary to sort the cytosol from the living cell. This mode permits the detection of various intracellular compo-

* Corresponding authors.

E-mail addresses: gdp116@163.com (D. Jiang), dechenjiang@nju.edu.cn (D. Jiang).

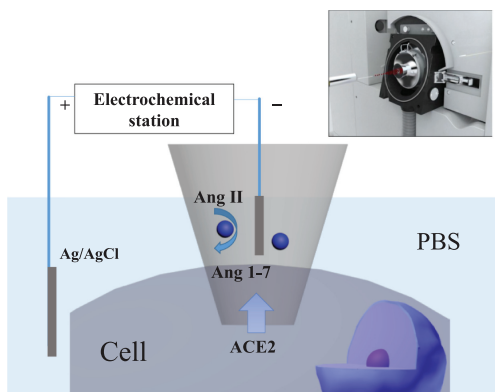


Fig. 1. The schematic nanokit coupled electro spray ionization mass spectrometry for the analysis of ACE2 activity in single living cells. The electrochemical sorting of the cytosol with the target ACE2 into the capillary followed by the reaction with the substrate. Upper right: The electro spray ionization mass spectroscopic analysis of the enzymatic activity.

nents, and thus, is widely applied in single-cell metabolite analysis. Considering its feature in the analysis of metabolites, this method should be suitable for the measurement of enzymatic products after the loading of substrates into the cell. Therefore, the proposed ESI based MS analysis will not rely on the detection on hydrogen peroxide, which could greatly expand the applications of our single cell nanokit analysis.

Here, nanokit coupled electro spray ionization mass spectrometry (nanokit-ESI-MS) is developed to measure the ACE2 activity in single living cell, utilizing the mass differentiation between the substrate and product. As illustrated in Fig. 1, angiotensin II (Ang II) is a polypeptide sequence (M.W. 1046.18) containing eight amino acids (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), which is filled into the microcapillary as the substrate of ACE2. After the capillary is inserted into one living hACE2-CHO cell with over-expressed ACE2 enzyme, the cytosol containing ACE2 enzyme is electrochemically sorted into the micro-capillary. Due to the extremely slow evaporation of liquid from the micro-sized orifice, the mixture of Ang II and ACE2 enzyme could be retained at the tip of capillary permitting the reaction for several hours. This catalytic process removes Phe from Ang II to generate Ang 1-7 with molecular weight of 899.00. Upon the application of a high voltage inside the capillary to initial the electro spray, the mass spectrum is recorded. Based on the difference of molecular weight, Ang 1-7 and Ang II are distinguished in the spectrum. Eventually, the peak intensities from both of them are used to calculate the activity of ACE2. The fluctuation of ACE2 activity after the cell inflammation is also investigated to elucidate the role of ACE2 during the inflammation.

Angiotensin converting enzyme 2 (ACE2), angiotensin II (Ang II), angiotensin 1-7 (Ang 1-7) were purchased from MCE (MedChem-Express) Company (Shanghai, China). hACE2-CHO cells were purchased from Antaiji Biotechnology Co (Beijing, China). Transforming growth factor β 3 (TGF β 3) and interleukin-1 β (IL1 β) were purchased from Novoprotein Co. (CA, USA). All other reagents were purchased from Sigma-Aldrich unless otherwise noted. Borosilicate glass tube (BF100-58-10) used in the experiment was purchased from Sutter Instrument (CA, USA). The ultra-pure water was obtained from the Milli-Q purification system, and the resistivity was ≥ 18.25 M Ω cm. hACE2-CHO cells were cultured in DMEM/high glucose medium supplemented with 15% FBS and 1% antibiotics (penicillin/streptomycin). The cells were cultured in a cell incubator containing 5% CO₂ at 37 °C in a humid atmosphere.

The nanospray mass spectrometry device consists of a drawn micro-capillary, a metal wire and a mass spectrometer (Agilent G6530B Q-TOF, USA). The positive ion mode is used with a drying temperature of 300 °C, the drying gas flow rate of 2.0 L/min,

the atomizer pressure of 0 Psig. The voltage applied at the voltage is 4000 V. The micro-manipulation is performed using a TransferMan®4r micromanipulator (Eppendorf, Germany) using a microscope model Olympus X73 (Olympus, Japan). Single-cell electrochemical tests are recorded using a MultiClamp700B patch clamp amplifier and an Axon Digidata 1550 data acquisition system (Molecular Devices, USA) with an Ag/AgCl electrode as a quasi-reference electrode.

