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Development of a surface plasmon resonance immunosensor and ELISA for 3-nitrotyrosine in human urine

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ABSTRACT

3-Nitrotyrosine (3-NT) is thought to be a relevant biomarker of nitrosative stress which is associated with many inflammatory and chronic diseases. It is necessary to develop confidential method for specific and sensitive 3-NT detection. In this paper, on the basis of anti-3-NT specific antibody, we developed a label-free indirect competitive surface plasmon resonance (SPR) immunosensor and ELISA for the detection of 3-NT. Under the optimized conditions, the SPR immunosensor can obtain a linear range of 0.17–6.07 μg/mL and a limit of detection (LOD) of 0.12 μg/mL while the ELISA can reach 0.33–9.94 μg/mL and a LOD of 0.24 μg/mL.The selectivity of 3-NT was also testified by six kinds of amino acid analogues. Besides, the developed SPR immunosensor was compared thoroughly with a conventional ELISA in spiked analysis of urine samples. Good recoveries and correlation between these two methods were observed (R^2 = 0.964). Therefore, it is concluded that the automated SPR platform can be applied to quantify 3-NT in biological samples with its sensitivity, accuracy, and real-timing.

1. Introduction

Reactive nitrogen species (RNS) derived from inflammatory cells can mediate the nitration of tyrosine to form 3-nitrotyrosine which has been identified as a stable final product [1]. Increased level of 3-NT has been associated with a wide range of diseases such as atherosclerosis [2], rheumatoid arthritis [3], Alzheimer [4], cardioplegia [5], diabetes [6,7], and so on. Therefore, 3-NT turns to be a clinically relevant biomarker of nitrosative stress and a sensitive, specific, and reliable method for its monitoring in biological samples which is necessary and important in understanding the etiology of these diseases.

Currently, green approaches have been established for determination of different bio(chemical) species in biological samples [8–10]. 3-NT has been determined in serum, urine and tissues by high-performance liquid chromatography (HPLC) (the limit of detection (LOD) reported as 1.86 ng/mL) [11], solid-phase extraction- molecularly imprinted polymer (SPE-MIP) (LOD of 0.7 μg/mL) [12], SPE-HPLC (LOD of 27.1 ng/mL) [13], realtime-tandem mass spectrometry (DART-MS/MS)(LOD of 2 μg/mL) [14]. Despite the advantage of being highly sensitive and selective, instrumental methods require extensive sample preparation and cleanup procedures, which is quite laborious and time-consuming. Moreover, the methods still need advanced infrastructure to support complex instrumentation, and specific expertise as well as well-trained operators. On the other hand, immunoassay methods have been proven to be the choice for easy, relatively inexpensive, and high-throughput screening.

In the published literature, in terms of 3-NT quantification, there are a variety of methods based on ELISA, such as the indirect, competitive, and sandwich-ELISA [15–17]. In some cases, the sandwich-ELISA only measures protein associated-3-NT for its limitation on binding sites. Although the reported sandwich-ELISA could reach a high sensitivity (LOD of 1.8 nM to 3-NT), it should be noted that the sandwich ELISA could not detect free 3NT in serum or plasma. It requires the presence of two epitopes of a molecule to be detectable by this ELISA [18]. As a matter of choice, enzyme-linked immunosorbent assay (ELISA) presents its merits as a simple, sensitive, specific and inexpensive tools for

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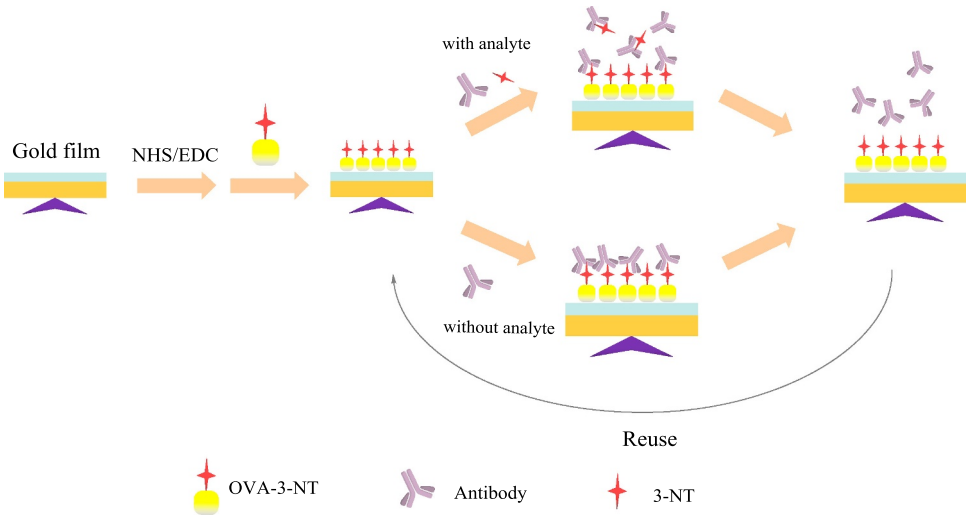


