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Sensitive immunoassays of nitrated fibrinogen in human biofluids

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ABSTRACT

Three new sandwich immunoassays for detection of nitrated biomarker have been established with potential applications in biomedical studies and clinical practice. In this study, nitrated human fibrinogen, a potential oxidative stress biomarker for several pathologies, was chosen as the target. To improve the sensitivity and overcome the interference caused by the complexity of human biofluids, we developed three sandwich strategies using various combinations of primary antibody and secondary antibody. All three strategies demonstrated high sensitivity and selectivity towards nitrated forms of fibrinogen in buffer, but their performances were dramatically reduced when tested with human plasma and serum samples. Systematically optimizations were carried out to investigate the effects of numerous factors, including sampling, coating, blocking, and immunoreactions. Our final optimization results indicate that two of these strategies retain sufficient sensitivity and selectivity for use as assays in human physiological samples. Specifically, detection limits reached the pM level and the linear response ranges were up to nM level with a correlation coefficient > 0.99. To our best knowledge, this is the first example of using an electrochemical immunoassay for a nitrated biomarker in a physiological fluid. This novel approach provides a rapid, sensitive, selective, cost efficient and robust bioassay for detection of oxidative stress in pathology and for clinical applications. Moreover, the sandwich strategies developed in this paper can be readily used to establish effective methods targeting other nitration biomarkers.

1. Introduction

Nitration of tyrosine residues in proteins is recognized as a signature of enhanced oxidative stress and inflammation [1–4]. This post-translational modification usually results in the alteration of protein spatial conformation and functions, thus inducing disorders and diseases [4–6]. Recent research has revealed that there are a variety of pathologies, such as cardiovascular disease [1,2,7], diabetes [8,9], hypertension [4,10], atherosclerosis [11,12], smoking [13] and aging [6,14], that are associated with increased oxidative stress. In particular, nitrated fibrinogen has been demonstrated as a potential biomarker for coronary artery disease [15–17], aging [14], lung cancer and stress response to smoking [13]. Accordingly, the nitrated proteins can serve as potential novel biomarkers for clinical diagnosis. However, the assay of nitrated protein is traditionally accomplished with immunohistochemical staining and western blotting, which are time consuming, laborious, and lacking precise quantitation [1,6,13,14]. The intrinsic limited selectivity also hinders them from monitoring specific nitrated biomarkers, which could provide valuable information for pathology research and disease diagnosis. To achieve better specificity and quantitation, several methods utilizing effective separation technologies, such as high-performance liquid chromatography (HPLC) and gas chromatography (GC), in combining with various detection systems, including absorbance [18,19], fluorescence [20], electrochemical assay [21,22] and mass spectrometry [14,23,24], have also been established to characterize the nitrated proteins in complex sample after pre-concentration [25]. However, these approaches require expensive instruments, labor-intensive pre-treatment and sophisticated data processing, thus cannot meet the requirements for clinical applications. Recently, some efforts have been devoted to establish an immunoassay based spectroscopic assay by using nitrated BSA as the model. One assay showed a detection limit in nM range and excellent selectivity, providing a fast colorimetric mean to evaluate the nitrated protein content [26]. However, this study did not analyze a real nitrated protein biomarker especially when they are presented in complex human biofluids such as serum or plasma. Another assay utilized a competitive ELISA (Enzyme-Linked ImmunoSorbent Assay) method to detect 3′-nitrotyrosine in several human plasma proteins but suffered from semi-quantitation and intrinsic non-selectivity [27]. All together, the development of a robust, rapid, and sensitive assay for nitrated proteins with high selectivity is greatly desired for monitoring nitrated protein biomarkers in biological and clinical samples such as human biofluids.

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In this work, electrochemical immunoassays of nitrated human fibrinogen in human serum and plasma using disposable screen-printed electrode (SPE) have been established employing three sandwich ELISA strategies. The sandwich ELISA has been widely used in immunology research, bioassay and clinical diagnosis [28,29]. However, to establish an effective sandwich ELISA for a given target biomarker, one of the key steps is to select the suitable pair of first-antibody and secondary antibody, which can specifically recognize the biomarker with high affinity but no cross-reaction. Especially in this work, there is no available antibody pair for a sandwich immunoassay of nitrated human fibrinogen. Hence, the pairing of antibodies and the optimization of immunoreactions are of central importance for developing the sandwich ELISA method to effectively detect the nitrated human fibrinogen. Moreover, a simple, economic, robust and sensitive signal readout technology should be employed to facilitate the future application and generalization of sandwich ELISA approaches developed in this paper. In this regard, the electrochemical assay was chosen to transfer and deliver the immunoreaction signal by using disposable screen-printed electrode [30–36]. Here we developed three sandwich strategies for electrochemical immunoassays of nitrated human fibrinogen and investigated their analysis capability in human serum and plasma samples. After careful optimization, two ELISA sandwich strategies can selectively and sensitively detect pM levels of nitrated fibrinogen spiked in diluted human plasma or serum. The success of developing nitrated human fibrinogen electrochemical immunoassay also provides meaningful and valuable knowledge for establishing convenient and practical bioassay approaches for other nitrated biomarkers, thus contributing to the study of oxidative stress induced pathological studies and clinical applications.