All the glass micro-capillary used in the experiment are drawn from a borosilicate glass tube (BF100-58-10; o.d. 1.00 mm, i.d. 0.58 mm) using with P-2000 laser puller (Sutter Instrument, CA, USA). The opening of the micro-capillary is characterized to be 2-3 μ m. To get good reproducibility, the dimension (including the opening size and inclination degree of the glass wall) of the capillary should be very close. Since the shape of the capillary controls the ionic current through its opening, only the capillaries with the similar ionic current are selected for the following experiments.

For the reaction of Ang II with the standard ACE2 enzyme, 100 μ g/mL Ang II is mixed with 600 ng/mL ACE2 in presence of 100 μ mol/L ZnCl₂, and reacted at 37 °C for a certain time. The aliquots are taken and filled into the micro-capillary for nanospray. To react with the enzyme from the cytosol, 10⁵ hACE2-CHO cells are lysed in pure water to get the cellular lysate. Then, 100 μ g/mL Ang II is reacted with the lysate in presence of 100 μ mol/L ZnCl₂ for 1 h before the nanospray.

In single cell analysis, a mixture of 100 μ g/mL Ang II and 100 μ mol/L ZnCl₂ is loaded into the capillary. Then, with the help of micromanipulation system, the tip of the capillary is positioned into a living hACE2-CHO cell. A voltage of -1 V is applied at Ag/AgCl wire inside the capillary to electrochemically sort the cytosol for 3 min. Afterward, the capillary is placed at 37 °C for 3 h to permit the reaction between Ang II and ACE2 enzyme. Finally, the capillary is positioned in front of the inlet of the mass spectrometry to initial the nanospray.

The mass spectrum of the standard Ang II and Ang 1-7 are measured by the filling of the chemicals into the microcapillary (i.d. 2-3 μ m) following ESI-MS. The peaks at the mass to charge ratio of 1046.54 and 523.77 are associated with Ang II (Fig. 2A), and 899.47, 450.24 and 300.50 are associated with Ang 1-7 (Fig. 2B). The peak intensities of Ang II and Ang 1-7 with varied concentrations are listed in Fig. S1 (Supporting information), which exhibits a slightly stronger ionization efficiency of Ang 1-7 than that of Ang II. To verify the analysis of enzymatic activity, Ang II is reacted with the pure ACE2 enzyme in the buffer at 37 °C. Then, the mixtures at different reaction time are filled into the capillary for MS analysis, respectively. At the time of 0.5 min, only the peaks corresponding to Ang II are observed exhibiting the substrate in the mixture only. After the reaction for 10 min, the peaks associated with Ang II (1046.54 and 523.77) and Ang 1-7 (450.24 and 300.50) appear illustrating the partial conversion of Ang II into Ang 1-7 (Fig. 3A). With the extended reaction time, the peaks associated with Ang II decrease gradually, while the peaks associated with Ang 1-7 increase. The removal of ACE2 enzyme from the solution results in the observation of the peaks of Ang II only after the incubation for 2 h (Fig. S2 in Supporting information). These results exhibit the continuous conversion of Ang II by ACE2 enzyme.

To quantify the conversion rate, the peak intensities from Ang II and Ang 1-7 at the varied reaction time are measured. The conversion rate is quantified using the peak intensities from Ang II and Ang 1-7 (Fig. 3B). The near-linear increase in the conversion ratio is observed, which exhibits the continuous reaction between ACE2 and Ang II. After the reaction for 3 h, approx. 40% of Ang II is converted into Ang 1-7. Since the initial concentration of Ang II is 100 μ g/mL and the volume of the mixture inside the capillary is 10 μ L, the catalytic activity of ACE2 is calculated to be 1.765 μ U.

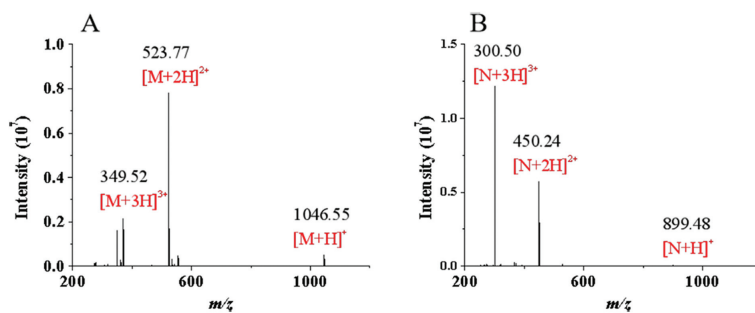


Fig. 2. The standard mass spectra of (A) Ang II and (B) Ang 1–7 using electrospray ionization. The concentrations for both of them are 100 $\mu\text{g/mL}$.

