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Simultaneous quantification of spike and nucleocapsid protein in inactivated COVID-19 vaccine bulk by liquid chromatography-tandem mass spectrometry

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ABSTRACT

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) vaccines are the most promising approach to control the COVID-19 pandemic. There are eminent needs to develop robust analytical methods to ensure quality control, as well as to evaluate the long-term efficacy and safety of vaccine. Although in vivo animal tests, such as serum-based ELISA, have been commonly used for quality control of vaccines, these methods have poor precision, are labor intensive, and require the availability of expensive, specific antibodies. Thus, there is growing interest to develop robust bioanalytical assays as alternatives for qualitative and quantitative evaluation of complex vaccine antigens. In this study, a liquid chromatography tandem mass spectrometry method was developed using optimized unique peptides for simultaneous determination of spike (S) and nucleocapsid (N) protein. Method sensitivity, linearity, repeatability, selectivity, and recovery were evaluated. The amount of S and N proteins in 9 batches of inactivated COVID-19 vaccines were quantified, and their compositions relative to total protein content were consistent. We believe this method can be applied for quality evaluation of other S and/or N protein based COVID-19 vaccine, and could be extended to other viral vector, and protein subunit-based vaccines.

1. Introduction

The COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has triggered a global public health crisis [1]. Vaccines are expected to be the most promising solution to control SARS-CoV-2, which lead to accelerated development of effective vaccines against SARS-CoV-2 [2,3]. Hitherto, seven different vaccines across different platforms have been rolled out in countries as of 18 February 2021[4], including inactivated virus and mRNA vaccine. As of May 24th, 1.467 billion doses of inactivated COVID-19 vaccines have been administered in China [5]. Tremendous efforts have been made to ramp up the production of COVID-19 vaccines. Enable to support this increasing demand, rapid and robust bioanalytical assays are needed to ensure the quality, efficacy, and safety of the vaccines.

In SARS-CoV-2, there are more than ten kinds of antigens reported [6]. Among them, spike (S) protein is the main antigen. Moreover, nucleocapsid (N) protein, a multifunctional protein which forms complexes with genomic RNA and plays a critical role in virus transcription and assembly, is the most abundant protein in SARS-CoV-2, poorly and non-neutralizing antibodies produced as result of immunogenic response to these proteins in inactivated COVID-19 vaccine could exacerbate subsequent infections via antibody-dependent enhancements (ADE) mechanism, which was also reported previously as a possible cause for enhanced infections of SARS-CoV and middle east respiratory syndrome coronavirus (MERS-CoV) [7–9]. Thus, quantifying the composition of S and N proteins are useful for determining the efficacy

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and safety of inactivated COVID-19 vaccine.

Traditionally, in vivo animal tests such as serum ELISA (enzyme linked immunosorbent assay) assays, have been used to measure produced antibodies as a result of immunization response to administered vaccines. However, these assays have many practical challenges, including inherent variability in their accuracy and precision as well as being technically challenging, labor intensive and time consuming to routinely run in an analytical laboratory [10,11]. For example, vaccine matrix such as adjuvant usually leaded to unstable results of ELSA methods. Thus, a complex desorption pretreatment was needed for vaccine before it was detected by ELISA method. More importantly, highly specific S and N antigens are needed as cross reactivity between similar analogues of S and N proteins could generate false positive responses. Thus, a more selective and robust bioanalytical method is needed to better characterize and quantify these vaccine antigens.

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) offers exciting and important opportunities to better characterize complex vaccine antigens due to its superior sensitivity and selectivity [12,13]. Multi-reaction monitor (MRM) based LC-MS/MS methods had been established previously for the determination of antigens in pneumococcal polysaccharide [14], diphtheria tetanus acellular pertussis (DTaP) vaccine [15], glycoconjugate vaccines [16] and nine-valent human papilloma virus vaccines [17]. Compared to MRM-based method, parallel reaction monitoring (PRM) based method provides a more precise characterization result as it allows parallel detection of all target product ions in a single analysis using a high resolution and accurate mass analyzer. In addition, PRM requires less method development and is potentially more specific than MRM; therefore, it is widely adopted for quantitative analysis of complex metrics, such as proteomics as well as translational studies [18,19]. Herein, we describe the development and application of a LC-PRM method for bioanalysis of S and N protein in 9 batches of inactivated COVID-19 vaccine bulk. We believe this method would be an attractive alternative to conventional serum-based ELISA for quality control of inactivated and other protein-based COVID-19 vaccines.