Fig. 1. The basic principle of SPR immunosensor.

analysis of various targeted analytes. However, ELISA is a heterogeneous method, which involves multiple washing steps, and long reaction time.

Surface plasmon resonance (SPR) is a label-free technique for its direct monitoring of ligands and receptor (or analyte) interaction [19]. When the analyte was specifically combined with the ligands, the weight on the gold firm was increasing and would cause the changes of refractive index (RI). Then, the detectors measured the changed SPR angle which was associated with RI. Hence, the SPR detectors could generate mass-dependent signals, and realize real-time monitoring. In this indirect competitive SPR immunosensor, if the concentration of the target analyte is high in the sample, it will combine and consume more antibody firstly, and then there will be less antibody that could bind with 3-NT-OVA on the surface of the sensor chip consequently. The less antibody binds with 3-NT-OVA, the less the weight on the surface of gold film, which results in a slight change of RI and, therefore, the RU is low. On the contrary, if the concentration of the target analyte is low, more antibody remain and 3-NT-OVA will combine with the antibody, which results in a distinct change of RI and, the RU is high. The basic principle of SPR immunosensor is depicted in Fig. 1. Compared with traditional immunoassay technology, SPR immunosensor has the features of simple structure, label-free property, fast detection speed, and automatic operation [20]. Besides, immunoassay based on SPR has been proven to be the choice for an easy, relatively inexpensive, and high-throughput screening of environmental contaminants [21,22], food additives [23,24], and biological metabolites [25].

In general, 3-NT exists in a free state or is protein-conjugated in biological fluid. When reactive nitrogen species (RNS) reacts with L-tyrosine and protein-associated Tyr, free 3-nitrotyrosine and protein-associated 3-nitrotyrosine are formed [26]. Most researchers adopted the nitration of tyrosine and carrier protein like BSA or KLH as antigen [27,28]. However, the nitration of tyrosine or other protein is a selective process that its immunogenicity is unpredictable. In some researches, antibody based on nitration of protein could not recognize free 3-NT or might have a low affinity for free 3-NT [27]. Therefore, it is important to produce a kind of antibody based on 3-NT that might be bound to free 3-NT.

Herein, based on the structure of 3-NT, we produced a specific antibody against to 3-NT with high titre. In the paper, a sensitive and specific label-free SPR immunosensor and ELISA were developed based on an indirect competitive assay. Under the optimal conditions, the developed SPR immunosensor was successfully applied to detect 3-NT in human urine samples and validated by ELISA. The developed SPR immunosensor was time-saving in analyzing a sample within 7 min, and more automated, accurate and reproducible than other reported methods. Moreover, it showed a more intuitive way for the binding between antibody and antigen, Therefore, the developed SPR immunosensor is a promising method to rapidly detect 3-NT in urine samples.

2. Material and methods

2.1. Materials and reagents

3-Nitrotyrosine (3-NT), 3-chlorotyrosine were purchased from Macklin (Shanghai, China). L-methionine, L-phenylalanine, tryptophan, L-cysteine, glycine were obtained from Sangon Biotech (Shanghai, China). Sensor chip CM5 research grade (BR-1000-14), 10 × HBS-EP buffer (BR-1001-88), NHS coupling solution (22-0526-53), and EDC coupling solution (22-0526-54) were obtained from GE Healthcare (Little Chalfont, U.K.). Freund's adjuvant was obtained from Sigma (Paris, France). Freund' s complete adjuvant, Freund's incomplete adjuvant, bovine serum albumin (BSA) and ovalbumin (OVA) were purchased from Sigma (St. Louis, MO, USA). Glutaraldehyde and ethanolamine were purchased from Damao chemical reagent factory (Tianjin, China).

