Rapid Fluorescence Polarization Immunoassay for Highly Sensitive Detection of 2,4,6-Trinitrotoluene

WENZONG LU[*], YUAN NI, GUANGFENG JIA, HAIXIAN PAN and CHEN ZHAO

Department of Biomedical Engineering, Xi'an Technological University, Xi'an 710021, Shaanxi Province, P.R. China

*Corresponding author: Tel: +86 29 86173358; E-mail: wenzonglu@126.com

Detection of explosives is of great significance in public security programmer and environmental science. In the present work, a fluorescence polarization immunoassay based on a monoclonal antibody for the detection of 2,4,6-trinitrotoluene was established and optimized. Fluorescein-labeled (tracer) was synthesized and purified by column chromatography. The immunoassay exhibited excellent sensitivity for the detection of 2,4,6-trinitrotoluene with good stability. The detection limit was 5 µg mL^{-1}, within a response time of 4 min. The cross-reactivity of the method to 1,3,5-trinitrohexahydro-1,3,5-triazine, 2,4,6-trinitrophenol, 2,4,6-trinitrobenzoic acid, 2-nitrotoluene and 2,4-dinitrotoluene, were very low (cross-reactivity < 0.01 %). The method developed is characterized by high specificity and reproducibility (coefficients of variance ranged from 2.7-6.5 % for interassay and from 1.6-4.4 % for intra-assay). The relative error value was less 1 % in assay of simulating environmental sample. The fluorescence polarization immunoassay method can be applied to the screening of environmental sample for 2,4,6-trinitrotoluene without complicated operation.

Keywords: Antibody, Explosive, Fluorescence polarization immunoassay, 2,4,6-Trinitrotoluene.

INTRODUCTION

The existence of explosive nitroaromatics such as the ubiquitous 2,4,6-trinitrotoluene (TNT) at various operational and defunct military installations and munitions testing grounds throughout the world[1]. Monitoring the concentration of explosive materials in environmental water is important for maintaining aquatic health and eventually ensuring safe drinking water supplies as well as tracking and locating explosives materials. The environmental protection agency deems 2,4,6-trinitrotoluene as toxic because it produces both toxic and mutagenic effects to the life including human, fish and microorganisms[2]. Consequently, several analytical methods have been developed for the detection and monitoring of explosive materials. Generally, laboratory assays for explosives have been performed using analytical instrumentation including mass spectrometric detection[3,4], ion mobility spectrometry[5,6], surface-enhanced Raman spectroscopy[7], terahertz spectroscopy[8], X-ray[9], surface plasmon resonance[10], *etc*. Although highly sensitive and reliable, they are time-consuming, involve multiple steps in sample preparation and analysis and require expensive equipment and skilled analysts.

Nowadays, with increasing development of immunoassays, these methods have been shown to be useful alternative for analysis of explosive materials in environmental samples.

Immunosensors have also become increasingly practical tools in environmental monitoring. Recently the use of immunochemical methods has gained widespread acceptance for measuring environmental pollutants, including 2,4,6-trinitrotoluene. The Environmental Protection Agency stated the detection of 2,4,6-trinitrotoluene using antibody-based detection systems has set sensitivity and specificity criteria for investigators using this technique[11]. There are a variety of ways in which antibody based sensors can be configured, including methods based on ceramic matrix (sol-gel) immunoaffinity purification[12], surface plasmon resonance[13], simple molecule-based fluorescent sensors[14], chemiluminescent ELISA and lateral-flow immunoassay[15], fluorescence resonance energy transfer[16]. Each technology has certain advantages and disadvantages, in regard to quantification, speed and cost; however, all are limited by either lack of sensitivity or speed for rapid detection of trace quantities of 2,4,6-trinitrotoluene.

Demands to increase speed and lower costs have lead to continued improvements in such assays. Because 2,4,6-trinitrotoluene is low molecular weight, immunoassays for its detection have generally been constructed in competitive heterogeneous formats. Fluorescence polarization immunoassay (FPIA) is a homogeneous format that does not require the separation of bound and free labels (tracer) and is getting attention as screening tool in environmental monitoring and food-safety

control due to its simplicity, rapidity, low cost and reliability. The aim of this study was the development of a rapid, sensitive and quantitative FP immunoassay for determination of 2,4,6-trinitrotoluene.