2. Materials and methods

2.1. Chemicals and materials

Reference proteins (S and N protein) were obtained from National Institutes for Food and Drug Control (Beijing, China). Nine batches of inactivated COVID-19 vaccine bulk were provided by Minhai Biotechnology Co., LTD (Beijing, China). Inactivated COVID-19 vaccine bulk was prepared according to following steps: 1) culture SARS-CoV-2 using Vero cell; 2) harvest virus and inactivated SARS-CoV-2 using β-propiolactone; 3) purify SARS-CoV-2 using ultrafiltration materials. All bathes of COVID-19 vaccine bulk samples were stored at 4 °C before use. Trypsin and chymotrypsin, ammonium bicarbonate (NH_4HCO_3), hydrochloride (HCl), dithiothreitol (DTT), iodoacetamide (IAM) were from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (FA) and trifluoroacetic acid (TFA) were purchased from ThermoFisher (Sunnyvale, CA, USA). Acetonitrile and 1.5 mL microcentrifuge tubes were obtained from Merck (Billerica, MA, USA). High purity water was prepared by a Milli-Q system (Bedford, MA, USA). RapiGest™ surfactant was purchased from Waters (Milford, MA, USA).

2.2. Sample preparation

A portion of vaccine bulk stock of 100 μL aliquot was diluted with 100 μL of 0.1% RapiGest™ (w/v) in 100 mM NH_4HCO_3 aqueous solution. The solution was heated at 60 °C for 15 mins, followed by addition of 10 μL of 500 mM DTT and then incubated at 60 °C for 30 mins. The sample was cooled down to room temperature before adding 10 μL of 1 mM IAM. The sample tube was placed in a drawer shielded from light for 30 mins. Sample was digested by adding 5 μL of trypsin stock solution and 670 μL of 100 mM NH_4HCO_3 and incubated overnight in a column oven with temperature set at 37 °C. Sample digestion was terminated by adding 5 μL of TFA and incubated at 37 °C for 30 mins. Then, the sample was centrifuged at 14,000*g* for 15 mins, and supernatant was collected and dried under a reduced vacuum. Finally, the sample was reconstituted with 100 μL water and analyzed by LC–MS.

Table 1
Flow rate and mobile phase gradient for peptide mapping and bioanalysis.

	Peptide mapping		Bioanalysis	
Flow rate	0.2 mL/min		0.3 mL/min	
Gradient	Time(min)	B%	Time(min)	B%
	0	3	0	5
	3	3	8	40
	85	32	8.1	90
	90	90	10	90
	95	90	10.1	5
	95.1	3	15	5
	100	3		

2.3. Instrument and LC-MS conditions

The LC-MS system is configured with a Thermo Scientific™ Vanquish™ Flex UHPLC (Waltham, MA, USA), and a Thermo Scientific™ Q Exactive™ Focus mass spectrometer (Waltham, MA, USA) equipped with a heated electrospray ionization (HESI) interface. This setup was used for both peptide mapping and bioanalysis of S and N proteins in vaccines. Mobile phases were 0.1% FA in water (A) and 0.1% FA in acetonitrile (B). 10 μL of samples were injected onto Bio C18 column (2.1 × 150 mm, 3 μm) with a column oven temperature set at 35 °C. LC flow rates and gradient conditions were outlined in Table 1. For all sample runs, a diverter valve was used to stream the effluent to waste for the first 2 min before switching back to MS for the remainder of runs. Data acquisition was performed with Xcalibur™ 4.4 software and data analysis and relative quantification was performed with Proteome Discoverer™ 2.5 software.

