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ELISA detection of semicarbazide based on a fast sample pretreatment method†

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A direct ELISA was established for the fast detection of semicarbazide (SEM) using a novel biotin derivative. Without a tedious extraction procedure, as low as 0.07 μg L^{-1} of SEM could be detected reproducibly. This assay has better recovery and accuracy than competitive ELISA.

Nitrofurazone belongs to the group of nitrofuran antibiotics, which has been widely used as a food additive for the treatment of gastrointestinal infections in cattle, pigs, poultry, fish and shrimps *etc.* It has been reported to cause tumors in experimental animals and participate in nitrofurazone-induced carcinogenesis.[1] Nitrofurazone and its metabolites are also involved in tumor initiation through *p*53 gene damage, and stimulating the proliferation of tumor cells.[2] Because of its carcinogenicity, the use of nitrofurazone in food-producing animals has been banned by food regulatory authorities, including those in Europe, USA and China.

Nitrofurazone is rapidly metabolized *in vivo* and has a short half-life in edible tissues. The parent drug breaks down in a few hours.[3] The decrease of nitrofurazone is accompanied by the accumulation of the side chain metabolite semicarbazide. SEM and protein-bound SEM are more stable and can persist in the body for several weeks.[4] Thus, the metabolite of nitrofurazone (**1a,1b**, Fig. S1, ESI†), semicarbazide, was used as a marker residue for the monitoring of illegal drug use.[5] Recently, it was found that SEM was also produced as a breakdown product of azodicarbonamide (ADC) (**1c**, Fig. S1, ESI†), a blowing agent used in the manufacture of plastic packaging materials,[6] and a product of the hypochlorite reaction in food processing.[7] ADC may cause allergic reactions in people who are sensitive to furazolidone (AZO) compounds, and SEM was also considered to be a potential carcinogenic agent that may cause tumor.[8] Due to their potential health risks, the use of ADC as a blowing agent in food packaging has been banned in the European Union since 2005.

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The first established method for the detection of SEM in food samples was based on liquid chromatography–mass spectrometry.[9] But SEM lacks absorbance in the UV region and possesses a low mass that locates in the range of MS background noise. Derivatisation and extraction are required for further analysis.[10] But matrix compounds with similar chemical property can also be extracted. In this case, extraction recovery and MS background may be compromised. Furthermore, expensive equipment and well trained operators are a pre-requisite for accurate detection. Therefore, the third-world countries which suffer more from the abuse of illegal drugs need more convenient and cheaper detection methods.

In a previous study, we prepared monoclonal antibodies capable of detecting the SEM derivative with 4-carboxybenzaldehyde (CBA). Antibody from a strain of hybridoma with high specificity was used to develop a competitive ELISA for the SEM analysis.[11] However, the extraction recovery rate and repeatability were not good. The residual organic extraction solvent also reduced the affinity of the antibody, thus compromising the accuracy and reproducibility of the test. Therefore, a simpler and more efficient assay for SEM detection is needed.

To address these limitations, we introduced a ligand–receptor pattern for the purification and sandwich detection of the SEM derivative. Biotin was used as the ligand in this study. Streptavidin (SA) was its natural receptor that can bind biotin with high affinity ($K_d \approx 10^{-14}$ mol L^{-1}).[12] Thus, using biotin modified CBA, we established a direct ELISA for quantitative analysis of small molecule SEM. The derivatisation agent CBA was first modified with biotin, which was then used for the derivatisation of SEM. The resultant biotinylated-SEM (**1d**, Fig. S1, ESI†) had two functional groups: the SEM-CBA moiety and the biotin moiety. The SEM-CBA moiety was captured by the antibody that was coated on the 96-well plate, and the biotin moiety in this molecule was further captured by a streptavidin–horseradish peroxidase conjugate (SA–HRP). Thus, the assay is established with a sandwich format (as shown in Fig. 1).