EXPERIMENTAL

Pyrenyldiazomethane (PDAM), 2-nitrotoluene, 2,4-dinitro-toluene, 2,4,6- trinitrophenol were purchased from Sigma-Aldrich (St Louis, MO, USA). 2,4,6-Trinitrotoluene and 1,3,5-trinitrohexahydro-1,3,5-triazine were obtained from China North Chemical Industries Group Co., Ltd. (China). 2,4,6-Trinitrobenzoic acid was purchased from Atomax Chemicals Co., Ltd. (China). Other chemicals used were of analytical grade. Monoclonal antibody of 2,4,6-trinitrotoluene in this study was custom in our Biomedical Engineering Lab.

Fluorescence polarization data were recorded with a Victor-2 (Perkin Elmer, USA).

Synthesis of fluorescent tracer: 2,4,6-Trinitrotoluene was conjugated to pyrenyldiazomethane according to the following procedure. 50 µg 2,4,6-trinitrobenzoic acid was added to 200 µL ethyl acetate solution of 0.1 % pyrenyldiazomethane. The mixture was stirred for 90 min at room temperature. The reaction mixture was applied to the C_{18} solid phase extraction column using 30 mL methanol-water, 1:1 (v/v), as eluent. Subsequently, the column was eluted by 10 mL methanol and then the eluate was collected. Fractions eluting were collected from the column and the solvent was removed by a combination of vacuum evaporation and lyophilization. Finally, the fluorescent tracer was stored in methanol at 4 ºC.

The tracer concentrations were estimated spectrophotometrically at 492 nm, assuming the absorbance in phosphate buffer (20 mmol L^{-1}, pH 7.2) to be the same as for fluorescein ($\varepsilon = 8.78 \times 10^4$ mol^{-1} L cm^{-1}). The tracer solution was further diluted in 20 mmol L^{-1} phosphate buffer and used for FPIA measurements.

Antibody titer value corresponding to the dilution of antibody which gave a 50 % tracer binding was determined from the corresponding dilution curve. Working solutions of 2,4,6-trinitrotoluene monoclonal antibody was prepared by diluting aliquots of the stock solutions with phosphate buffer.

2,4,6-Trinitrotoluene standard solutions: 50 mg 2,4,6-trinitrotoluene dissolved in 3 mL concentrated sulfuric acid (98.3 %) and diluted with water to 1000 mL. The concentration of standard solution of 2,4,6-trinitrotoluene was 0.05 mg mL^{-1}.

Fluorescence polarization immunoassay: Antibody dilution curve was obtained by incubating 2,4,6-trinitrotoluene antibody concentrations (dilution of antiserum ranged from 1/50 to 1/8000) with an appropriate amount of fluorescent tracer (2 nmol L^{-1}) in a total volume of 0.5 mL for 1 min at room temperature. The fluorescence polarization (P) value was defined by the equation $P = (I_v - I_h)/(I_v + I_h)$, where I_v and I_h were the intensities of fluorescence of the tracer along the vertical axis and the horizontal axis, respectively, were measured for each dilution of antiserum. The polarization is commonly expressed in millipolarization units (mP).

The competitive FPIA calibration curve was constructed with 2,4,6-trinitrotoluene at the concentrations 1, 10, 30, 100, 300 and 1000 µg mL^{-1}. The 2,4,6-trinitrotoluene standard (50 µL) was mixed with the tracer and antibody solution in phosphate buffer in a final volume of 0.5 mL and the mP signal was measured. The data were plotted against analyte concentration and a four-parameter equation was used to fit the experimental sigmoidal curve in Origin 7.0 for Windows. The assay limit of detection (LOD) was determined from the calibration curve by the blank signal and the 3-fold confidence interval and converted into the analyte concentration. IC_{50} is the analyte concentration resulting in 50 % inhibition of tracer binding. In these experiments to evaluate the kinetics of the competition, measurements were made after holding at room temperature for time ranging from 30 s and 10 min.

Tracers working solutions were prepared by diluting aliquots of the tracer stock solutions with methanol. For each tracer, the optimum tracer concentration in the test solution was determined as the dilution of the stock solution producing a total fluorescence intensity equal to 2- to 3-fold the blank signal measured against phosphate buffer.

Cross-reactivity of the fluorescence polarization immunoassay: The cross-reactivity of the optimized FPIA was determined for structurally related (2-nitrotoluene, 2,4-dinitro-toluene, 2,4,6-trinitrobenzoic acid, 2,4,6-trinitrophenol, 1,3,5-trinitrohexahydro-1,3,5-triazine). The data for anti-2,4,6-trinitrotoluene was calculated according to the equation $CR = IC_{50\ (TNT)}/IC_{50(cross-reactant)} \times 100$ %, where IC_{50} was the concentration of analyte or cross-reacting compound that inhibits 50 % of fluorescent tracer binding.