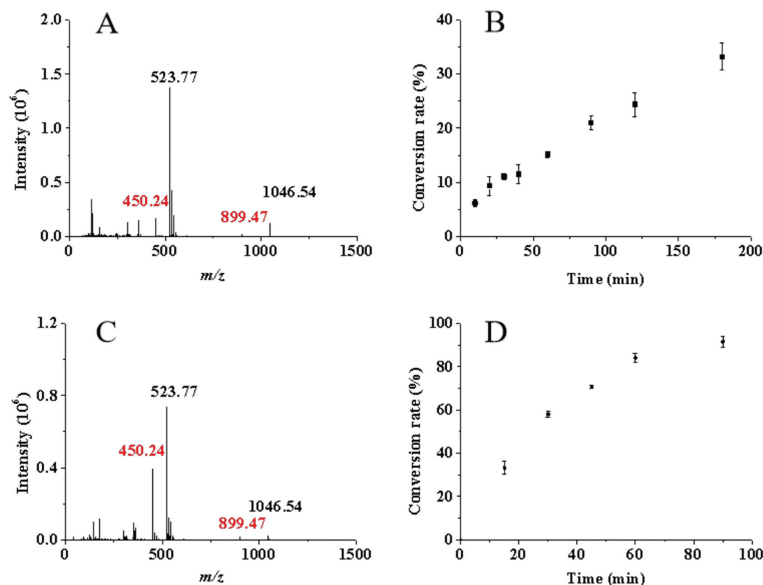


Fig. 3. (A) The typical mass spectrum of the mixture after the reaction of Ang II (100 $\mu\text{g/mL}$) with the standard ACE 2 enzyme for 15 min. (B) The conversion ratio of Ang II in presence of ACE2 enzyme. The error bar presents the standard deviation from three independent measurements. (C) The typical mass spectrum of the mixture after the reaction of Ang II (100 $\mu\text{g/mL}$) with the cellular lysate for 15 min. (D) The conversion ratio of Ang II in presence of the cellular lysate for 90 min.

For the investigation of ACE2 activity in living cells, hACE2-CHO cells with a high expression of ACE2 enzyme is used as the model [26]. The cell lysate is obtained, and then, mixed with Ang II. Fig. 3C illustrates the typical mass spectrum after 15 min of reaction, exhibiting the peaks associated with Ang II and Ang 1–7. The peak intensity of Ang II is as high as 10^6 so that the peaks from many other intracellular molecules are invisible. The observation of Ang 1–7 from the mixture with the cell lysates confirms that ACE2 in these cells could convert Ang II effectively. The relationship between the conversion ratio and the reaction time (Fig. 3D) displays that over 80% Ang II is converted by the enzyme in 1 h. The enzymatic activity from the cellular lysate is calculated to be 1.06 mU, which is much higher than that from the commercial ACE2 enzymes. As comparison, A549 cells that do not express ACE2 enzymes are used for the control experiment (Fig. S3 in Supporting information). Only 3.14% Ang II is converted into Ang 1–7 after 3 h, which exhibits an obviously low enzymatic activity in A549 cells. Both of the experimental results supports the detection of the enzymatic activity from ACE2 expressed cells.

After validating the existence of ACE2 in the cells, the nanokit coupled ESI-MS is applied for the analysis of ACE2 activity in single living cells. With the help of the micro-manipulation system, the tip of the capillary is inserted into one cell (Fig. 4A). The cytosol in the cell is sorted into the capillary using the electrochemical pump following our previously reported protocol [18,19]. It is noted that ACE2 basically is produced inside the cells and then goes to the

plasma membrane, however, part of it is still present inside the cell [27]. Therefore, this process can sort some ACE2 inside the capillary. Moreover, during the penetration of the capillary into the cell, a small piece of cell membrane containing ACE2 is loaded into the capillary, as well. Both of these parts will hydrolyse angiotensin II inside the capillary to generate angiotensin 1–7. Considering that the sorted amount of ACE2 from single cell is less than that from the cell lysate, the reaction time is extended to 3 h to maximize the conversion of Ang II. In this period, no obvious liquid loss is observed from the capillary exhibiting the same reaction volume. The slow volatilization of buffer from the orifice of capillary guarantees the reaction between the sorted enzyme from single cell and the substrate inside the capillary. Then, the mass spectrum is collected (Fig. 4B) that exhibits the peak of (450.24 and 899.47) from Ang 1–7. Since Ang 1–7 is not the cellular content, the observation of Ang 1–7 from the mixture inside the capillary provides the direct evidence about the reaction between ACE2 and Ang II. The result exhibits that the loaded enzyme from single cell can react the pre-filled substrate to complete the nanokit reaction. The same procedure is applied at single living A549 cells and no peak associated with Ang 1–7 is observed (Fig. S4 in Supporting information). This result illustrates the absence of the enzymatic reaction inside the cells without highly expressed ACE2.