The synthetic strategy for biotinylated aldehyde derivative **1**, in which the biotin group and the CBA group are linked by a triethylene glycol group, is shown in Fig. 2 according to reported

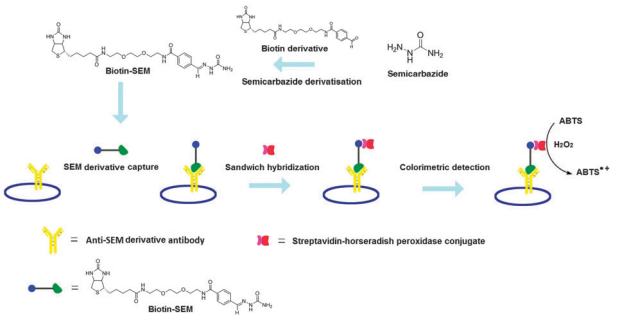

Fig. 1 Schematic presentation of the direct ELISA for SEM detection.

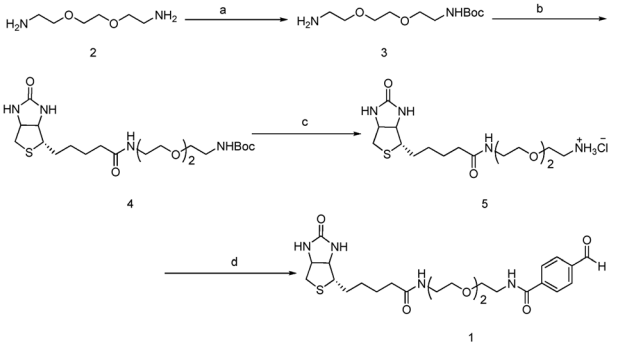

Fig. 2 Synthetic strategy for biotinylated aldehyde derivative **1a**. Reagents and conditions: (a) $(Boc)_2O$, DIPEA, DCM; (b) biotin, HATU, DIPEA, DMF; (c) HCl, DCM; (d) 4-carboxybenz-aldehyde, HATU, DIPEA, DMF.

methods with some modifications.[13] Mono-*N*-Boc protected amine **3** was first prepared starting from commercially available diamine **2** in anhydrous DCM. The mono-Boc protected amine **3** was then coupled to biotin in the presence of the HATU–DIPEA system, an effective activating agent used in peptide synthesis. Deprotection of Boc protected biotinylated amide **4** in a HCl–dioxane system yielded amine **5**. Compound **5** was then coupled with 4-carboxybenzaldehyde in a HATU–DIPEA system to give compound **1** with 40.5% overall yield starting from **3** (ESI†).

To quantitatively analyze SEM, a standard curve or calibration plot was needed. In the present study, it was obtained by performing a calibration experiment over serially diluted SEM standard solutions (SEM spiked ultra-pure water) with a concentration range of 0–70 μg L^{-1}. SEM standard solution was first derivatised with biotinylated aldehyde for 4 hours and the SEM derivative was analyzed using the present assay.

High backgrounds were observed in our preliminary results. We speculated that the unreacted aldehyde group in biotinylated aldehyde covalently linked to free amino groups in the antibody or bovine serum albumin (BSA) that was coated on the plate. Besides, the pH of the derivatisation solution was too low (pH ≈ 5) for the proper functioning of the antibody. Referring to the extraction method used in LC-MS/MS analysis, we employed a chimeric phosphate buffered saline-Tris (hydroxymethyl) aminomethane-HCl buffer (PBS-Tris buffer, pH = 7.5) as the hybridization buffer. The results were consistent with our hypothesis and low backgrounds were obtained. Meanwhile, PBS buffer and Tris buffer with the same pH were also tested, but neither of their performance was comparable to that of the PBS-Tris buffer. The low background can