The biological interaction analyzer (BIAcore T200) was from GE Healthcare (Pittsburgh, USA). Microplate reader (Multiskan FC) was from Thermo Fisher Scientific (Waltham, USA).

2.2. Production of antibody against 3-nitrotyrosine

The immunogen was prepared by conjugating 3-NT to the carrier protein, BSA, with the active ester method with minor modification [29]. Hapten 3-NT (22 mg) was dissolved in 0.1 M hydrochloric acid (5 mL) with pH 0.1 M sodium hydroxide adjust pH to 4–6. To this solution, EDC-HCl (60.7 mg) and NHS (15.2 mg) dissolved in H_2O were dropwise added, respectively. And the formation of the mixed solution was allowed to proceed overnight at RT. The solution was then added dropwise to an ice-cold solution of BSA (113 mg) in 5 mL of PBS with stirring overnight at 4 °C. Finally, the mixture was dialyzed against PBS for 3 days at 4 °C. Meanwhile, the 3-NT-OVA conjugate was synthesized as coating antigen for ELISA and detection antigen for SPR. The 3-NT-OVA was prepared by the use of glutaraldehyde crosslinking according to the previously reports [30]. Both antigens were identified by SDS-PAGE (data not shown).

Female New Zealand white rabbit was immunized with 3-NT-BSA using the scheme described previously [31] with minor modification.

Briefly, 500 μg of the 3-NT-BSA dissolved in 500 μL of PBS was emulsified with Freund's complete adjuvant (1/1, v/v) and injected intradermally at multiple sites on the back of the rabbit. After a month, the rabbit was boosted with an additional 500 μg of the conjugate emulsified with Freund's incomplete adjuvant (1/1, v/v). Four booster immunizations was performed at two weeks intervals and then the rabbit serum was collected. The serum was centrifuged and stored at − 20 °C until use.

2.3. Indirect competitive ELISA immunosensor

The titre of anti-3-NT polyclonal antibody was determined by ELISA. Under the optimized conditions, Indirect competitive ELISA was performed as follow, The microplates were coated with 100 μL/well of 3-NT-OVA (5 μg/mL) in coating buffer at 37 °C for 2 h. After discarding the buffer and washing the microplates five times with PBST (PBS containing 0.5% Tween-20), the non-specific binding was blocked with 270 μL/well of 3% skim milk power (w/v) in PBS at 37 °C for 1 h. After another washing step, 50 μL/well of anti-3NT pAb diluted 1:4000 in PBS was incubated together with 50 μL/well of standard 3-NT, or its analogues (0, 0.01, 0.1, 1, 10, 100, 1000 μg/mL) for 1 h. After washing again, 100 μL/well of goat anti-rabbit IgG-HRP (diluted in 1:5000) was added and incubated at 37 °C for 1 h. After another washing with PBST, 100 μL/well of the colorimetric substrate TMB was added and the plate was incubated for 15 min. After stopping the color development with 0.5 M sulfuric acid (50 μL/well), the absorbance $A_{405\,nm}$ was measured.

2.4. Immobilization of 3-NT-OVA conjugate on the SPR chip surface

The 3NT-OVA conjugates were immobilized onto the surface of the CM5 sensor chip by active ester method. Firstly, 0.4 M EDC solution and 0.1 M NHS solution was set to injection into the flow cell at a steady 30 μL/min rate for 15 min. And then 100 μg/mL 3-NT-OVA in acetate buffer (pH = 4.0) was injected into the flow cell for 15 min. Next, 1 M ethanolamine solution (pH = 8.5) was injected into the flow cell to block unreacted carboxyl group. During the whole process, HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20) was used as the running buffer.

2.5. Indirect competitive SPR immunosensor

The measurement procedure in this research was designed based on the principle of competitive immunoassay. The 3-NT standard working solution in HBS-EP buffer at different concentrations (0, 0.01, 0.1, 1, 10, 100, 1000 μg/mL) was initially pre-incubated with the anti-3-NT-PAb diluted in 1:100 for 30 min. After rinsing the chip surface to a steady baseline with running buffer at 30 μL/min, the mixture was injected into the flow cell at 30 μL/min rate for 3 min individually to allow the unbound Abs to bind with the 3-NT-OVA conjugates that were immobilized on the surface of the sensor chip. Next, 0.01 M Gly-HCl (pH 1.5) was chosen as regeneration buffer as previously reported [24,32] that would be injected into the flow cell at a rate of 30 μL/min for 2 min and then rinsed with running buffer for the next measurement.