2. Experimental

2.1. Reagents and materials

Human fibrinogen was purchased from Sigma (F4883). Polyclonal human fibrinogen antibody was obtained from Millipore Corporation (AB7144F) while horseradish peroxidase (HRP) labeled goat anti-human fibrinogen was ordered from Rockland Immunochemicals, Inc. (200-103-240). Several nitrotyrosine antibodies were tested in this paper. Goat anti-nitrotyrosine antiserum was purchased from Academy Biomedical Company. Goat anti-nitrotyrosine was ordered from Mederidian Life Science Inc. Two monoclonal mouse anti-nitrotyrosine was obtained from Abcam Inc. (ab7048) and Cayman Chemical Company (189542). Two HRP conjugated anti-nitrotyrosine antibodies were used, in which one was provided by Abcam Inc. (ab27648) and another was obtained from Millipore Corporation (16-227). Human serum and plasmas were purchased from Golden West Biologicals Inc. Three human plasmas were treated with different coagulants including EDTA, heparin, sodium citrate. The 1% casein in PBS buffer was provided by Bio-Rad. All other chemicals were purchased from Sigma–Aldrich. All buffers and reagent solutions were prepared with purified water, which was produced from a high purity water system (NANOPure UV, Barnstead).

2.2. Apparatus and electrodes

The square wave voltammetry (SWV) measurement was performed by a μAutoLab Type III (Metrohm Autolab B.V., Netherlands). The disposable screen-printed carbon electrode (SPE) was purchased from DropSens (Oviedo, Spain). The current peak height was then calculated using software General Purpose Electrochemical System (Ver. 4.9).

2.3. Preparation of nitrated human fibrinogen

Human fibrinogen (1 mg/mL) in phosphate buffered saline (pH 7.4) was nitrated by bolus addition of 1 mM authentic peroxynitrite (R&S Systems, Minneapolis, MN) according to recommendations of the manufacturers. The volume of added peroxynitrite was <1% of the total volume of the incubation mixture. The nitrotyrosine content of nitrated fibrinogen was measured from its absorbance at 420 nm as compared with that at 280 nm using extinction coefficients of 4300 M^{-1} cm^{-1} and 5.14×10^5 M^{-1} cm^{-1}, respectively. In detail, 100 μL of reaction solution was mixed with 10 μL of 0.5 M pH 10 Na_2CO_3–$NaHCO_3$ buffer in 100 μL of quartz cuvette to measure absorbance using an Ultrospec 2100 Pro spectrophotometer (Biochrom Ltd., Cambridge, UK). Nitrated fibrinogen was stored as a stock solution of 1 mg/mL in PBS at −80 °C.

2.4. Analysis of native and in vitro-nitrated human fibrinogen using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS)

Human fibrinogen protein samples were denatured with 50% trifluoroethanol (TFE) in 50 mM NH_4HCO_3 buffer (pH 8.0) for 2 h at 60 °C followed by reduction with 5 mM dithiothreitol (DTT) for 0.5 h at 37 °C. Then proteins were digested into peptides by sequencing grade modified porcine trypsin (Promega, Madison, WI) at a trypsin:protein ratio of 1:50 (w/w) for 3 h at 37 °C. Digests were lyophilized and redissolved in 100 μL 50 mM NH_4HCO_3 for LC–MS/MS analysis.

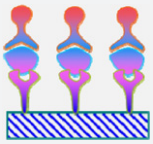
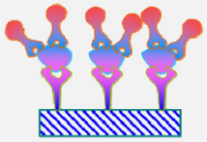
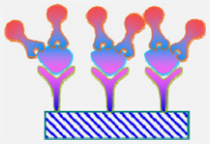
LC–MS/MS analysis was performed using a high pressure capillary LC system coupled on line to a linear ion trap mass spectrometer (LTQ; ThermoElectron) via an electrospray ionization interface. The obtained MS/MS spectra were identified by database searching using the SEQUEST algorithm against a fasta file containing the sequences of all subunits of fibrinogen. Nitrotyrosine peptides were identified by allowing dynamic nitration modification (+44.9851 Da) on tyrosine residues. The details of LC–MS/MS analysis and peptide identifications are similar as previously reported [37].

2.5. Sandwich ELISA strategies

Three sandwich ELISA strategies have been designed and tested using anti-fibrinogen and anti-nitrotyrosine antibodies as first or secondary antibody. The constructions of three sandwich strategies were listed in Table 1.

For each strategy, the primary antibody was coated overnight at 4 °C with 50 μL solution (20 μg/mL in PBS buffer) in each well of 96-well plate. The primary antibody solution was then removed before adding 250 μL PBS buffer in each well, shaking the solution at 250 rpm for 5 min to wash away uncoated antibody. After this washing step, the plate was blocked by adding 250 μL 1% casein in PBS buffer in each well shaking at 100 rpm under 32 °C for 1 h. Then each well was washed three times with 250 μL PBS buffer before addition of 50 μL of the analyte prepared with 0.5% BSA in PBS buffer, after which the plate was incubated at 32 °C for 1 h with shaking at 100 rpm. The unbound analyte was then discarded and the plate was washed three times followed by 1 h incubation with 50 μL of horseradish peroxidase (HRP) labeled secondary antibodies, which were prepared in 0.2% casein in PBS buffer at a final concentration of 2–4 μg/mL. The solution was discarded and the plate was washed again with PBS buffer for three times to remove the unbound secondary antibody. After the final washes, the micro-well plate was ready for electrochemical or spectroscopic analysis.

Table 1
Three sandwich strategies using various antibodies combinations.

	Strategy A	Strategy B	Strategy C
			
Primary antibody	Anti-nitrotyrosine	Anti-nitrotyrosine	Anti-human fibrinogen
Secondary antibody (HRP labeled)	Anti-human fibrinogen	Anti-nitrotyrosine	Anti-nitrotyrosine

2.6. Electrochemical detection and spectroscopic analysis

Electrochemical detection was used to detect the nitrated fibrinogen for the high sensitivity and convenience of electrochemical analysis. To perform the electrochemical detection, 50 μL of *O*-phenylenediamine (OPD) solution (0.2 mM in PBS buffer with 20 μM hydrogen peroxide) was added into each well and allowed to react for 10 min before the square wave voltammetry (SWV) measurement was performed. Here, HRP catalyzed the oxidization of OPD in the presence of hydrogen peroxide and the enzymatic product is 2,3-diaminophenazine (DAP), which can be detected by electrode [38]. After 10 min of the HRP catalysis reaction, 50 μL of OPD solution was transferred to a disposable screen-printed carbon electrode (SPE) (DropSens, Oviedo, Spain) and a μAutoLab (Type III) was used to perform SWV measurement. Typical SWV curves were scanned from 0.1 to −0.3 V with 4 mV step, 25 mV pulse, and 15 Hz frequency.