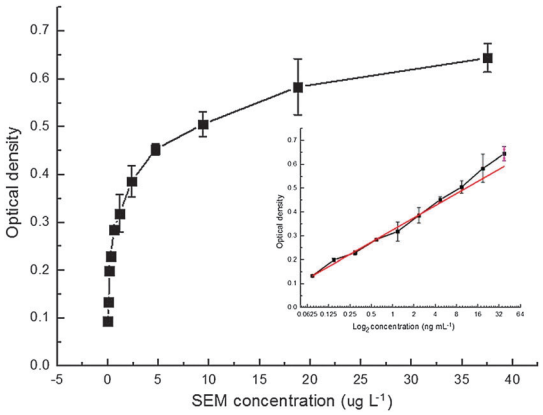

Fig. 3 Standard curve of the assay. Optical densities are obtained from serially diluted SEM standard solutions (0 μg L^{-1}, 0.07 μg L^{-1}, 0.14 μg L^{-1}, 0.29 μg L^{-1}, 0.58 μg L^{-1}, 1.17 μg L^{-1}, 2.34 μg L^{-1}, 4.68 μg L^{-1}, 9.37 μg L^{-1}, 18.75 μg L^{-1}, 37.5 μg L^{-1}).

be attributed to the following two reasons: the primary amine group in Tris blocks the free aldehyde group in biotinylated aldehyde and PBS buffer provides a suitable environment for the proper functioning of the antibody.

After optimizing the parameters of this assay, a standard curve was obtained (Fig. 3). It was then applied to curve fitting using Originpro (Originpro 8.5). The resulting calibration plot exhibited a well-defined linear relationship between the SEM concentration and optical density, with a dynamic range of 0.07–40 μg L^{-1}. The coefficient of variation ranged from 1% to 8%. The mean optical density of the blank was 0.093, and the standard deviation was 0.006. Thus, the limit of detection (LOD) is 0.07 μg L^{-1}, which is calculated based on the triple standard deviation from the mean of blanks (mean + 3SD). We have established a competitive ELISA using the same antibody in a previous study. The sensitivity of the present method is better than that of the competitive ELISA (Fig. S3, ESI†), and is comparable to those of LC-MS/MS.[14]

Recovery is another important parameter for the detection of SEM. In this study, skimmed milk power purchased at a local supermarket was used as the sample for evaluation of the performance. Serially diluted SEM stock solutions were added to 1 g of milk powder, and the volume was adjusted to 10 mL with ultra-pure water. The final SEM concentration ranged from 0 μg kg^{-1} to 37.5 μg kg^{-1}. Derivatisation was performed at 37 °C for 4 hours. After derivatisation, the mixture was centrifuged at 5000 rpm for 10 min to remove the insoluble substance. The supernatant was subjected to the present assay.

All the SEM spiked samples showed positive results and all the blank samples showed negative results. The recovery ranged from 96% to 113% (Fig. 4), which were much better than those in indirect competitive ELISA (20–100%, data not shown). The high recovery can be explained by the simplified treatment of the SEM derivative. Without several steps of extraction and clean-up, the biotinylated molecule is directly added to the plate and captured by antibodies, which means no SEM derivative is lost in this process.

The selectivity of this method is dependent on the specificity of the antibody. Several veterinary drugs, related derivatives and metabolites were tested in this study. The test was conducted according to the procedures described above.

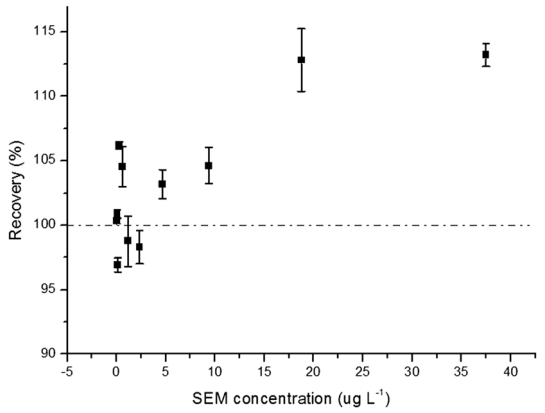

Fig. 4 Distribution of the recovery of spiked samples between 0 μg kg^{-1} and 37.5 μg kg^{-1} (0 μg kg^{-1}, 0.07 μg kg^{-1}, 0.14 μg kg^{-1}, 0.29 μg kg^{-1}, 0.58 μg kg^{-1}, 1.17 μg kg^{-1}, 2.34 μg kg^{-1}, 4.68 μg kg^{-1}, 9.37 μg kg^{-1}, 18.75 μg kg^{-1}, 37.5 μg kg^{-1}).