2,4,6-Trinitrotoluene immunoassay in surface water samples: Environmental samples of surface water were spiked with 2,4,6-trinitrotoluene standard solution. The actual concentration of water samples was 10, 20, 200, 500 and 1000 µg mL^{-1}, respectively. Fluorescent tracer (2 nmol L^{-1}) and working solutions of 2,4,6-trinitrotoluene (1/1600) were used in the assay. The mP signal of samples was measured and the measured concentration of samples was calculated by the aforementioned competitive FPIA calibration curve. Accuracy of the immunoassay was estimated by relative error values.

Statistical analysis: Statistical evaluation of data (means and standard deviations) was performed using Origin version 7.0 (OriginLab Corporation, Northampton, MA, USA).

RESULTS AND DISCUSSION

Assessment of antibody binding with fluorescent tracers: Fluorescence polarization immunoassay is a solution phase assay. Fluorescence polarization instruments indirectly measure the rate of rotation of a fluorescent molecule in solution. Because the rate of rotation is related with the size of the molecules, lager molecules rotate slower and give a higher signal than smaller molecules[17]. The lowest tracer concentration should be selected for a sensitive assay because FPIA is a competitive assay. For TNT-PDAM, the optimum concentration was 2 nmol L^{-1} in accordance to fluorescence intensity approximately ten times higher than the background signal from buffer.

Antibody dilution curve was recorded for assessment of antibody affinity toward the labeled antigens and antibody titer values were determined (Fig. 1). The value of the antibody titer corresponding to 50 % tracer binding is dependent on tracer structure. Anti 2,4,6-trinitrotoluene showed the higher affinity toward TNT-PDAM by the titer value (1/1600).
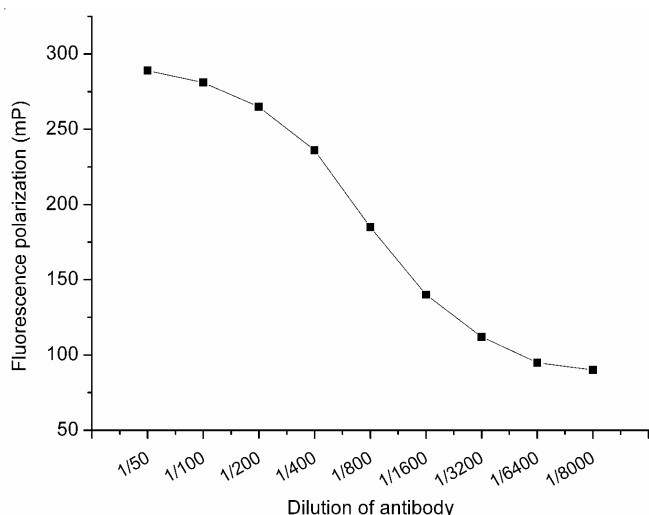
Fig. 1. 2,4,6-Trinitrotoluene antibody dilution curve of tracer

Optimization of the fluorescence polarization immuno-assay: The FPIA was used to measure the competition between the tracer and unlabeled 2,4,6-trinitrotoluene in solution for binding the antibody. The effect of the antibody-tracer incubation time on fluorescence polarization response was investigated. The time required for the fluorescence polarization signal to reach equilibrium was 4 min for competitive FPIA (Fig. 2). The absence of long incubation times and washing steps reduced the time of analysis as compared to other antibody-based methods. Actually, the overall time consumed in 2,4,6-trinitrotoluene analysis with the optimized immunoassay procedure was less than 10 min, thus allowing the performance of the analysis with a very high sample throughput.
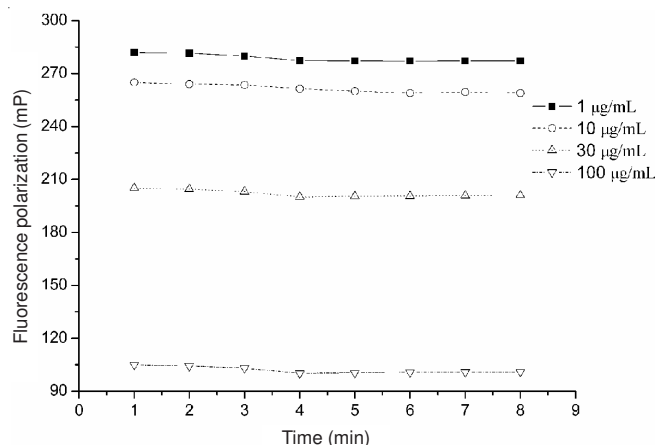