Spectroscopic analysis was employed in the optimization of experimental conditions for its speed and high throughout. The plate was developed with the addition of 100 μL TMB solution in each well and incubated at 32 °C with shaking at 100 rpm for 10–20 min to allow development of sufficient color intensity. Samples under optimal experimental conditions present a deeper blue color.

2.7. Optimization of physiological sample assay

To analyze the physiological samples, the experimental conditions were further optimized to reduce or eliminate the effects from complex components in samples. The optimization of several key factors, such as blocking, sampling, antibody coating, anticoagulant, dilution, etc., have been investigated to improve the selectivity and sensitivity of the physiological sample assay.

To prepare spiked samples, the human plasma or serum samples were diluted with buffer to decrease interferences from complex physiological fluids. Two factors, the dilution ratio and dilution buffer have been optimized. The human plasma or serum was diluted for 4, 10 and 20 times with dilution buffer, respectively. The dilution buffers used for sampling optimization were PBS buffer, 0.5% BSA and 1% casein.

The effects of anticoagulants on the immunoassay were also investigated. Human serum and three human plasmas with different anticoagulation treatment with EDTA, heparin and sodium citrate were used to prepare spiked samples for determining those effects.

To reduce nonspecific absorbance, the blocking is enhanced in a straight forward way. After overnight coating, 250 μL of blocking agents (3% BSA or 1% casein) were added into each well to block the uncoated plate well surface. The plate was then shaken at 100 rpm and 32 °C for 1–3 h. Other steps were completed as described in the previous section. The effects of blocking agents and incubation time on immunoassay were investigated in these experiments.

The effects of antibody coating conditions on nitrated fibrinogen assay were also studied. The antibody concentration and coating duration were optimized to obtain higher efficiency of immunoreaction. 20 and 100 μg/mL antibody in PBS buffer were added into each well with volume of 50 μL. The coating step was processed at 4 °C for 15, 40 and 64 h.

The effect of immunoreaction duration was also investigated. After coating of the primary antibody, 50 μL of human plasma or serum samples were introduced into each well to perform the immunoreaction. The plate was then shaked at 100 rpm 32 °C for 30, 45 and 60 min before secondary antibody staining. The rest procedures were the same as described before.

2.8. The assays of nitrated fibrinogen in biofluid samples

Nitrated fibrinogen at desired concentrations was spiked into human plasma or serum samples which were diluted 20 times with 0.5% BSA. The final concentration of fibrinogen or nitrated fibrinogen was ranging from 3.0 pM to 30 nM.

The optimized strategies B and C were used to determine the amount of nitrated fibrinogen spiked in physiological samples. The coating solution for the strategy B was prepared with anti-nitrotyrosine antibody in PBS buffer at the concentration of 20 μg/mL. For strategy C, the coating solution was prepared with anti-human fibrinogen antibody in PBS buffer at the concentration of 100 μg/mL. 50 μL coating solution was added into each well and coated for 40 h at 4 °C. The coating solution was then removed before washing the plate. After a washing step the plate was blocked by adding 250 μL 1% casein in PBS buffer in each well and shaken at 100 rpm at 32 °C for 2 h. After three times additional washing, 50 μL of physiological sample was added and incubated at 32 °C for 30 min with shaking at 100 rpm. The unbound analyte was then discarded and the plate was washed three times before 1 h incubation with 50 μL of HRP labeled anti-nitrotyrosine, which were prepared in 0.2% casein PBS buffer at the final concentration of 2 μg/mL. The solution was discarded and the plate was washed again with 250 μL/well PBS buffer for three times to remove the unbound antibody. The micro-well plate was then subject to electrochemical analysis.

3. Results and discussion

3.1. Nitration of fibrinogen

The extent of nitration in fibrinogen was evaluated by spectroscopic measurements of absorbance at 280 and 420 nm. According

Table 2
LC–MS/MS identified tyrosine sites (in both nitrated and native peptide forms) in an *in vitro*-nitrated fibrinogen. Count data represent the number of MS/MS spectra identified either the nitrated or native peptides.