Except for nitrofurazone, none of these chemical compounds had cross-reactivity with this antibody, including those structurally related to SEM-CBA and the derivatisation agent CBA (Fig. S2, ESI†). Nitrofurazone has an imidazole group that resembles the benzene ring in SEM-CBA, which can explain the low cross-reactivity.

We also synthesized and tested biotinylated aldehyde substrates using one ethylene group and a tetraethylene glycol group as the linker, respectively. The sensitivity of this method using ethylene as the linker was lower than that of the others, while the tetraethylene glycol one had the same sensitivity compared to the triethylene glycol compound **1** (data not shown). The low sensitivity can be explained by the steric hindrance between antibody and streptavidin. These results suggested that the linker has to be long enough to position the biotin moiety distant from the reactive aldehyde group.[15]

In summary, a novel biotin derivative, carrying the 4-carboxybenzaldehyde moiety, was synthesized with a triethylene glycol linker. It was used for the derivatisation of SEM in food samples. Based on the resulting chemical agent that possessed both biotin and SEM-CBA moieties, a direct ELISA was then established for the quantitative detection of SEM.

The present assay we developed has the following advantages. First, it is a novel sandwich like ELISA for the detection of small molecules. It is important for the monitoring of high risk chemicals, such as toxins and narcotic drugs. For example, small molecules with an amine group can first react with the *N*-hydroxysuccinimide-activated derivatisation agent that is modified with biotin. The resulting compound is directly captured by its antibody. Therefore, small molecules can be detected without the need of their conjugated hapten. Besides, the sensitivity and reproducibility within batches can also be improved. Second, using the biotinylated aldehyde, the pretreatment of the SEM contaminated sample can be simplified. Without extraction, no organic extraction solvent is involved in the hybridization buffer, which renders the assay with high accuracy and reproducibility. Besides, this biotinylated aldehyde can also be used in LC-MS/MS methods as a derivatisation agent. The biotin tagged derivative molecule can easily be purified using a streptavidin affinity column, similar to the purification of the his-tagged protein using a nickel alloy column. A short incubation in hot water will reversibly break the interaction between biotin and streptavidin without denaturing streptavidin, allowing the collection of the derivative and re-use of the streptavidin column.[16] The limitation of this method is the complexity of the preparation of the derivatisation agent. The overall yield is about 40%, which still needs further improvement.

In conclusion, we have established a direct ELISA for the detection of small molecule SEM based on our synthesized biotinylated aldehyde. It not only simplifies and improves the ELISA based assay but also provides a possibility for the improvement of the LC-MS/MS performance. The method reported in this communication is inspiring toward its applications in other small molecule detection.

