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RESEARCH ARTICLE

Fluorescent competitive assay for melamine using dummy molecularly imprinted polymers as antibody mimics

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Abstract

A fluorescent competitive assay for melamine was first developed utilizing dummy molecularly imprinted polymers (DMIPs) as artificial antibodies. This method is based on the competition between fluorescent substances and the unlabeled analyte for binding sites in synthesized DMIPs and the decreased binding of fluorescent substances to DMIPs due to increased concentrations of melamine in the solutions. DMIPs for melamine were synthesized under a hot water bath in the presence of the initiator azobisisobutyronitrile (AIBN) using 2,4-diamino-6-methyl-1,3,5-triazine (DAMT) as a dummy template, methacrylic acid (MAA) as a functional monomer, and ethylene glycol dimethacrylate (EGDMA) as a crosslinking agent. The adsorption capacity and selectivity of DMIPs for melamine were evaluated by the isothermal adsorption curve and Scatchard analysis. The evaluation results showed that the synthesized DMIPs had specific recognition sites for melamine and the maximum adsorption amount was 1 066.33 μg g^{-1}. Later, 5-(4,6-dichlorotriazinyl) amino fluorescein (DTAF) with a triazine ring, which slightly resembles melamine, was selected as the fluorescent substance. The fluorescent competitive assay using DMIPs as the antibody mimics was finally established by selecting and optimizing the reaction solvents, DMIPs amount, DTAF concentration, and incubation time. The optimal detection system showed a linear response within range of 0.05–40 mg L^{-1} and the limit of detection (LOD) was 1.23 μg L^{-1}. It was successfully applied to the detection of melamine in spiked milk samples with satisfactory recoveries (71.9 to 86.3%). According to the comparative analysis, the result of optimized fluorescent competitive assay revealed excellent agreement with the HPLC-MS/MS result for melamine.

Keywords: dummy molecularly imprinted polymers, melamine, fluorescent competitive assay, artificial antibody

1. Introduction

Melamine (2,4,6-triamino-1,3,5-triazine), also referred to as three amines, is an important chemical material in the production of melamine resins as a kind of organic compounds with a nitrogen heterocyclic ring, which are widely used in wood processing, plastics, paint, papermaking, textile, leather, electric and medicine industries. Melamine detection has

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become a hot topic since the discovery of kidney stones in children caused by melamine-contaminated infant formula powders in China and pet death due to melamine-contaminated feeds in America. The determination of melamine in complex matrices has been reported by confirmatory methods such as liquid chromatography (LC) (Ali *et al*. 2008), gas chromatography-mass spectrometry (GC-MS) (Xu *et al*. 2009), liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Sancho *et al*. 2005) and rapid methods such as the visible color change approach based on gold nanoparticles (Ai *et al*. 2009), chemiluminescence immunoassay (CLIA) (Zhang *et al*. 2011) and enzyme-linked immune sorbent assay (ELISA) (Garber 2008) based on antibodies. Most confirmatory methods are known to be expensive and time-consuming (Lin 2009), and a few methods such as LC with a UV detection system are easily influenced by the complicated matrixes of samples in its sensitivity and accuracy (Tittlemier 2010). Meanwhile, although rapid methods such as CLIA and ELISA are time-effective, the required biological antibodies are unstable, and they are sensitive to storage conditions and used only under aqueous solutions (Ye and Mosbach 2008) with limited applications. Therefore, the methods that can detect melamine quickly, accurately and at a low cost are highly demanded.