Fibrinogen subunits	SWISS-PROT ID	Tyrosine site	Count of nitro-Tyr	Count of Tyr	Identified peptide sequence
Alpha chain	P02671-1	Y-95	2	3	K.NSLFEY*QK.N
		Y-127		1	R.GDFSSANNRDNTYNR.V
		Y-197	1	6	R.EVDLKDY*EDQQK.Q
		Y-277	11	3	R.GGSTSY*GTGSETESPR.N
		Y-579	2		R.GKSSSY*SK.Q
		Y-589	6		K.SSSY*SKQFTSSTSY*NR.G
		Y-713	6		R.GFGSLNDEGEGEFWLGNDY*LHLLTQR.G
		Y-737		2	R.VELEDWAGNEAYAEYHFR.V
		Y-740		2	R.VELEDWAGNEAYAEYHFR.V
		Y-835	2	2	R.NNSPY*EIENGVVWVSFR.G
Beta chain	P02675	Y-71	15	7	R.EEAPSLRPAPPPISGGGY*R.A
		Y-147	2	2	R.NSVDELNNNVEAVSQTSSSSFQY*MY*LLK.D
		Y-149	2	2	R.NSVDELNNNVEAVSQTSSSSFQY*MY*LLK.D
		Y-172	12	19	K.QVKDNENVVNEY*SSELEK.H
		Y-182	9	13	K.HQLY*IDETVNSNIPTNLR.V
		Y-255		16	R.KGGETSEMYLIQPDSSVKPYR.V
		Y-266		13	R.KGGETSEMYLIQPDSSVKPYR.V
		Y-299		4	R.KWDPYKQGFGNVATNTDGK.N
		Y-356	6	20	K.AHY*GGFTVQNEANK.Y
		Y-368	3	9	K.Y*QISVNK.Y
		Y-408		1	R.TMTIHNGMFFSTYDR.D
		Y-434	2	10	K.EDGGGWWY*NR.C
		Y-446		13	R.YYWGGQYTWDMAK.H
		Y-447		13	R.YYWGGQYTWDMAK.H
		Y-452	2	13	R.YYWGGQY*TWDMAK.H
		Y-475	2		K.GSWY*SMR.K
Gamma chain	P02679-1	Y-44	2	1	R.FGSY*CPTTCGIADFLSTYQTK.V
		Y-58		1	R.FGSYCPTTCGIADFLSTYQTK.V
		Y-94	1		K.AIQLTY*NPDESSKPN.M
		Y-122		3	K.YEASILTHDSSIR.Y
		Y-135	9	4	R.Y*LQEIYNSNNQK.I
		Y-140	5	4	R.YLQEIY*NSNNQK.I
		Y-193		3	K.QSGLYFIKPLK.A
		Y-237		5	K.NWIQYK.E
		Y-270	1	13	K.IHLISTQSAIPY*ALR.V
		Y-300	2	4	K.VGPEADKY*R.L
		Y-304	2	18	R.LTY*AY*FAGGDAGDAFDGFDFGDDPSDK.F
		Y-306	2	19	R.LTY*AY*FAGGDAGDAFDGFDFGDDPSDK.F
		Y-389	12	11	K.ASTPNGY*DNGIIWATWK.T

to the absorbance at 280 and 420 nm, the average nitration content per fibrinogen molecule can be determined by calculating the molar ratio of nitrotyrosine to fibrinogen, determined here to be 14.9. Fibrinogen is composed of dimers of three different polypeptide chains ($\alpha_2\beta_2\gamma_2$) with 25, 21, and 24 tyrosines, respectively; thus, the observed stoichiometry of ~15 nitrotyrosines per mol of fibrinogen suggests an average of 11% of the tyrosines are nitrated.

The sites and extents of fibrinogen nitration were also characterized with LC–MS/MS. As shown in Table 2, 26 different nitrotyrosine sites were identified as nitrated within the three fibrinogen subunits among a total of 39 observed tyrosine residues.

3.2. Sandwich immunoassays of nitrated fibrinogen

Three sandwich electrochemical immunoassays have been developed according to the properties of nitrated fibrinogen and the potential challenges from analyzing physiological samples. In detail, the strategy A used anti-nitrotyrosine as the primary antibody to capture all nitrated protein and then used anti-human fibrinogen to stain the captured nitrated fibrinogen. This strategy is straightforward, but may suffer from nonspecific absorption of native fibrinogen, which will bind to anti-human fibrinogen producing a high nonspecific signal. Therefore we also designed strategy C using anti-human fibrinogen as the primary antibody to capture both nitrated and intact fibrinogen before using anti-nitrotyrosine to selectively stain the nitrated fibrinogen. In addition, strategy C may have higher sensitivity for multiple second antibodies may bind to one nitrated fibrinogen since it may have multiple nitration sites. Moreover, strategy B constructed using anti-nitrotyrosine as both the primary antibody and secondary antibody also based on the fact that one nitrated fibrinogen might have multiple nitration sites. Strategy B can be used as a general approach to detect nitrated proteins.

Three best antibody pairs were selected for three sandwich strategies respectively according to their sensitivity, excellent selectivity and cross-reactivity. Strategy A used Abcam produced monoclonal mouse anti-nitrotyrosine (ab7048) and Rockland HRP labeled goat anti-human fibrinogen (200-103-240). Strategy B used Abcam produced monoclonal mouse anti-nitrotyrosine (ab7048) and HRP conjugated goat polyclonal anti-nitrotyrosine (ab27648). Strategy C selected Chemicon's polyclonal human fibrinogen antibody (AB7144F) and Abcam's HsRP conjugated goat polyclonal anti-nitrotyrosine (ab27648).

All three approaches demonstrated high sensitivity and excellent selectivity. OPD was employed as the substrate of HRP and electroimmunoassay. In PBS buffer (pH 7.4), the OPD produces a measurable current peak in voltammetric scan, which provides a sensitive signal and effectively avoids the interferences from other substrates in analyte solution. This is very important for the assay performed with complex samples including physiological fluids such as human serum and plasma, which will be studied in this work.