2.6. Selectivity of SPR immunosensor

After the optimization, the specificity of the developed ELISA and SPR immunosensor was investigated by using various kinds of amino acid with similar structures to 3-NT. The cross-reactivity (CR) was calculated according to the following equation:

$$CR\ \% = [IC_{50}(3-NT)/IC_{50}(cross-reactant)] \times 100\%.$$

2.7. Spiked samples analysis

Human urine samples were collected from the volunteers in laboratory. The collected urine samples (10 mL of each sample) were centrifuged at 13,000 rpm for 10 min at 4 °C to remove any solid debris. The resulting supernatant was then filtered through a syringe filter (0.22 μm) and immediately stored at − 80 °C until use. 3-NT dissolved in PBS and HBS-EP buffer were spiked into the above samples to get three final concentrations of 50, 250, 500 ng/mL, respectively. And the recoveries were determined by ELISA and SPR immunosensor.

3. Results and discussion

3.1. Production of Anti-3NT polyclonal antibody

As a micromolecule, 3-NT is difficult to produce immune response directly as an immunogen. Therefore, the synthesis of 3-NT and carrier protein is essential to enhance the immunogenicity of the 3-NT. On the basis of the structure of 3-NT, we designed two coupling ways which specific to its amino and carboxyl groups, as immunogen and coating antigen, respectively. In this study, after routine immunization, we collected the rabbit serum and determined its titre. The titre of anti-3-NT pAb was determined by indirect ELISA, and the highest titre (1:64,000) was observed in the experiment to meet the requirements for further use.

3.2. Immobilization of the 3-NT-OVA conjugate

It is quite important to combine more 3-NT-OVA as detecting antigen to the surface of CM-5 sensor chip, so that it could generate a stronger signal when the pre-incubated remaining antibody flowed through the sensor. Acetate buffer solutions with different pH values (4.0, 4.5, 5.0, and 5.5) were used to prepare the 3-NT-OVA solution (100 μg/mL) to optimize the immobilization on the chip surface, and 50 mM NaOH was used as regeneration buffer. Fig. 2**a** showed the results of 3-NT-OVA immobilization under different pH conditions in 10 mM acetate buffer solution. It was found obviously that when the pH reached 5.5 or 5.0, there was hardly any increasing signal of binding. When the pH reached to 4.5 or 4.0, it could be observed that the sensor chip had an increasing signal which demonstrated that 3-NT-OVA could bind with the activated carboxyl dextran on the surface of the CM-5 sensor chip in that condition. In order to achieve more binding between sensor chip and 3-NT-OVA, we chose pH 4.0 for the later immobilization.

After the activated by EDC/NHS for 15 min, the sensor chip was flowed with 3-NT-OVA and diluted in acetate buffer solution (100 μg/mL, pH 4.0) for 15 min 3-NT-OVA was set to link with the sensor chip as much as it could. As Fig. 2**b** showed, 3-NT-OVA immobilization could reach the 4106 RU value after being blocked with ethanolamine, which was sufficient for the further analysis.

3.3. Optimization of the Ab concentration ab concentration

In an immunology, the concentration of the antibody was a crucial parameter that affected sensitivity, reproducibility and stability of the antibody. Insufficient antibody would result in a weak SPR signal and narrow detection range, while excessive antibody would affect the sensitivity of the immunosensor. Optimization was carried out to analyze the dilution of antibody (1:800, 1:400, 1:200, 1:100) without or with 10 μg/mL free 3-NT.

As is showed in Fig. 3**a**, when there was no 3-NT in solution, the SPR response increased from 25.6 to 180.4 RU and the Ab concentration increased from 1/800 to 1/100, signifying that the amount of Ab bound on the chip surface was growing, which was in line with the principle of antigen-antibody binding. When free 3-NT (10 μg/mL) was in the mixed solution, the SPR response decreased to 7.4 and 52.2 RU, respectively.