2.4. MS parameters

For all LCMS runs, positive HESI was used with spray voltage set at 3.8 kV. Both sheath gas and aux gas flow were set at 35 and 10, respectively. Ion transfer capillary temperature was set at 250 °C and aux gas heater temperature was set at 350 °C.

For peptide mapping experiments, full MS scans were acquired with mass range of 300–3000 *m/z* and mass resolution of 70,000. The AGC target value was set at 3e6 and the maximum injection time was set at 200 ms. Peaks were fragmented using higher-energy collisional dissociation (HCD) with normalized collision energy (NCE) set at 27%. MS/MS spectra were obtained with mass resolution set at 17,500, AGC target set at 1.0e4, and dynamic exclusion set at 10.0 s.

For the quantitative analysis of N and S proteins, PRM was used with mass resolution set at 17,500 and isolation window set at 1.6 *m/z*. Peaks were fragmented using HCD with NCE set at 22%. Spectrum data type was profile.

2.5. Determination of total protein content by Lowry protein assay

Total protein content was determined by using the Lowry protein assay which was previously described in Chinese Pharmacopeia method [20]. Bovine serum albumin (BSA) was used as the standard, and inactivated COVID-19 vaccine was determined at 650 nm. Measured concentrations were corrected to account for dilutions.

2.6. Database search

For unique peptides analysis, the MS/MS raw file was searched

Table 2
Information of signature peptides of N and S protein.

	Peptide	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	-Collision Energy (%)
S	QIAPGQTGK	3.15	450.25	587.3148	22
	FLPFQQFGR*	7.98	570.30	440.2272	22
N	NPANNAAIVLQLPQGTTLPK*	7.61	687.39	841.4778	22
	ADETQALPQR	4.08	564.79	400.2303	22

* This is quantifier transition and the other one is for confirmation.

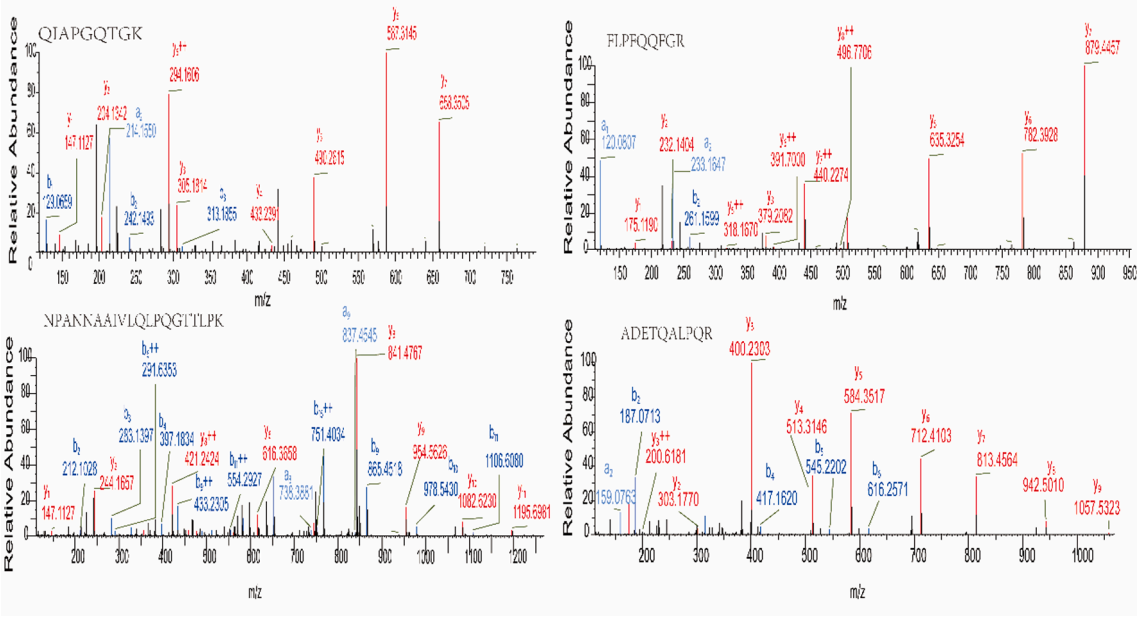

Fig. 1. The representative MS/MS spectra of signature peptides for S and N protein quantitative analysis. Up: MS/MS spectra for quantifier peptide (left) and qualifier peptide (right) of S protein. Bottom: MS/MS spectra for quantifier peptide (left) and qualifier peptide (right) of N protein.