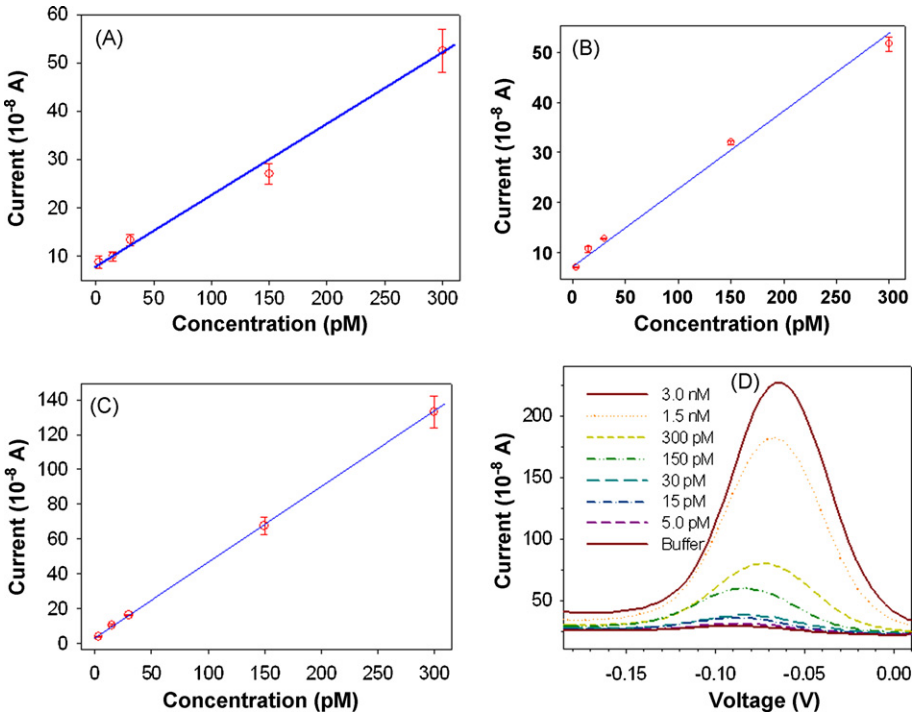


Fig. 1. Results of the three sandwich strategies based electrochemical immunoassay of nitrated fibrinogen. (A), (B) and (C) are the calibration curves of current peak height as a function of the concentration of nitrated fibrinogen from strategy A, strategy B and strategy C, respectively. (D) Voltammograms of nitrated fibrinogen with increasing concentration from bottom to top (strategy B).

Fig. 1(A–C) shows the calibration curves of the three approaches. Fig. 1D shows the typical voltammograms of the strategy B with increasing concentration of nitrated fibrinogen.

Strategy A, as shown in Fig. 1A, has a linear detection range up to 0.3 nM and a detection limit of 13.5 pM (S/N = 3).

Strategy B, has a linear detection range up to 0.3 nM and a detection limit of 2.4 pM (S/N = 3) (Fig. 1B). Compared with the first strategy, this approach provides better sensitivity. This lower detection limit is a benefit based on the fact that one nitrated fibrinogen protein molecule has multiple nitrotyrosine residues. In strategy A, each captured nitrated fibrinogen would only bind one secondary antibody to produce limited signal. In comparison to the first strategy, each captured nitrated fibrinogen in the second strategy can bind with multiple HRP labeled secondary antibodies, thus giving a stronger signal for the electrochemical immunoassay.

For the strategy C, it has a linear detection range up to 0.3 nM and a detection limit of 0.9 pM (S/N = 3) (Fig. 1C), which is the highest sensitivity among three sandwiches. This high sensitivity is benefitted from the fact that each arrested fibrinogen only occupies one binding site of the primary antibody, which results in a higher capture efficiency of analyte comparing with the first and second strategies. In addition, the captured nitrated fibrinogen can also bind with multiple HRP labeled secondary antibodies, thus producing high electrochemical signal.

The selectivity of these three strategies have also been investigated and compared and the results are presented in Fig. 2. When tested with 3.0 nM fibrinogen samples, no obvious signals were observed in SWV scanning. All three approaches presented excellent selectivity to distinguish the intact fibrinogen and nitrated fibrinogen. The exceptional selectivity is due to the excellent specificity of anti-nitrotyrosine antibody and the optimized blocking and sampling processes.

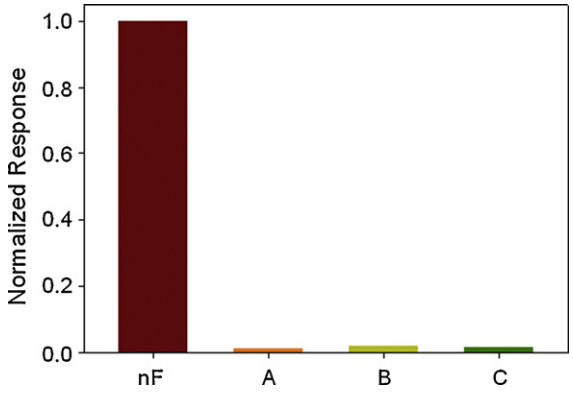


Fig. 2. Selectivity of three strategies. The response of each strategy was normalized according to nitrated human fibrinogen samples, respectively. The bars labeled with nF represent the response to nitrated fibrinogen while A, B and C indicate the responses towards unmodified fibrinogen in three strategies.

3.3. Optimizations for detection in biofluid samples

When tested with human biofluid samples, i.e. plasma and serum, using the same experimental conditions for buffer assays, each strategy encountered significant challenges. The sensitivity of all three approaches was dramatically decreased due to significant disturbance of immunoreactions. In addition, the large amount of fibrinogen in human plasma and serum nonspecifically adhered on 96-well plate during incubation, resulting in notable interference to the strategies A and C. As a result, the specificity of the strategy A was sacrificed since its secondary antibody, i.e. HRP linked anti-human fibrinogen, could be captured by nonspecific absorbed fibrinogen. Meanwhile, the sensitivity of the strategy C was significantly reduced because of the extremely high ratio of native fibrinogen to nitrated fibrinogen in the samples. Therefore, most binding sites of the primary antibody, anti-human fibrinogen, were occupied by native fibrinogen, directly competing with nitrated fibrinogen and reducing the sensitivity. At the same time, other abundant proteins in the samples also showed considerable negative effects on the sensitivity of all three sandwich strategies. As