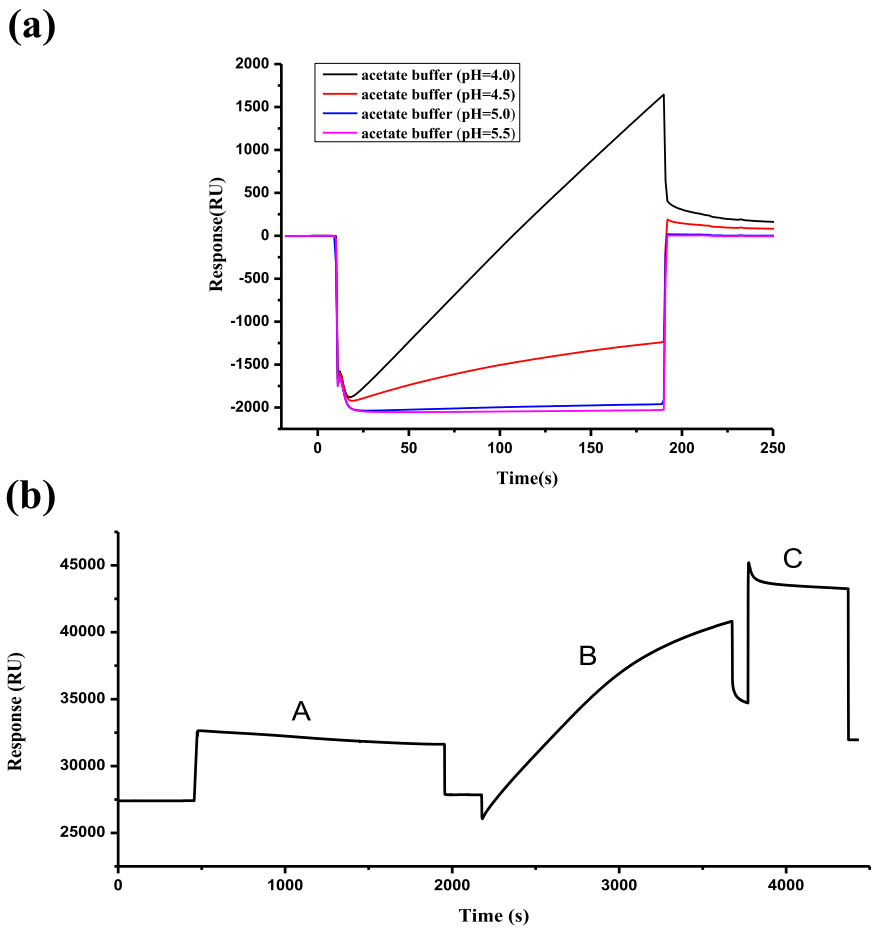


Fig. 2. (a) The effect of different pH in pre-immobilization process of 3-NT-OVA conjugate on the SPR chip surface. (b) The immobilization process of 3-NT-OVA conjugate on the SPR chip surface. A, The pro-combined process; B, EDC/NHS activation process; C, 3-NT-OVA immobilization; D, ethanolamine blocking process.

Because of the competition binding inhibition from free-3-NT and the 3-NT-OVA conjugated on the chip surface. As is shown in Fig. 3**b**, δRU (RU-RU_0) were also calculated which might be the level of response range of each dilution. And the inhibition rates were from 0.25 to 0.28. Taking into consideration of the cost of pAb to avoid waste and the performance of binding antigen, pAb diluted in 1:200 was selected for competition analysis in this research.

3.4. Cross-reactivities

Several analogues were used to further validate the specificity of the SPR immunosensor. As is shown in Table 1, the immunosensor yielded highly specific recognition to 3-NT compared to other types of amino acid. Moreover, the IC_{50} (concentration corresponding to Bi/B0 = 50%) of the 3-NT was calculated to be 1.01 μg/mL. In addition, the IC_{50} of