Different from the partial conversion of Ang II by the enzyme in cell lysate, the loaded enzyme from single cell is insufficient and could convert a small amount of substrate. As a result, the peak in-

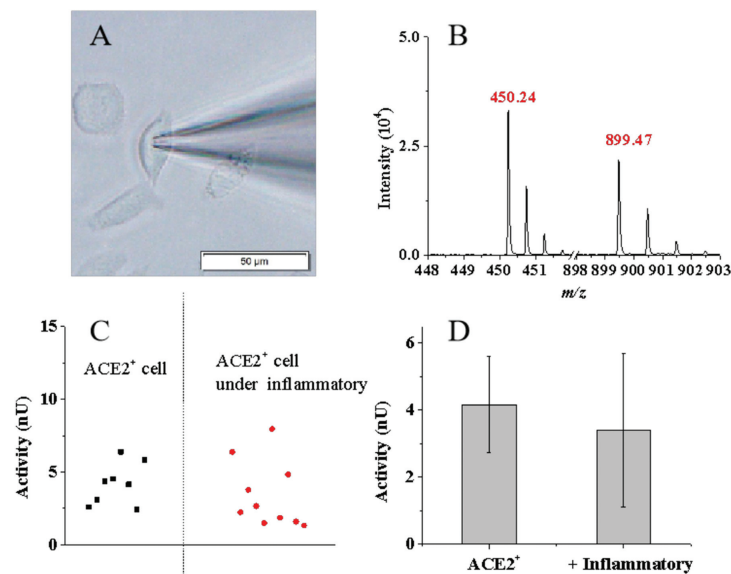


Fig. 4. (A) The bright field image to exhibit the penetration of the microcapillary into one living hACE2-CHO cell for the electrochemical loading of cytosol. The scale bar is 50 μm . (B) The mass spectrum collected from the reaction mixture inside the capillary exhibiting the peaks of Ang 1–7. (C) The enzymatic activity from individual hACE2 (ACE2⁺) cells and the cells during the inflammatory. Each spot presents the result from one cell. (D) The statistic enzymatic activity from single cells. The error bar presents the standard deviation from single cell analysis.

tensity of Ang II is almost the same, which is not shown in the spectrum. To calculate the enzymatic activity in single cell, the generated amount of Ang 1–7 is determined based on the calibration curve of the peak intensity from the standard (Fig. S1 in Supporting information). Fig. 4C lists the ACE2 activities in 8 cells. The average enzymatic activity from single cell is determined to be 4.2 ± 1.4 nU. The relative standard deviation is calculated to be 33.3%, which is obviously larger than that from the reaction with the enzyme in cellular lysate (2.5% from Fig. 3D). The observation of a large deviation from single cell analysis provides a clear evidence about the cellular heterogeneity in the ACE2 activity. As compared with our previous electrochemical strategy [18,19], this nanokit coupled ESI-MS method does not require any electrochemical byproduct, and can be applied on almost all the enzymatic reaction to determine its activity.

Coronavirus can specifically recognize and bind ACE2 at the cellular membrane, and then, enter the interior of cells to trigger inflammatory response [28]. In order to investigate the alteration of ACE2 activity under the inflammatory, hACE2-CHO cells are treated with inflammatory factors (tgfb3 and IL1B) for 24 h [29]. After that, the nanokit coupled ESI-MS method is carried out to measure the ACE2 catalytic activity at single cells. The activities from 10 cells are listed in Fig. 4C, as well. The statistical analysis of the activities with and without inflammatory (Fig. 4D) does not exhibit significant difference. This result provides a clue that the inflammatory state might not affect the intracellular ACE2 catalytic activity. The previous proteome study has revealed that ACE2 expression in the COVID-19 patients does not change [30]. Therefore, it might only function as the carrier for the SARS-CoV-2. In our study, the information about the independence between its activity and the cellular inflammatory will provide more information for the understanding of this enzymes in the disease.

In conclusion, the nanokit coupled ESI-MS method is firstly developed to determine the enzymatic activity in single cell. The loading of the cytosol, the reaction of the target enzymes and the following ESI-MS are integrated into one capillary, which is suitable for single cell analysis. Since this strategy relies on the mass difference between the substrate and the product, it can be applied on the analysis of various enzymes without any restriction.

More importantly, this method could be easily developed for the measurement of multiple enzyme activity in one cell by the incorporation of all these kits into one capillary. More detail characterization of this method is being conducted in the laboratory so that the quantitative measurement is feasible at single cells.

