APPLICATION NOTE

High Throughput Detection of Tetracycline Residues in Milk Using Graphene or Graphene Oxide as MALDI-TOF MS Matrix

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Abstract

In this work, a new pre-analysis method for tetracyclines (TCs) detection from the milk samples was established. As a good accomplishment for the existing accurate quantification strategies for TCs detection, the new pre-analysis method was demonstrated to be simple, sensitive, fast, cost effective, and high throughput, which would do a great favor to the routine quality pre-analysis of TCs from milk samples. Graphene or graphene oxide was utilized, for the first time, as a dual-platform to enrich and detect the TCs by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). All together, four TCs were chosen as models: tetracycline, oxytetracycline, demeclocycline, and chlortetracycline. Due to the excellent electronic, thermal, and mechanical properties, graphene and graphene oxide were successfully applied as matrices for MALDI-TOF MS with free background inference in low mass range. Meanwhile, graphene or graphene oxide has a large surface area and strong interaction force with the analytes. By taking the advantage of these features, TCs were effectively enriched with the limit of detection (LOD) as low as 2 nM.

Key words: Graphene, Graphene oxide, Tetracyclines, Enrichment and ionization, MALDI-TOF MS

Introduction

In veterinary medicine, antibiotics are widely used in animals for the treatment and prevention of diseases [1]. However, antibiotic residues may have direct toxic effects [2, 3]. Therefore, the fact that almost all food-production animals receive medication attracts public health concerns a lot. As one typical case, incorrect use of antibiotics in cow breeding can contaminate milk products destined for human consumption. Tetracyclines (TCs) are widely used for the treatment of cow breeding [4]. Their basic chemical structure is built by a hydronaphthacene backbone consisting of four fused rings [5]. Various techniques for the detection and

quantification of TCs have been reported in the literature. Traditional analysis methods include microbial growth inhibition assays, microbial receptor assays, enzymatic colorimetric assays, receptor binding assays, chromatographic methods, and immunoassays [6, 7]. Although the conventional methods provide extraordinarily accurate quantitative information, they do not fully meet the demand for the pre-analysis. Rapid, high throughput, sensitive qualitative detection is the basic principle for the pre-analysis of TCs from the milk samples.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was widely used for simplifying the sample pretreatment steps and labor saving. However, in the low mass ranges ($m/z < 600$ Da), the strong background interferences from conventional α -cyano-4-hydroxycinnamic acid (CHCA) or 2,5-dihydroxybenzoic acid (DHB) matrix severely obscure the detection of small molecules by MALDI-TOF MS [8]. Recently, various matrix substances have been tried successfully to solve the

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background interference defect, such as porous silicon [9, 10] and carbon-based materials [11, 12]. Among these, graphene and graphene oxide attracted our attention most.

Being one of the most exciting research topics in the multi-dimensional carbon nano-material family, graphene was first obtained in 2004 by a surprisingly simple technique [13]. These one-atom-thick and two-dimensional layers of sp^2 -bonded carbon materials exhibit many unusual properties, such as high surface area, large thermal conductivity, superior mechanical properties, and excellent electronic transport properties [13, 14]. Up to now, graphene and graphene oxide have been successfully utilized as MALDI-TOF MS matrix and enrichment to detect small molecules by our research group [15, 16]. The reason for their enrichment ability mainly lies in the interaction between carbon atoms and analytes. Thus, the unique chemical structure of graphene and graphene oxide may produce a more powerful capture of TCs.

To the best of our knowledge, the possibility of utilizing graphene and graphene oxide to detect TCs from the milk samples has hardly been tried before. Hence, in this work, graphene and graphene oxide were utilized, for the first time, to detect TCs as MALDI matrix and enrichment medium at the same time. The aim of this study was to develop a simple, sensitive, rapid, cost effective and high throughput MALDI-TOF MS method for TCs detection from the milk sample as pre-analysis. The workflow is illustrated in Figure 1. By utilizing graphene or graphene oxide as matrix for the above mentioned component identification from milk sample, satisfactory results were obtained with low limit of detection (LOD) and high throughput, which has never been reported before.

Material and Methods

Trifluoroacetic acid (TFA) was chromatographic grade (Merk, Darmstadt, Germany). Ethanol was analytical grade. Tetracycline (TC), oxytetracycline (OTC), demeclocycline

(DC), chlortetracycline (CTC), citric acid monohydrate, and sodium citrate dehydrate were provided by Sigma-Aldrich Chemical (St. Louis, MO, USA). Graphene and graphene oxide was from Shanghai Boson Technology Co., Ltd. Aliquots of 1 mL milk sample were mixed with 0.5 mL of 20 % TFA and 5 mL of citrate buffer (0.4 M, pH=4). The mixture was centrifuged at 4000 rpm for 15 min and the supernatants were stored for further analysis [17]. Additionally, for the fortified samples, 100 μ L of TCs standard working solution was added to 1 mL milk sample before protein deposition. The final concentration of the TCs standard was 10 nM. For matrices preparation, 1 mg of graphene or graphene oxide was dispersed in a 1 mL solution of ethanol/water (1:1, vol/vol) containing 0.1 % TFA and sonicated for 3 min. One μ L of the matrices was pipetted onto the MALDI target quickly and left in the air to form a thin layer; 0.5 μ L solution of analyte was then pipetted onto the layer of matrix and analyzed by MALDI-TOF MS. For enrichment step, 2 mg of materials were rinsed twice with 50 % (vol/vol) ethanol and sonicated for 3 min. Ten μ L of the suspension was pipetted immediately into two 100 μ L of analyte solution and sonicated for 15 min. After centrifugation at 13,000 rpm for 10 min, the supernatants were removed, and the pellets of graphene were suspended again in a 5 μ L solution of ethanol in H_2O (1/1, vol/vol) containing 0.1 % TFA. Finally, about 1 μ L of the suspension of graphene was pipetted onto MALDI sample target. The sample target was again left at room temperature for evaporation of the solvent and for further analysis by MALDI-TOF MS.