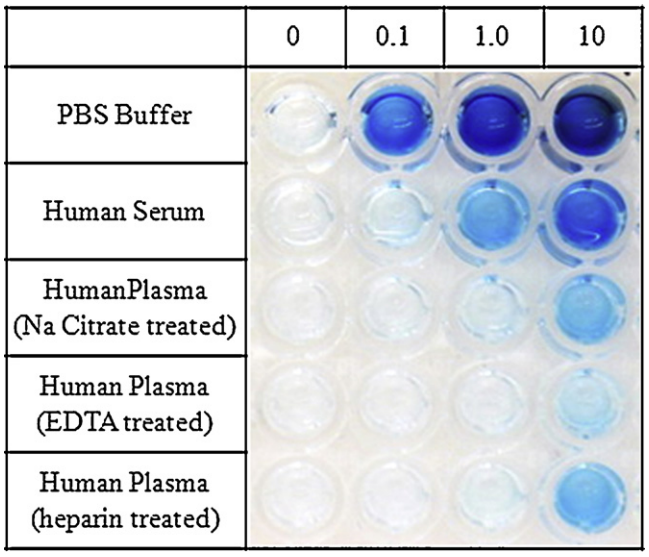


Fig. 3. Results of strategy C sandwich immunoassay testing with nitrated fibrinogen prepared in different samples. The concentrations of nitrated fibrinogen in samples are 0, 0.3, 3.0 and 30 nM from left to right, respectively.

an example, the result of sandwich C based immunoassay testing with different samples was shown in Fig. 3.

To overcome these challenges and improve the sensitivity and selectivity of immunoassays, many factors have been investigated and optimized. Strategy A showed the worst performance even after all the optimization attempts, mainly due to the inability to reduce the nonspecific absorption of fibrinogen. The optimizations of strategies B and C explored effects of many factors involved in each step of these sandwich immunoassays. For primary antibody coating on 96-well plate, the antibody concentration and coating duration were investigated. The results showed that the higher antibody concentration and longer coating time would lead to higher density of coated antibody, thus higher efficiency of capturing nitrated fibrinogen and better sensitivity. Finally, the optimized primary antibody concentration was 20 μg/mL for strategy B and 100 μg/mL for strategy C, while the coating time was optimized to 40 h.

The blocking step is one of the key procedures to decrease the nonspecific absorption from biofluids. Several blocking reagents including 1% casein, 1% BSA, 3% BSA and 3% BSA with 1% PEG 6000 were used in optimizing the blocking step. The experiment results showed that 1% casein produced the best blocking effect and also kept the reactivity of sequential immunoreactions. However, the blocking time was extended to 2 h instead of regular 1 h for assay performed in buffer.

To improve the nitrated fibrinogen capture efficiency in immunoreaction, the dilution buffer, dilution ratio, incubation duration were optimized. To prepare the samples, 0.5% BSA, 3% BSA and 1% casein were used to dilute human plasma and serum. The results suggested that 0.5% BSA dilution buffer provided acceptable capture efficiency and also decreased the nonspecific absorption. Compared with 4 and 10 times dilution, 20 times dilution samples gave higher sensitivity and selectivity. Proper incubation duration, i.e. 30 min, resulted in lower nonspecific absorption and satisfied efficiency of capturing nitrated fibrinogen comparing with 45 min and 1 h incubation. Regarding anticoagulants, the EDTA treated human plasma prepared samples gave the lowest signal comparing with sodium citrate and heparin treated human plasmas. The samples prepared with diluted human serum delivered the highest sensitivity, which was based on the fact that most fibrinogen was subtracted during human serum production.

The optimization of immunoreactions between captured nitrated fibrinogen and HRP conjugated anti-nitrotyrosine was focusing on the selection of buffer and antibody concentration. Two buffers, 0.5% BSA and 0.2% casein, were employed to prepare antibody solution and 1% casein buffer presented satisfied sensitivity as well as minimize the nonspecific absorption of antibody on 96-well plate. The 2 μg/mL antibody prepared in 0.2% casein gave high signal and, at the same time, kept low background, which were important for improving the sensitivity of assay.

After systematic optimization of each step in sandwich immunoassay of nitrated fibrinogen, the spiked biofluid samples and PBS buffer samples were tested and developed with 100 μL TMB color solution in each well. The plate image is shown as Fig. 4.

According to Fig. 4, the immunoassay with both strategies in human serum samples gave about one order lower signal compared with buffer samples. The strategy C assay of human plasma samples presented about one order lower signal than in human serum sample, while the strategy B assay delivered similar signal with both physiological fluids samples. Compared to the results shown in Fig. 3, the buffer samples presented similar results, whereas the signal from human serum and plasma samples has been increased more than one to two orders of magnitude after optimization, which is a significant improvement for nitrated fibrinogen assay in authentic biological samples.

3.4. Fibrinogen assays for biofluid samples

Using optimized reaction conditions, three electrochemical immunoassays were performed to analyze the nitrated fibrinogen spiked in the human serum and human plasma samples. Two assays employed strategy C to test human serum and plasma samples and the results are shown in Fig. 5A and B. Since strategy B presented similar performance for human serum and plasma samples, only the results of human plasma samples were presented in Fig. 5C.

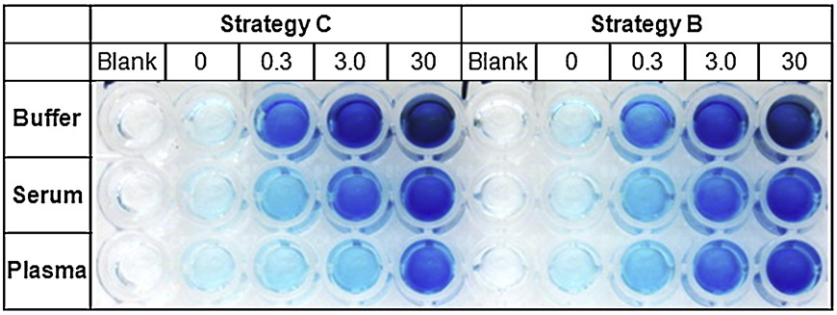


Fig. 4. The image of developed plate using optimized conditions. The blank samples means there were no immunoreactions in these wells. The concentration of spiked nitrated fibrinogen was labeled in nM. The samples were prepared in 0.5% BSA (top), 20 times diluted human serum in 0.5% BSA (middle) and 20 times diluted human plasma in 0.5% BSA (bottom).