Declaration of competing interest

The authors report no declarations of interest.

Acknowledgments

This work was supported by Ministry of Science and Technology of China (No. 2017YFA0700500), the National Natural Science Foundation of China (Nos. 22025403 and 21974060), Kuanren Talents Program of the Second Affiliated Hospital of Chongqing Medical University, and Young and Middle-aged Senior Medical Talents Studio of Chongqing.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ccl.2022.05.036.

References

- [1] P. Zhou, X.W. Yang, et al., *Nature* 579 (2020) 270–273.
- [2] X. Yang, Y. Yu, J. Xu, et al., *Lancet Respir. Med.* 8 (2020) 475–481.
- [3] J. Wysocki, M.H. Ye, M.J. Soler, et al., *Diabetes* 55 (2006) 2132–2139.
- [4] F.X. Xiao, K.D. Burns, *Methods Mol. Biol.* 1527 (2017) 101–115.
- [5] Y.Y. Wu, L. Zhao, Y.R. Chang, et al., *Chin. Chem. Lett.* 32 (2021) 3446–3449.
- [6] M. Donoghue, F. Hsieh, E. Baronas, et al., *Circ. Res.* 87 (2000) E1–E9.
- [7] R. Santos, W. Sampaio, A. Alzamora, et al., *Physiol. Rev.* 98 (2018) 505–553.
- [8] R. Hu, S.L. Li, H.T. Bai, et al., *Chin. Chem. Lett.* 27 (2016) 545–549.
- [9] R. Kumar, C.M. Thomas, Q.C. Yong, et al., *Clin. Sci.* 123 (2012) 273–284.
- [10] N. Baumgarth, M. Roederer, *J. Immunol. Methods* 243 (2000) 77–97.
- [11] Q.L. Li, P.L. Chen, Y.Y. Fan, et al., *Anal. Chem.* 88 (2016) 8610–8616.
- [12] M. Mondal, R.J. Liao, L. Xiao, et al., *Angew. Chem. Int. Ed.* 56 (2017) 2636–2639.
- [13] Q. Smith, K.L. Wright, L. Ashton, *Analyst* 141 (2016) 3590–3600.
- [14] X.C. Li, S. Majidi, J. Duneval, et al., *Angew. Chem. Int. Ed.* 54 (2015) 11978–11982.
- [15] A.L. Wang, A. Ghaderi, H. Zhou, et al., *Nat. Biotechnol.* 32 (2014) 473–478.
- [16] X.K. Chen, X.D. Zhang, F.G. Wu, *Chin. Chem. Lett.* 32 (2021) 3048–3052.
- [17] D. Cheng, Y. Pan, B.C. Yin, *Chin. Chem. Lett.* 28 (2017) 1987–1990.

- [18] R.R. Pan, M.C. Xu, D.C. Jiang, et al., Proc. Natl. Acad. Sci. U. S. A. 113 (2016) 11436–11440.
- [19] R.R. Pan, M.C. Xu, J.D. Burgess, et al., Proc. Natl. Acad. Sci. U. S. A. 115 (2018) 4087–4092.
- [20] K.K. Hu, T.D.K. Nguyen, S. Rabasco, P.E. Oomen, A.G. Ewing, Anal. Chem. 93 (2021) 41–71.
- [21] X.Y. Gong, Y.Y. Zhao, S.Q. Cai, et al., Anal. Chem. 86 (2014) 3809–3816.
- [22] S.S. Rubakhin, E.V. Romanova, P. Nemes, J.V. Sweedler, Nat. Methods 8 (2011) S20–S29.
- [23] A. Amantonico, P.L. Urban, S.R. Fagerer, et al., Anal. Chem. 82 (2010) 7394–7400.
- [24] J. Brison, M.A. Robinson, D.S.W. Benoit, et al., Anal. Chem. 85 (2013) 10869–10877.
- [25] C. Giesen, H.A.O. Wang, D. Schapiro, et al., Nat. Methods 11 (2014) 417–422.
- [26] X.Y. Ou, Y. Liu, X.B. Lei, et al., Nat. Commun. 12 (2021) 2144.
- [27] F.J. Warner, R.A. Lew, A.L. Smith, et al., J. Biol. Chem. 280 (2005) 39353–39362.
- [28] C.H.T. Kwong, J.F. Mu, S.K. Li, et al., Chin. Chem. Lett. 32 (2021) 3019–3022.
- [29] B.L. Petrellaab, M.P. Vincentiab, Cancer Lett. 325 (2012) 220–226.
- [30] B. Shen, X. Yi, Y.T. Sun, et al., Cell 182 (2020) 59–72.