against a combination of structural protein (S, N, M and E) database (4 proteins), a Uniprot SARS-CoV-2 database (61 proteins), Uniport human database (20,324 proteins) and Uniport monkey database (2403 proteins). Preference settings were shown below: the mass tolerance was set at 10 ppm and MS/MS tolerance was set at 0.05 Da. Enzyme was trypsin with an allowance for two missed cleavage sites. Carboxyamidomethylation (C, 57.0215 Da) was selected as fixed modification. Oxidization (M, 15.9949 Da) and deamidation (N and Q, 15.9949 Da) were selected as variable modification. The FDR value was set at 0.01.

3. Results and discussion

3.1. Digestion optimization

Reliable quantification of S and N proteins requires selection of unique signature peptides that are specific to the proteolytic proteins and are free of endogenous interferences from the inactivated COVID-19 vaccines. Hence, both tryptic and chymotryptic digestion methods were explored. With chymotryptic digestion, only 5 peptides from S protein and 3 peptides from N protein yielded adequate MS response (i.e., signal intensities higher than $1e^8$). With tryptic digestion, not only it yields more peptides (e.g., 39 and 33 peptides for S and N protein respectively), signal intensities of the resultant peptides were higher as well. Therefore, trypsin was chosen to digest inactivated COVID-19 vaccine bulk for the remaining studies.

3.2. Protein identification

Given the complex formulation of the inactivated COVID-19 vaccine, the ability to identify all proteins and quantify their relative abundance are important for vaccine characterization as well as quality control during production scaling and pre- and clinical studies. With tryptic digestion, 39 proteins (shown in Supporting Information 1) were identified from inactivated COVID-19 vaccine bulk including N protein, S protein, M protein and some host cell proteins (HCP). However, E protein, an important structural protein, was not detected which may have been filtered during the sample purification step. As for S proteins, 39 unique peptides were identified whereas 33 unique peptides were identified in N proteins.

Among these unique peptides, 23/39 and 18/33 peptides suffer poor sensitivity as well as presence of post-translational modifications and unstable amino acid (such as methionine or cysteine) in their sequences. Five copies of S and N protein with the same amount was digested. The relative standard deviation (RSD%) of target peptide peak area obtained these samples was used to evaluate the repeatability of target peptides. Peptides with RSD% values of peak area less than 20% were used to evaluate peptide linearity. Five copies of S and N protein mixture with five different concentration was digested and the digestion was analyzed to evaluate linearity of target peptides. As a result, only 6 peptides of S protein and 4 peptides of N protein had good linearity and repeatability. Among those, the 2 most sensitive peptides, from different regions (i.e., one located at the receptor binding domain or RBD, and the other located at non-RBD portion of the protein), were selected for quantitative analysis of S and N proteins. Table 2 listed the peptide sequences and PRM transitions for S and N proteins, and sample tandem mass spectra of peptides were shown in Fig. 1. The ability to simultaneously detect and quantify both peptides for each protein will provide confidence in the determined amount and serve a good indicator for batch-to-

Table 3
Validation of the established method.