Molecular imprinting has attracted the interests of researchers all over the world as a technique for preparing molecularly imprinted polymers (MIPs) with a given template. MIPs are generally synthesized in the following procedure: (i) The template molecule and functional monomer are pre-assembled (covalent interaction) or self-assembled (non-covalent interaction) to form a complex; (ii) the cross-linking agent is added to polymerize with the template-functional monomer complex in the presence of an initiator; (iii) the template molecule is removed by physical or chemical methods to obtain MIPs that have the binding sites with an embedded template of the original shape, size and functional groups. Nowadays, MIPs possess the advantages of low cost, convenient synthesis, high stability to harsh chemical and physical conditions, excellent reusability and tailored binding sites for the template. In addition, they have been studied and applied in many fields, such as solid phase extraction (SPE) (Li *et al*. 2009; Pavlovic *et al*. 2015; Su *et al*. 2015), sensors (Sainz-Gonzalo *et al*. 2011; Anirudhan and Alexander 2015), drug delivery devices (Rostamizadeh *et al*. 2012; Asadi *et al*. 2014), catalysis (Abbate *et al*. 2011) and antibody mimics (Levi *et al*. 1997; Piletsky *et al*. 2001; Benito-Pena *et al*. 2006; Nicholls *et al*. 2006; Lu *et al*. 2007).

MIPs possess many binding properties apparently similar to those of natural antibodies such as marked selectivity towards related ligands (antigens for antibodies; template molecules for MIPs), and the binding properties are resulted from multiple reversible non-covalent interactions and characterized by well-defined thermodynamics and kinetics (Vlatakis *et al*. 1993). Therefore, many molecular imprinting investigators aim at replacing natural antibodies with artificial MIPs using so-called “molecularly imprinted sorbent assays” (MIAs). Researchers have reported only assays based on labeled (except some florescence-based assays), competitive, homogenous or heterogeneous formats (Vlatakis *et al*. 1993). In the applications of MIAs, there are different types of labeled substances, including radiochemical tracers, enzymatic tracers and fluorescent tracers. However, radiochemical tracers are hardly used for their high cost, hazards and legal restrictions on radionuclides, and the utilization of enzymatic tracers is also limited due to inactivation of organic solvents, slow diffusion velocity and assay kinetics in the micro/nanometer-sized polymers. Fluorescent tracers are less problematic than radiotracers and more stable and solvent-compatible than enzymes.

Fluorescent MIAs could possibly be used in three approaches (Haupt *et al*. 1998): (i) The polymer is imprinted with the target analyte, and a fluorescence-labeled derivative of the analyte is used for detection. (ii) The polymer is imprinted with the target analyte, whereas an unrelated probe that can bind to the polymer is used for detection. (iii) The polymer is imprinted with the fluorescence-labeled or otherwise derivatized analyte, and an unlabeled analyte competes with the labeled analyte for the binding sites on the polymer upon analysis. The first fluorescent MIA reported in literature was a competitive approach for triazine using 5-(4,6-dichlorotriazinyl) amino fluorescein (DTAF) as a fluorescent analog of the analyte by Piletsky and co-workers (Piletsky *et al*. 1997). However, some drawbacks hindered the development of this approach, such as the difficulty in synthesizing and purifying efficient fluorescent tracers as conjugates between fluorescent tags and analytes, and the complexity in preparing imprinted polymers that can bind to both the analyte and fluorescent tracers in the same manner. Therefore, only some analytes were detected and reported based on fluorescent MIAs, such as triazine (Piletsky *et al*. 1997, 2001), pentachlorophenol (Nicholls *et al*. 2006), 2,4-dichlorophenoxyacetic acid (Haupt *et al*. 1998; Lu *et al*. 2007), chloramphenicol (Levi *et al*. 1997; Mc-Niven *et al*. 1998; Suarez-Rodriguez and Diaz-Garcia 2001) and penicillins (Benito-Pena *et al*. 2006; Urraca *et al*. 2007). In this paper, we selected DTAF as a competitive fluorescent substance because it has the same triazine ring as melamine, as shown in Fig. 1.

To address template leaking in the applications of MIPs, the dummy molecular imprinting technique using a structural analog of the targeted compound as the template has been considered highly effective (Chen *et al*. 2011). So far, cyromazine (Curcio *et al*. 2010), 2,4,6-trimethoxy-1,3,5-triazine (He *et al*. 2009) and 2-(4,6-diamino-1,3,5-triazin-2-ylamino)