Fig. 2. Kinetics process of fluorescence polarization immunoassay for 2,4,6-trinitrotoluene

Calibration curve for 2,4,6-trinitrotoluene was obtained with different concentrations in a combination with TNT-PDAM fluorescent tracer (Fig. 3). The calibration curve had an LOD of 5 μg mL^{-1} and IC$_{50}$ above 39 μg mL^{-1}. It should be noticed that LOD is commonly used as a measure of immuno-assay sensitivity. 2,4,6-Trinitrotoluene detection using llama antibodies and a two-step competitive fluid array immunoassay and utilizing the same two-step competitive assay format the dynamic range of the monoclonal antibody was found to have a broad range (1 ng/mL to 1 mg/mL)[18]. The FPIA was characterized by good reproducibility; coefficients of variance (CVs) ranged from 1.6-4.4 % within assay and from 2.7 to 6.5 % between assays (Table-1).
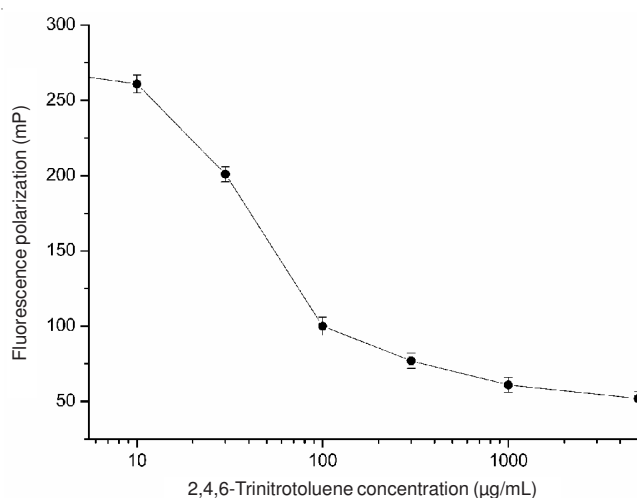

Fig. 3. Calibration curve for 2,4,6-trinitrotoluene by competitive fluorescence polarization immunoassay

Cross-reactivity of the fluorescence polarization immunoassay: To assess the specificity of developed assay, anti 2,4,6-trinitrotoluene was tested for its cross-reactivity (CR) with structurally related compounds, including 2-nitrotoluene, 2, 4-dinitrotoluene, 2,4,6-trinitrobenzoic acid, 1,3,5-trinitro-hexahydro-1,3,5-triazine and 2,4,6-trinitrophenol (Table-2). A very low cross-reactivity (CR < 0.01 %; IC$_{50}$ > 10^5 μg mL^{-1}) was observed for nitroaromatic compounds except for 2,4,6-trinitrotoluene. The antibody demonstrated high specificity for 2,4,6-trinitrotoluene (IC$_{50}$ = 39 μg mL^{-1}). The antigen-antibody complex formation is a complicated interaction and it is easily affected by slight differences in chemical structure. Monoclonal antibody has high specificity because it possesses highly homogenerous physico-chemical properties, single biological activity and makes only one kind of interaction with antigen epitope. Cross-reactivity is the reaction between an

TABLE-1
RSD OF INTRA-ASSAY AND INTER-ASSAY FOR FLUORESCENCE POLARIZATION IMMUNOASSAY (n = 5)

2,4,6-Trinitrotoluene (μg mL^{-1})	Intra-assay RSD			Inter-assay RSD		
	FP (mP)	SD (mP)	RSD (%)	FP (mP)	SD (mP)	RSD (%)
10	261	5.2	2.0	259	7.1	2.7
30	201	3.3	1.6	203	6.8	3.3
100	100	2.9	2.9	98	6.4	6.5
300	77	2.6	3.4	78	4.7	6.0
1000	61	2.7	4.4	62	4.0	6.5
Average	–	–	2.9	–	–	5.0

TABLE-2
CROSS-REACTIVITY OF 2,4,6-TRINITROTOLUENE
ANTIBODY WITH NITROAROMATICS

Chemical	IC$_{50}$ (μg mL^{-1})	Cross-reactivity (%)
2,4,6-Trinitrotoluene	39.3	100
2-Nitrotoluene	> 10 000	< 0.1
2,4-Dinitrotoluene	> 10 000	< 0.1
2,4,6-Trinitrophenol	> 10 000	< 0.1
2,4,6-Trinitrobenzoic acid	> 10 000	< 0.1
1,3,5-Trinitrohexahydro-1,3,5-triazine	> 10 000	< 0.1

antibody and an antigen that differs from the immunogen and is also a commonly evaluated parameter for the validation of immune and protein binding based assays such as ELISA. In this case it is normally quantified by comparing the assays response to a range of similar analytes and expressed as a percentage.