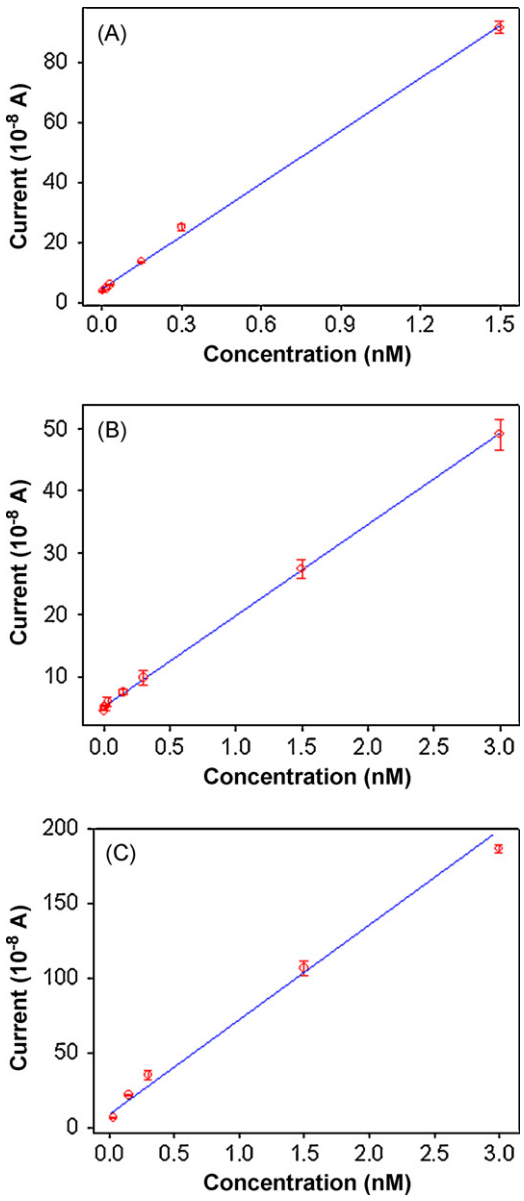


Fig. 5. Strategies B and C based electrochemical immunoassay of nitrated fibrinogen spiked in human plasma and serum. (A) and (B) are calibration curves of strategy C assay of human serum samples and plasma samples, respectively. (C) Calibration curve of strategy B assay of human plasma.

Compared with assays for buffer samples, the linear detection ranges were significantly expanded up to 1.5–3.0 nM, which was 5 or 10 times higher than the buffer samples, and the regression coefficients of all three assays were > 0.99. On the other hand, the slope of the calibration curve in low concentration range was smaller than in buffer samples, resulting in a higher detection limit. Strategy C can detect 6.9 pM nitrated fibrinogen spiked in human serum samples and 20.7 pM in human plasma samples, while strategy B assay can detect 3.9 pM nitrated fibrinogen spiked in human serum. These results corresponded to the spectroscopic results shown in Fig. 4. Considering the high concentration (0.3%, i.e. 9.0 μM) of fibrinogen in human plasma [39], the sensitivity of these assays is sufficient to detect as low as 0.1% of nitration of fibrinogen in human plasma samples even if only one nitration site is present in the protein. Moreover, the exceptional selectivity of these assays also can be confirmed by the fact that the extreme high concentration of fibrinogen in plasma did not introduce high background. The improved electrochemical immunoassay provides a rapid nitrated fibrinogen assay for biofluids and other biological samples with excellent selectivity and high sensitivity. In particular, strategy B provides a more general assay suitable for measuring total nitration in biological samples.

4. Conclusion

Electrochemical immunoassays, based on three sandwich strategies for detection of nitrated human fibrinogen, as well as total nitration, have been established. To our best knowledge, this is the first example of an electrochemical immunoassay analyzing nitrated proteins in biofluids. In this study, nitrated human fibrinogen, a potential oxidative stress biomarker for several pathologies, was chosen as the analyte. All three sandwich strategies immunoassays demonstrated high sensitivity and excellent specificity for nitrated fibrinogen analysis in the buffer sample. A series of optimizations were carried out to improve the analysis performance of these immunoassays in biofluids. After careful optimizations, the sensitivity of these assays for human serum and plasma samples has been improved more than two orders without sacrificing selectivity. The detect limits reached pM level and the linear response ranges were up to 1.5 or 3.0 nM. All together, the performance of these sandwich electrochemical immunoassays is sufficient for nitrated fibrinogen detection in human serum and plasma samples. The success of sandwich electrochemical immunoassay of nitrated fibrinogen found a novel approach to establish rapid, sensitive, selective, cost efficient and robust bioassay for oxidative stress pathology study and clinical applications. Moreover, the sandwich strategies developed in this paper can be readily used to establish effective bioassay targeting other nitration biomarkers and total protein nitration.