Protein	Peptide	linear	R^2	LLOQ (μg/mL)	Intra-day%	Inter-day%
S	QIAPGQTGK	A = −10850.6 + 9078.48C	0.9934	0.1	3.4	1.9
	FLPFQQFGR*	A = −11184.9 + 5121.8C	0.9982	0.1	2.9	7.3
N	NPANNAAIVLQLPQGTTLPK	A = −30179.5 + 98122.8C	0.9989	0.1	2.2	2.1
	ADETQALPQR	A = −2989.9 + 14598.6C	0.9989	0.1	3.7	1.2

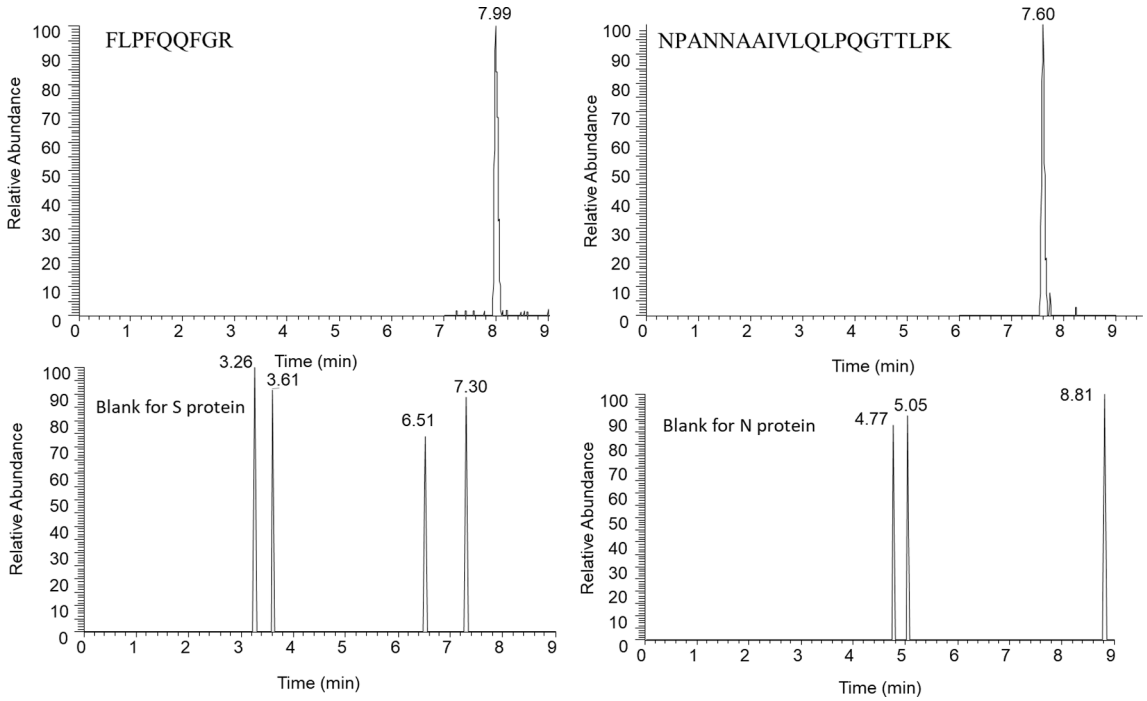

Fig. 2. The extracted ion chromatograms (XIC) for the quantifier ions. Left: The XIC for the quantifier ion of S protein at LLOQ (up) and at blank matrix (bottom). Right: The XIC for the quantifier ion of N protein at LLOQ (up) and at blank matrix (bottom).

batch reproducibility and digestion efficiency.

3.3. Validation of LC-MS/MS

Linearity, sensitivity, selectivity, precision, and recovery were evaluated using the optimized PRM method for quantitation of S and N protein in inactivated COVID-19 vaccine bulk.

3.3.1. Linearity and accuracy

The linearity of the quantification method was measured by selecting 9 different concentrations tested across the range 1–25 μg/mL for S protein and 10–80 μg/mL for N protein. The linear regression fit was applied with 1/C weight. As shown in Table 3, the method demonstrated good linearity across tested concentration 1–25 μg/ml and 10–80 μg/ml with the regression correlation coefficient $R^2 \geq 0.993$ for signature peptides of S and N protein, respectively. The PRM method exhibits excellent accuracy, all within the range of 91.4–110.1% across check standards.