Results and Discussion

Although great effort have been made to detect low molecular weight substances by MALDI-TOF-MS, there are few reports on the TCs detection using MALDI-TOF MS. Hence, in this work, TC (m/z 444), OTC (m/z 460), DC (m/z 464), and CTC (m/z 478) were selected for MALDI-

Figure 1. Flow chart of tetracyclines analysis by utilizing graphene or graphene oxide as enrichment adsorbent and MALDI-TOF MS matrix

Table 1. LOD Values and RSDs (%) of the Four TCs with Graphene or Graphene Oxide as the Enrichment Adsorbents in the Negative Ion Mode

	Graphene (RSD, %)	Graphene oxide (RSD, %)
Before enrichment		
Tetracycline	720 nM (5.48 %)	180 nM (2.41 %)
Oxytetracycline	950 nM (5.85 %)	190 nM (2.37 %)
Chlortetracycline	1260 nM (6.27 %)	210 nM (2.95 %)
Demeclocycline	900 nM (6.03 %)	200 nM (2.78 %)
After enrichment		
Tetracycline	15 nM (6.62 %)	2 nM (3.54 %)
Oxytetracycline	10 nM (6.91 %)	2 nM (4.71 %)
Chlortetracycline	10 nM (7.15 %)	2.5 nM (3.69 %)
Demeclocycline	15 nM (7.36 %)	2 nM (5.28 %)

TOF MS analysis. The performance of graphene and graphene oxide were first investigated as matrices, both in positive and negative ion modes (Figure S1, S2, found in Supplemental Data). Compared with the results utilizing CHCA and DHB as matrix (Figure S3, S4, Supplemental Data), no obvious background interferences were observed. As for positive ion mode, multiple ions such as sodium adduct $[M+Na]^+$ and potassium adduct $[M+K]^+$ were simultaneously observed. While for negative ion mode, only one characteristic ion of $[M-H]^-$ was observed for each TC. Similar to those of carbon nano-tube composites, graphene and graphene oxide are electrically- and thermally-conducting, in which electrons

shoot along with minimal resistance. All the characteristics suggested that graphene and graphene oxide were the desired matrices for TCs detection by MALDI-TOF MS.

Efforts have been made to optimize the detection results by adjusting pH. No significant improvement was observed for matrix graphene (data not shown). Meanwhile, for graphene oxide, the background interferences were cleared by the addition of 0.1 % TFA (Figure S5, Supplemental Data). Since graphene oxide has a carboxyl-rich structure, the presence of 0.1 %TFA may enhance the ionization ability by aiding in π - π interactions between analytes and graphene oxide. The detection reproducibility and sensitivity on two matrices in both positive and negative ion modes were investigated (Figure S6, Table S1, Supplemental Data). The statistics results indicated that for the four TCs, higher signal intensity and better shot-to-shot reproducibility can be obtained in the negative ion mode by using graphene oxide as matrix. As electron-rich hydrophobic materials with high surface areas, graphene and graphene oxide might also serve as marvelous pre-enrichment materials for TCs from the milk samples. To testify this hypothesis, both graphene and graphene oxide were utilized as the enrichment materials. Enrichment conditions were optimized for both graphene and graphene oxide. The result is shown in Figure S7 (Supplemental Data). Fifteen minutes was chosen as the optimum incubation time for graphene and graphene to enrich the targeted TCs. The

Figure 2. MALDI-TOF-MS profiles (in negative ion mode) of milk sample with GO as enrichment and ionization material without (a) or and with (b) spiked with four standard TCs. All TCs spiked in the milk were at the concentration of 10 nM each

satisfactory results of the limit of detection (LOD) before and after enrichment were obtained (Table 1). Graphene oxide demonstrated itself to be more effective than graphene when performed as pre-enrichment material.

The tandem MS spectra were further obtained for TCs identification from the milk samples as a mini-database (Figure S8, Supplemental Data). The milk samples with or without spiked TCs (10 nM for each tetracycline) were treated for proteins deposition at first. Graphene oxide was added to the treated samples for 15 min and then acted as MALDI-TOF MS matrix directly. The MALDI MS results were further analyzed by tandem MS. All the tandem MS signals were compared with the mini-database mentioned above and tetracycline were identified. The MALDI MS results were shown in Figure 2 and the tandem MS results were shown in Figure S9. The results indicated that no TCs were detected in the milk without spiked standard samples. All four TCs spiked in the milk were successfully detected by MALDI MS and identified by tandem MS with mini database. This kind of utilization fully demonstrated the excellent characteristics of graphene oxide as an enrichment absorber and ionization matrix.

In the case of screening large numbers of milk samples, a rough but high throughput identification procedure was what the working staffs wanted. The dual capability of graphene and graphene oxide as enrichment adsorbents and MALDI matrices would offer a powerful and rapid technique for the detection of TCs before accurate quantification by HPLC or immunoassay. After the pre-analysis by MALDI-TOF MS, the suspected samples with the detection of TCs can be further accurately quantified by HPLC or immunoassay.

Conclusions

In this work, both graphene and graphene oxide were utilized to enrich and ionize four TCs. By exhibiting the advantages such as simple sample preparation, efficient analytes enrichment, enhanced detection limit, eliminated background ions interference, as well as improved shot-to-shot reproducibility, both graphene and graphene oxide have successfully demonstrated their great potential for selective enrichment of target analytes and the attainment of direct mass spectrometric readouts, even from very complex media.

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