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Notes and references

1 (*a*) F. W. Kari, J. E. Huff, J. Leininger, J. K. Haseman and S. L. Eustis, *Food Chem. Toxicol.*, 1989, **27**, 129; (*b*) M. Takahashi, S. Iizuka, T. Watanabe, M. Yoshida, J. Ando, K. Wakabayashi and A. Maekawa, *Cancer Lett.*, 2000, **156**, 177.
2 Y. Hiraku, A. Sekine, H. Nabeshi, K. Midorikawa, M. Murata, Y. Kumagai and S. Kawanishi, *Cancer Lett.*, 2004, **215**, 141.
3 (*a*) R. J. McCracken and D. G. Kennedy, *J. Chromatogr., B: Biomed. Appl.*, 1997, **691**, 87; (*b*) A. Leitner, P. Zöllner and W. Lindner, *J. Chromatogr., A*, 2001, **939**, 49.
4 K. M. Cooper and D. G. Kennedy, *Analyst*, 2005, **130**, 466.
5 (*a*) J. G. Bendall, *Food Addit. Contam., Part A*, 2009, **26**, 47; (*b*) A. Leitner, P. Zöllner and W. Lindner, *J. Chromatogr., A*, 2001, **939**, 49.
6 (*a*) A. S. Pereira, J. L. Donato and G. De Nucci, *Food Addit. Contam.*, 2004, **21**, 63; (*b*) R. H. Stadler, P. Mottier, P. Guy, E. Gremaud, N. Varga, S. Lalljie, R. Whitaker, J. Kintscher, V. Dudler, W. A. Read and L. Castle, *Analyst*, 2004, **129**, 276.
7 (*a*) K. Hoenicke, R. Gatermann, L. Hartig, M. Mandix and S. Otte, *Food Addit. Contam.*, 2004, **21**, 526; (*b*) A. Becalski, B. P. Lau, D. Lewis and S. W. Seaman, *J. Agric. Food Chem.*, 2004, **52**, 5730.
8 (*a*) K. Hoenicke, R. Gatermann, L. Hartig, M. Mandix and S. Otte, *Mutat. Res.*, 2003, **536**, 91; (*b*) E. R. Nestmann, B. S. Lynch, K. Musa-Veloso, G. H. Goodfellow, E. Cheng, L. A. Haighton and V. M. Lee-Brotherton, *Food Addit. Contam.*, 2005, **22**, 875.
9 A. Leitner, P. Zollner and W. Lindner, *J. Chromatogr., A*, 2001, **939**, 49.
10 (*a*) S. Effkemann and F. Feldhusen, *Anal. Bioanal. Chem.*, 2004, **378**, 842; (*b*) P. Edder, S. Vargas, D. Ortelli and C. Corvi, *Clin. Chem. Lab. Med.*, 2003, **41**, 1608; (*c*) S. P. Khong, E. Gremaud, R. Richoz, T. Delatour, P. A. Guy, R. H. Stadler and P. Mottier, *J. Agric. Food Chem.*, 2004, **52**, 5309; (*d*) L. Saari and K. Peltonen, *Food Addit. Contam.*, 2004, **21**, 825; (*e*) S. Szilagyi and B. de la Calle, *Anal. Chim. Acta*, 2006, **572**, 113.
11 A. Gao, Q. Chen, Y. Cheng, J. Lei and L. Zeng, *Anal. Chim. Acta*, 2007, **592**, 58.
12 N. M. Green, *Adv. Protein Chem.*, 1975, **29**, 85.
13 (*a*) S. Fusz, S. G. Srivatsan, D. Ackermann and M. Famulok, *J. Org. Chem.*, 2008, **73**, 5069; (*b*) F. Friscourt, P. A. Ledin, N. E. Mbua, H. R. Flanagan-Steet, M. A. Wolfert, R. Steet and G. J. Boons, *J. Am. Chem. Soc.*, 2012, **134**, 5381; (*c*) G. Sabatino, M. Chinol, G. Paganelli, S. Papi, M. Chelli, G. Leone, A. M. Papini, A. De Luca and M. Ginanneschi, *J. Med. Chem.*, 2003, **46**, 3170; (*d*) A. Eisenfuhr, P. S. Arora, G. Sengle, L. R. Takaoka, J. S. Nowick and M. Famulok, *Bioorg. Med. Chem.*, 2003, **11**, 235.
14 (*a*) A. Becalski, B. P. Lau, D. Lewis and S. W. Seaman, *J. Agric. Food Chem.*, 2004, **52**, 5730; (*b*) L. Saari and K. Peltonen, *Food Addit. Contam.*, 2004, **21**, 825.
15 (*a*) S. Fusz, S. G. Srivatsan, D. Ackermann and M. Famulok, *J. Org. Chem.*, 2008, **73**, 5069; (*b*) G. Sabatino, M. Chinol, G. Paganelli, S. Papi, M. Chelli, G. Leone, A. M. Papini and A. De Luca, *J. Med. Chem.*, 2003, **46**, 3170; (*c*) M. Famulok, *J. Med. Chem.*, 2009, **52**, 6951.
16 A. Holmberg, A. Blomstergren, O. Nord, M. Lukacs, J. Lundeberg and M. Uhlen, *Electrophoresis*, 2005, **26**(3), 501–510.