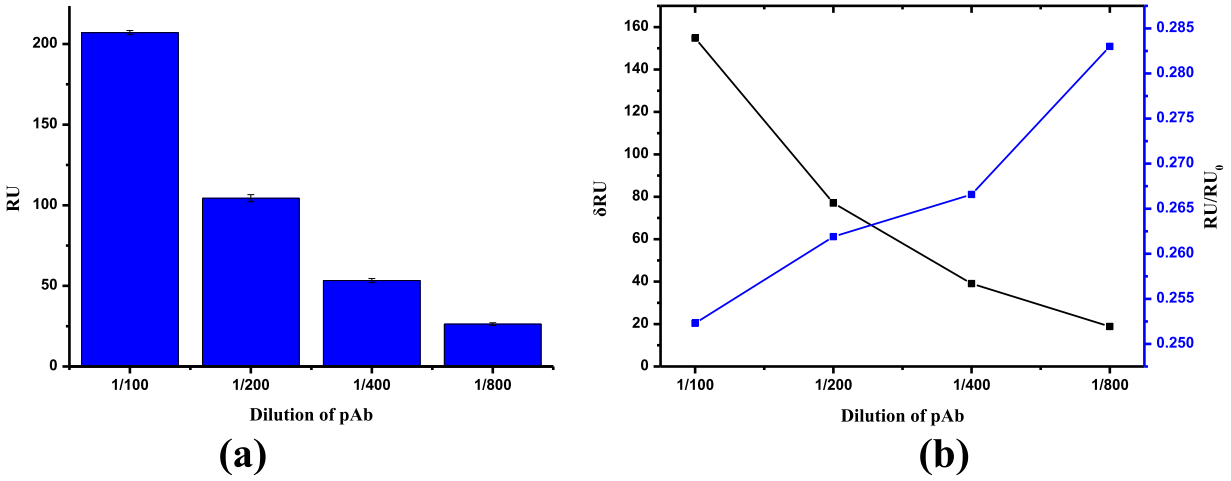
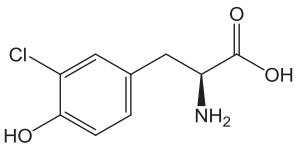
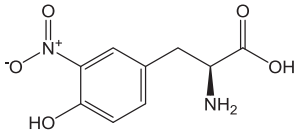
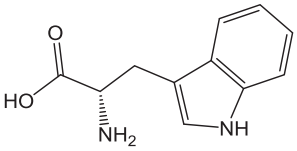
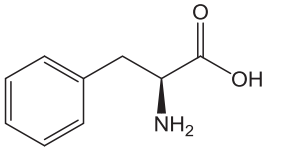
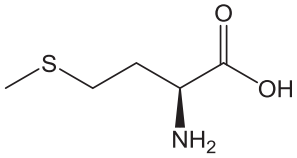
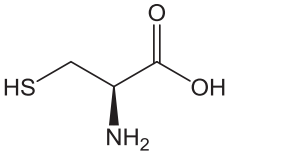
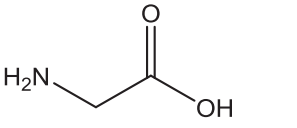


Fig. 3. Results of optimization of antibody concentration in detection process. (a) results of different dilution of pAb without 3-NT; (b) results of different dilution of pAb with 10 μg/mL 3-NT.

Table 1
Cross-reactivity of the antibody to 3-NT and related compounds by SPR immunosensor.

Chemical	Structure	IC_{50}(μg/mL)	CR(%)
3-nitrotyrosine		1.01	100
3-chlorotyrosine		> 1000	< 0.1
Tryptophan		> 1000	< 0.1
Phenylalanine		> 1000	< 0.1
Methionine		> 1000	< 0.1
Cysteine		> 1000	< 0.1
Glycine		> 1000	< 0.1

other six analogues was higher than 1 mg/mL and the cross-reactivity was below 0.1%. As a result, there were no problems with the sensitivity or specificity for the developed immunosensor.

3.5. Indirect competitive ELISA and comparison between the two immunosensors

An indirect competitive ELISA was also developed for 3-NT detection in human urine, with pAb diluted at 1:8000 and HRP-labeled secondary antibody diluted at 1:5000. Fig. 4**a** showed the sensorgram of the indirect competitive SPR immunosensor. When there was more 3-NT in pre-incubated solution, less antibody could be combined with 3-NT-OVA on the surface of the sensor, and resulting in lower response unit (RU) value.

After optimization, both SPR immunosensor and ELISA standard curves for 3-NT were constructed. The four-parameter logistic equations were y = 0.64/[1 + (x/1.82)^0.82] + 0.36 while the value of R^2 was 0.9989 for ELISA, and y = 0.83/[1 + (x/1.01)^0.77] + 0.21 while the value of R^2 was 0.9992. As is shown in Fig. 4b, for ELISA, the IC_{50} value was 1.82 μg/mL, LOD was 0.24 μg/mL and the working range was 0.33–9.94 μg/mL, and SPR immunosensor attained a linear range of 0.17–6.07 μg/mL and LOD of 0.12 μg/mL. Although the two immunosensors provided similar linear detection ranges, the SPR immunosensor provided twice lower LOD, and was more advantageous in labeld reagents and analysis time. Moreover, the whole analysis can be completed with automatic injection and signal generation. As is shown in Table 2, our method had its comparative advantage over some ELISA methods in terms of sensitivity, duration time and reproducibility. Even if sandwich ELISA methods (reported as LOD of 1.8 nM [17]) had much higher sensitivity, they were involved with time-consuming procedures and could not detect free 3NT in serum or plasma. A SPR immunoassay was also reported for 3-NT [33], which had higher sensitivity than our method but provided an alkanethiol monolayer with hydrophobic surface which might not be satisfying for the interactions of biological molecules. In addition, our method was preformed with less time and higher reuse cycles that might be more cost-effective [24]. Besides, our method had better sensitivity than some reported chromatographic methods. HPLC methods were performed with much higher sensitivity, but they needed expensive instruments and time-consuming procedures while our method was more time-saving and automated.