3.3.2. Sensitivity and selectivity

The lower limit of quantitation (LLOQ), which was defined as the lowest concentration level measured with 20% precision and 80%-120% accuracy, was determined to be 0.1 μg/ml for S and N protein (Table 3). Sample extracted ion chromatograms (XIC) of quantifier ions at LLOQ level (top) and in blank (bottom) for S and N protein were shown in Fig. 2. This method demonstrates high selectivity towards S and N proteins as no endogenous interferences were observed in blank samples which contained all components in vaccine, except inactivated SARS-Cov-2.

3.3.3. Precision

Intra-day precision (n = 5) was evaluated by analysis of QC samples on the same day. The concentrations of S and N protein in QC samples were 5 and 20 μg/mL, respectively. Inter-day precision was determined by repeated analyses of the QC samples in five consecutive days. As shown in Table 3, the method exhibits excellent reproducibility as both intra-day and inter-day precision are well within 8% for quantified S and N proteins.

3.3.4. Recovery

Recovery values of target peptides in inactivated COVID-19 vaccine bulk was used to evaluate matrix effect. Recovery was calculated by % recovery = Determined amount (spiked amount) /Theoretical (sample + reference sample) * 100%. Reference sample was the digestion of the mixture of S and N protein (10 μg/mL). The reference sample was prepared by mixing the standard solution of S and N protein and then digested. The concentration of S and N protein in reference sample was 10 μg/mL. Sample is the digestion of inactivated COVID-19 vaccine bulk which contained 10 μg/mL − 40 μg/mL S and N protein. Spiked sample is the digestion of the mixture of sample and reference sample. Spiked amount was the amount of S and N protein standards added to inactivated COVID-19 vaccines. The amount of S and N protein in spiked sample and sample were determined by linear equation obtained in Section 3.3.1. The recovery of S and N protein determined was in the

Table 4
Quantitative results of S and N protein in nine batches of inactivated COVID-19 vaccine bulk.

c	Total protein [a]	Concentration (μg/mL)		Ratio of protein [b]		Other[c]
	(μg/mL)	S	N	S	N	
1	47.9	6.6	16.6	13.8%	34.7%	51.5%
2	60.3	13.2	34.8	21.8%	57.8%	20.4%
3	69.1	13.6	26.6	19.7%	38.5%	41.8%
4	31.4	4.2	17.1	13.4%	54.5%	32.2%
5	88.3	18.5	60.6	20.9%	68.6%	10.4%
6	53.5	8.7	34.0	16.4%	63.6%	20.0%
7	76.9	11.8	45.4	15.4%	59.0%	25.6%
8	90.8	14.4	53.6	15.9%	59.1%	25.1%
9	161.9	20.4	75.6	12.6%	46.7%	40.7%
RSD%				20.3%	21.2%	

a: The amount of total protein was determined by Lowry method described in section 2.5. b: Ratio of S and N protein $= \frac{C^{*}Mr}{\text{total protein}}$*100%, C and Mr was molar concentration and molecular weight of target protein, respectively. c: Ratio of other protein = 100% − (ratio of S + ratio of N).

range of 86%-115%, suggesting that the method exhibited excellent accuracy.

3.4. Quantification of S and N protein in inactivated COVID-19 vaccine bulk

The optimized PRM method was applied to quantify S and N proteins in nine batches of inactivated COVID-19 vaccines. The results were shown in Table 4. According to the results in Table 4, the concentrations of S and N protein in inactivated COVID 19 vaccine bulk are higher than 4 and 15 μg/mL, respectively. This means that the sensitivity of the current LC-MS/MS method is adequate for targeted quantitation of S and N protein in inactivated COVID 19 vaccine bulk.

As shown in Table 4, although the determined amount of S and N proteins in each batch vary slightly, the composition (i.e., % S and N protein relative to the total protein concentration) are rather consistent, with a relative standard deviation around 20% across 9 batches. The possible reason that could account for such variation is that the inactivated SARS-CoV-2 virus (bulk) was damaged during purification process leading to loss of some proteins, such as E protein. This was verified via electron microscopy imaging of the vaccines (Fig. 3), showing the damaged inactivated SARS-CoV-2 species. The protein composition study in Section 3.2 also verified that no E protein was detected in inactivated COVID-19 vaccine bulk. The total amount of S and N proteins contributed to about 48.5%-89.6% of the total proteins in inactivated COVID-19 vaccine bulk, and other proteins accounted for about 10.4%-51.5% in inactivated COVID-19 vaccine bulk, consistent with other reporting [6]. Despite many reported articles on SARS-CoV-2 viral proteins, the roles of these other proteins remain unclear. We believe current LC-MS/MS method could be extended to quantify these proteins when researchers gain better understanding on these viral proteins and how they could impact the safety and efficacy of the vaccines.