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References

[1] A. Borbely, A. Toth, I. Edes, L. Virag, J.G. Papp, A. Varro, W.J. Paulus, J. van der Velden, G.J.M. Stienen, Z. Papp, Cardiovasc. Res. 67 (2005) 225.
[2] M.J. Mihm, C.M. Coyle, B.L. Schanbacher, D.M. Weinstein, J.A. Bauer, Cardiovasc. Res. 49 (2001) 798.
[3] P. Nowak, B. Wachowicz, Platelets 13 (2002) 293.
[4] P. Pacher, J.S. Beckman, L. Liaudet, Physiol. Rev. 87 (2007) 315.
[5] J.E. Mahaney, J. Russ, L. Debelka, C. Bergman, N. Schneider, T. Knyushko, D. Bigelow, Biophys. J. (2007) 146A.
[6] S.Q. Xu, J. Ying, B.B. Jiang, W. Guo, T. Adachi, V. Sharov, H. Lazar, J. Menzoian, T.V. Knyushko, D. Bigelow, C. Schoneich, R.A. Cohen, Am. J. Physiol.-Heart C 290 (2006) H2220.
[7] G. Peluffo, R. Radi, Cardiovasc. Res. 75 (2007) 291.
[8] N. Ahmed, R. Babaei-Jadidi, S.K. Howell, P.J. Beisswenger, P.J. Thornalley, Diabetologia 48 (2005) 1590.
[9] Z.L. Xie, M.H. Zou, Diabetes 57 (2008) A181.
[10] H.M. Bosse, S. Bachmann, Hypertension 30 (1997) 948.
[11] J.S. Beckmann, Y.Z. Ye, P.G. Anderson, J. Chen, M.A. Accavitti, M.M. Tarpey, C.R. White, J.S. Beckman, Biol. Chem. Hoppe Seyler 375 (1994) 81.
[12] D. Mittar, R.S. Deeb, F.X. Zhao, D.P. Haijar, R.K. Upmacis, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 22.
[13] B. Pignatelli, C.Q. Li, P. Boffetta, Q.P. Chen, W. Ahrens, F. Nyberg, A. Mukeria, I. Bruske-Hohlfeld, C. Fortes, V. Constantinescu, H. Ischiropoulos, H. Ohshima, Cancer Res. 61 (2001) 778.

[14] J. Kanski, A. Behring, J. Pelling, C. Schoneich, Am. J. Physiol.-Heart C 288 (2005) H371.
[15] H. Ischiropoulos, Nitric Oxide-Biol. Chem. 11 (2004) 34.
[16] P. Nowak, H.M. Zbikowska, M. Ponczek, J. Kolodziejczyk, B. Wachowicz, Thromb. Res. 121 (2007) 163.
[17] C. Vadseth, J.M. Souza, L. Thomson, A. Seagraves, C. Nagaswami, T. Scheiner, J. Torbet, G. Vilaire, J.S. Bennett, J.C. Murciano, V. Muzykantov, M.S. Penn, S.L. Hazen, J.W. Weisel, H. Ischiropoulos, J. Biol. Chem. 279 (2004) 8820.
[18] J.P. Crow, H. Ischiropoulos, Nitric Oxide, Pt B, Academic Press Inc., San Diego, 1996, p. 185.
[19] G.D. Cymes, C. WolfensteinTodel, Biochim. Biophys. Acta-Protein Struct. Mol. Enzym. 1294 (1996) 31.
[20] Y. Ohkura, M. Kai, H. Nohta, J. Chromatogr. B 659 (1994) 85.
[21] M.F. Beal, W.R. Matson, K.J. Swartz, P.H. Gamache, E.D. Bird, J. Neurochem. 55 (1990) 1327.
[22] M.K. Shigenaga, H.H. Lee, B.C. Blount, S. Christen, E.T. Shigeno, H. Yip, B.N. Ames, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 3211.
[23] J.R. Crowley, K. Yarasheski, C. Leeuwenburgh, J. Turk, J.W. Heinecke, Anal. Biochem. 259 (1998) 127.
[24] C. Leeuwenburgh, M.M. Hardy, S.L. Hazen, P. Wagner, S. Ohishi, U.P. Steinbrecher, J.W. Heinecke, J. Biol. Chem. 272 (1997) 1433.
[25] C. Herce-Pagliai, S. Kotecha, D.E.G. Shuker, Nitric Oxide-Biol. Chem. 2 (1998) 324.
[26] T. Franze, M.G. Weller, R. Niessner, U. Poschl, Analyst 128 (2003) 824.
[27] J. Khan, D.M. Brennan, N. Bradley, B.R. Gao, R. Bruckdorfer, M. Jacobs, Biochem. J. 330 (1998) 795.
[28] L. Asensio, I. Gonzalez, T. Garcia, R. Martin, Food Control 19 (2008) 1.
[29] E. Liu, G.S. Eisenbarth, Clin. Immunol. 125 (2007) 120.
[30] G. Liu, Y.Y. Lin, J. Wang, H. Wu, C.M. Wai, Y. Lin, Anal. Chem. 79 (2007) 7644.
[31] J. Wang, G. Liu, M.H. Engelhard, Y. Lin, Anal. Chem. 78 (2006) 6974.
[32] M. az-Gonzalez, M.B. Gonzalez-Garcia, A. Costa-Garcia, Electroanalysis 17 (2005) 1901.
[33] G.D. Liu, Y.H. Lin, Talanta 74 (2007) 308.
[34] J. Wang, G.D. Liu, H. Wu, Y.H. Lin, Small 4 (2008) 82.
[35] Y.Y. Lin, J. Wang, G.D. Liu, H. Wu, C.M. Wai, Y.H. Lin, Biosens. Bioelectron. 23 (2008) 1659.
[36] A. Warsinke, A. Benkert, F.W. Scheller, Fresenius J. Anal. Chem. 366 (2000) 622.
[37] C.A. Sacksteder, W.J. Qian, T.V. Knyushko, H.X. Wang, M.H. Chin, G. Lacan, W.P. Melega, D.G. Camp, R.D. Smith, D.J. Smith, T.C. Squier, D.J. Bigelow, Biochemistry 45 (2006) 8009.
[38] I. Losito, F. Palmisano, P.G. Zambonin, Anal. Chem. 75 (2003) 4988.
[39] C.D. Krohn, O. Reikeras, S. Bjornsen, F. Brosstad, Blood Coagul. Fibrinol. 10 (1999) 167.