3.6. Recovery study in human urine

In this paper, the matrix effect was evaluated in artificial urine which was prepared according to the previously report [34], so as to ensure the accuracy and sensitivity of the SPR analysis. The obtained matrix calibration curves are shown in Fig. 5. It was observed that the calibration curve for the artificial urine was corresponding with the standard curve in the error tolerance range. The IC_{50} values for the

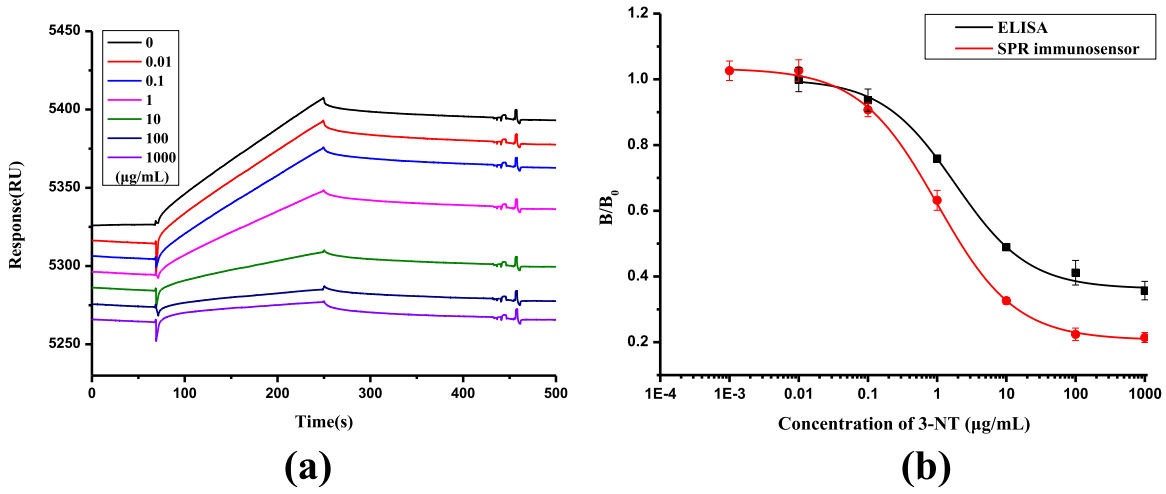


Fig. 4. (a) The detecting process of 3-NT-OVA conjugate on the SPR chip surface. (b) SPR immunosensor and ELISA calibration curve for 3-NT.

Table 2
Comparison between different methods used for the determination of free 3-NT in biological samples.

Method	LOD (μg/mL)	Linear range (μg/mL)	Analysis time (min)	Reuse cycles	Reference
HPLC	1.86×10^{-3}	N.S.	> 30 min	N.S.	[11]
SPE-MIP	0.70	2.50–55	> 30 min	N.S.	[12]
SPE-HPLC	0.03	N.S.	> 30 min	N.S.	[13]
DART-MS/MS	2	4–100	> 30 min	N.S.	[14]
Competive ELISA	N.S.	2.26–2260	> 120 min	No	[15]
SAM-SPR	1.86×10^{-3}	0.03–4.80	9 min	200 cycles	[31]
Indirect ELISA	0.24	0.33–9.94	> 120 min	No	This work
SPR immunosensor	0.12	0.17–6.07	7 min	≥300 cycles	This work

SAM: Self-assembly monolayer *N.S.*: not state.