S protein amount in the studied samples was also measured by an ELISA method developed by the manufacture (Fig. 4). The RSD value of

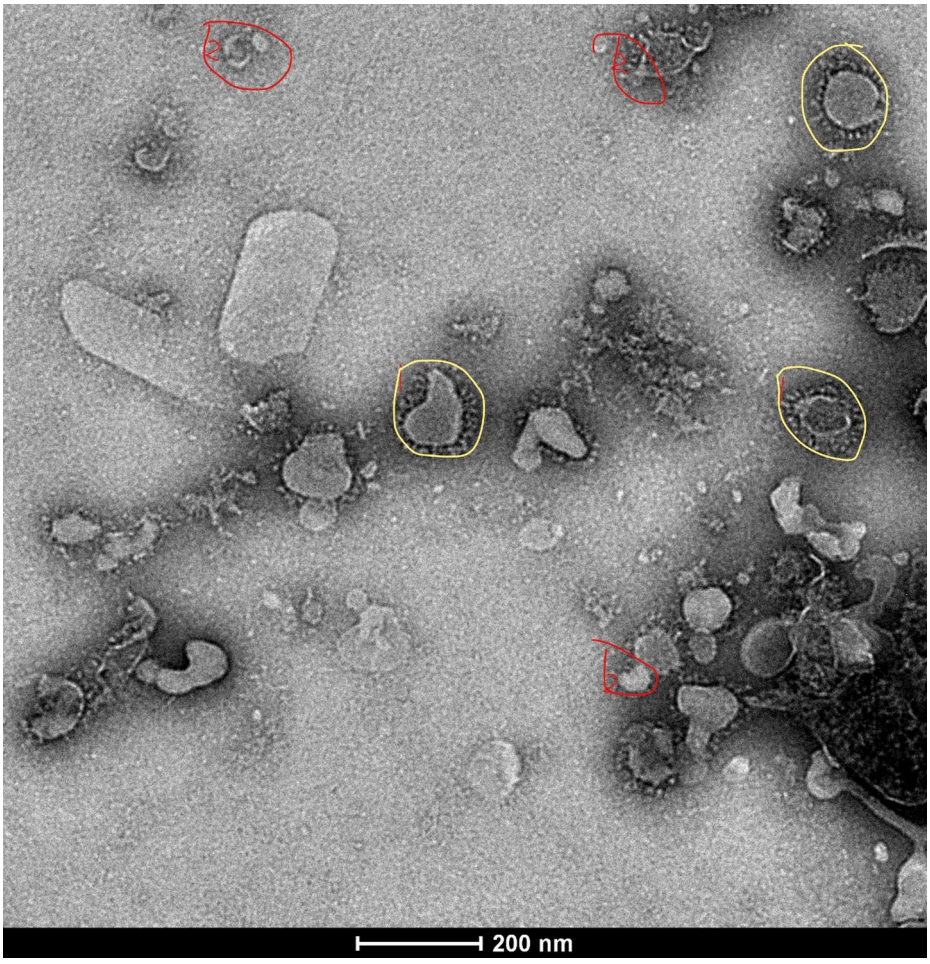

Fig. 3. The electron microscopy image of inactivated COVID-19 vaccine bulk. Yellow cycle: intact virus; Red cycle: damaged virus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