2,4,6-Trinitrotoluene immunoassay in surface water samples: To simulate environmental samples, surface water was spiked with 2,4,6-trinitrotoluene and analyzed using the optimized fluorescence polarization immunoassay. As shown in Table-3, the relative error value was less 1 % at different concentration in the assay, suggesting the immunoassay was reliable and applied to environmental 2,4,6-trinitrotoluene detection.

TABLE-3
RELATIVE ERROR VALUES OF THE 2,4,6-TRINITROTOLUENE
IMMUNOASSAY IN SURFACE WATER SAMPLES

Concentration of TNT (μg mL^{-1})		Relative error (%)
Actual	Measured	
10	9.91	0.90
20	19.87	0.65
200	198.31	0.85
500	495.79	0.84
1000	991.32	0.87

Conclusion

In this study, the FPIA method based on a monoclonal antibody for the detection of 2,4,6-trinitrotoluene was developed and optimized. The method was characterized with an LOD value of 5 μg mL^{-1} and IC$_{50}$ of 39 μg mL^{-1}. The method was highly specific and reproducible and the time of assay procedure was less than 10 min. On the basis of these initial results, this FPIA appears to meet the 2,4,6-trinitrotoluene detection of environmental samples without complicated operation.

REFERENCES

1. A.A. Adams, P.T. Charles, J.R. Deschamps and A.W. Kusterbeck, *Anal. Chem.*, **83**, 8411 (2011).
2. E.P. Agency, Health Advisory for TNT, Criteria and Standard Division, Office of Drinking Water, Washington, DC, USA (1989).
3. B. Balan, C. Vijayakumar, M. Tsuji, A. Saeki and S. Seki, *J. Phys. Chem. B*, **116**, 10371 (2012).
4. F. Rowell, J. Seviour, A.Y. Lim, C.G. Elumbaring-Salazar, J. Loke and J. Ma, *Forensic Sci. Int.*, **221**, 84 (2012).
5. M. Najarro, M.E. Davila Morris, M.E. Staymates, R. Fletcher and G. Gillen, *Analyst*, **137**, 2614 (2012).
6. M. Tabrizchi and V. Ilbeigi, *J. Hazard Mater.*, **176**, 692 (2010).
7. H. Wackerbarth, C. Salb, L. Gundrum, M. Niederkruger, K. Christou, V. Beushausen and W. Viöl, *Appl. Opt.*, **49**, 4362 (2010).
8. M.R. Leahy-Hoppa, M.J. Fitch and R. Osiander, *Anal. Bioanal. Chem.*, **395**, 247 (2009).
9. K. Wells and D.A. Bradley, *Appl. Radiat. Isot.*, **70**, 1729 (2012).
10. D.R. Shankaran, K.V. Gobi, T. Sakai, K. Matsumoto, K. Toko and N. Miura, *Biosens. Bioelectron.*, **20**, 1750 (2005).
11. E.S. Bromage, T. Lackie, M.A. Unger, J. Ye and S.L. Kaattari, *Biosens. Bioelectron.*, **22**, 2532 (2007).
12. M. Altstein, A. Bronshtein, B. Glattstein, A. Zeichner, T. Tamiri and J. Almog, *Anal. Chem.*, **73**, 2461 (2001).
13. Y. Mizuta, T. Onodera, P. Singh, K. Matsumoto, N. Miura and K. Toko, *Biosens Bioelectron.*, **24**, 191 (2008).
14. G.V. Zyryanov, M.A. Palacios and P. Anzenbacher Jr., *Org. Lett.*, **10**, 3681 (2008).
15. S. Girotti, S. Eremin, A. Montoya, M.J. Moreno, P. Caputo, M. D'Elia, L. Ripani, F.S. Romolo and E. Maiolini, *Anal. Bioanal. Chem.*, **396**, 687 (2010).
16. L. Feng, H. Li, Y. Qu and C. Lu, *Chem. Commun.*, **48**, 4633 (2012).
17. C.M. Maragos, *Mycotoxin Res.*, **22**, 96 (2006).
18. G.P. Anderson and E.R. Goldman, *J. Immunol. Methods*, **339**, 47 (2008).