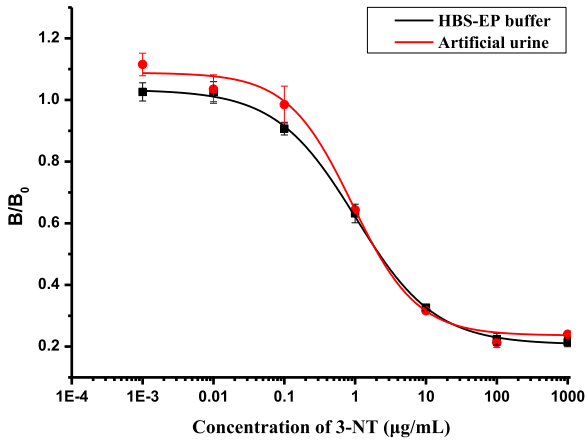


Fig. 5. Calibration curves in HBS-EP buffer and artificial urine with the SPR immunosensor.

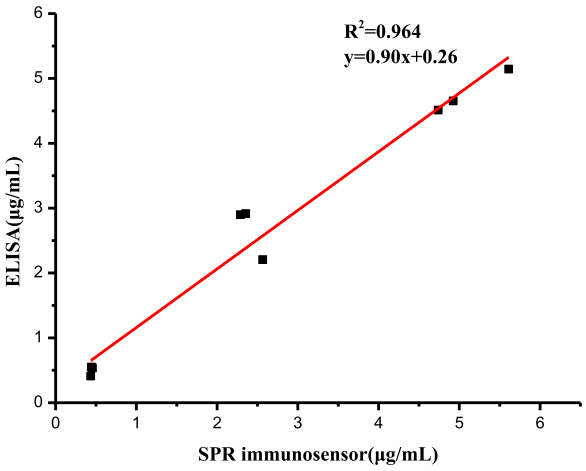


Fig. 6. Correlation between SPR immunosensor and ELISA results for human urine spiked with 3-NT.

artificial urine was 0.91 while the buffer was 1.01 μg/mL. Next, the newly-developed SPR immunosensor for the spiked samples was validated by a comparison with the results of ELISA. The recoveries of 3-NT from human urine determined by SPR immunosensor were in a range of 82.1–116.6%, and by ELISA in a range of 86.8–112.2%, respectively (Table 3). Both methods were acceptable for spiked study and showed good recoveries and correlated well with each other (Fig. 6). The developed SPR immunosensor was demonstrated to be a valid method to detect 3-NT in human urine.

4. Conclusion

In this study, the biosensor to combine immunoassay with SPR technique for the selective determination of 3-nitrotyrosine concentration has been developed. The sensor works on the basis of a highly selective interaction between specific antibody and 3-NT. We have established the SPR immunosensor and ELISA for free 3-NT determination. The SPR immunosensor has presented itself with high sensitivity, selectivity, and good stability. The SPR immunosensor was thoroughly compared with an indirect competitive ELISA in spiked human urine sample, and was demonstrated more advantageous in terms of detection limit, reagent consumption, analysis time, and operation automation than conventional ELISA. In short, the proposed SPR immunosensor could be considered as a promising method and sensing platform for real-time monitoring and quantitation of 3-NT residue in the human urine samples.

Table 3
Recovery of 3-NT from spiked human urine samples by ELISA and SPR immunosensor.

Samples	Spiked level (μg/mL)	ELISA (n = 3)			SPR immunosensor (n = 2)		
		Founded (μg/mL)	Recovery (%)	CV (%)	Founded (μg/mL)	Recovery (%)	CV (%)
Sample 1	0.5	0.43 ± 0.01	86.8	2.3	0.41 ± 0.01	82.1	2.5
	2.5	2.57 ± 0.14	102.6	5.4	2.21 ± 0.14	88.3	6.5
	5	5.61 ± 0.23	112.2	4.1	5.14 ± 0.25	102.9	4.9
Sample 2	0.5	0.46 ± 0.04	91.3	8.9	0.54 ± 0.01	107.2	2.0
	2.5	2.29 ± 0.11	91.6	4.8	2.90 ± 0.10	116.0	3.5
	5	4.74 ± 0.44	94.8	9.2	4.51 ± 0.12	90.2	2.6
Sample 3	0.5	0.44 ± 0.06	88.7	13.6	0.55 ± 0.00	110.0	0.8
	2.5	2.35 ± 0.26	94.2	11.2	2.91 ± 0.10	116.6	3.5
	5	4.93 ± 0.19	98.5	3.9	4.65 ± 0.24	93.1	5.2

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Conflict of interest

The authors declare that there is no conflict of interests to publish this paper.

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