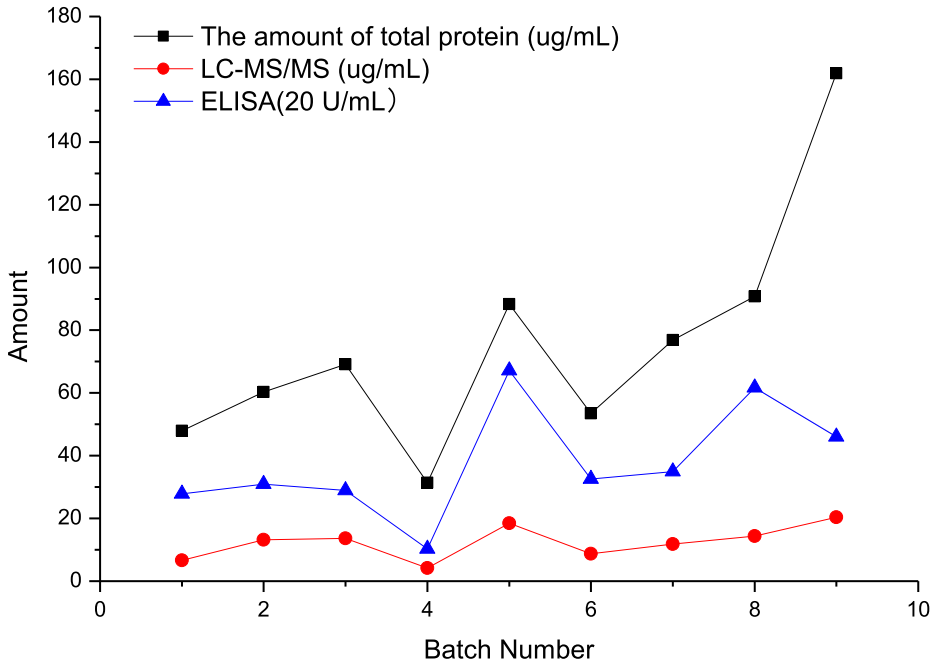

Fig. 4. The amount of total protein obtained with Lowry method and S protein amount obtained with the current LC-MS/MS method and an ELISA method.

this ELISA method was in the range of 6–8%. Details of this method was shown in Supporting Information 2. As shown in Fig. 4, the S protein amount obtained with the LC-MS/MS method agrees well with the one of total protein for all samples. The amount of S protein increased with the one of total protein. Except No.9 sample, the amount of S protein obtained with the developed LC-MS/MS method agrees well with the one obtained with the ELISA method. For No.9 sample, the result obtained with the developed LC-MS/MS method is very different from the one obtained with the ELISA method. Detected by the ELISA method, the amount of S protein in No.9 sample is lower than the one in No.8 sample, probably due to the interference from matrix which inhibited interactions between S protein and its antibody. Because of the high selectivity for LC-MS/MS, such interference has little effect on the determination of target protein. Thus, the LC-MS/MS method consistent the results obtained with ELISA method for most samples, while LC-MS/MS method is more robust due to its high selectivity.

4. Conclusions

In this study, a PRM based LC-MS/MS method was developed and applied for quantitative determination of S and N protein in inactivated COVID-19 vaccine bulk. The quantitative method was optimized and then used to determine the amount of S and N proteins simultaneously in inactivated COVID-19 vaccine bulk. The batch-to-batch stability of inactivated COVID-19 vaccine was studied in terms of the RSD% of S and N proteins. The LC-MS/MS method consistent the results obtained with ELISA method for most samples, while LC-MS/MS method is more robust due to its high selectivity.

Besides inactivated COVID-19 vaccine, this method can also be used to determine S and/or N protein, as well as HCPs in other protein based COVID-19 vaccines.

CRediT authorship contribution statement

Zhen Long: Conceptualization, Writing - original draft, Methodology. **Chen Wei:** Methodology, Resources, Validation, Project administration. **Xuefang Dong:** Conceptualization, Writing - review & editing, Methodology. **Xiuling Li:** Conceptualization, Supervision. **Hao Yang:** Writing - review & editing. **Haiqing Deng:** Resources. **Xiao Ma:** Conceptualization, Supervision. **Shanshan Yin:** Resources. **Yingzi Qi:** Writing - review & editing. **Tao Bo:** Writing - review & editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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