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Multiclonal plastic antibodies for selective aflatoxin extraction from food samples

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ABSTRACT

Herein, we focused on developing a new generation of monolithic columns for extracting aflatoxin from real food samples by combining the superior features of molecularly imprinted polymers and cryogels. To accomplish this, we designed multiclonal plastic antibodies through simultaneous imprinting of aflatoxin subtypes B1, B2, G1, and G2. We applied Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), and spectrofluorimetry to characterize the materials, and conducted selectivity studies using ochratoxin A and aflatoxin M1 (a metabolite of aflatoxin B1), as well as other aflatoxins, under competitive conditions. We determined optimal aflatoxin extraction conditions in terms of concentration, flow rate, temperature, and embedded particle amount as up to 25 ng/mL for each species, 0.43 mL/min, 7.0, 30 °C, and 200 mg, respectively. These multiclonal plastic antibodies showed imprinting efficiencies against ochratoxin A and aflatoxin M1 of 1.84 and 26.39, respectively, even under competitive conditions. Finally, we tested reusability, repeatability, reproducibility, and robustness of columns throughout inter- and intra-column variation studies.

1. Introduction

Whenever mycotoxins are mentioned, aflatoxin is the first one that comes to mind because it has been intensively examined. Aflatoxins are synthesized by *Aspergillus flavus*, *Aspergillus parasiticus*, and some subspecies of *Aspergillus nomius* (Chiavaro et al., 2001). These fungal metabolites, mainly consisting of aflatoxin B1, B2, G1, G2, M1, and M2, may acutely and/or chronically poison humans and animals depending on the exposure dose and duration (Jaimez et al., 2000; Ren et al., 2007; Tanaka et al., 2015). Acute exposure in animals directly affects the liver (Bryden, 2012) and results in other symptoms such as anorexia, hemolytic anemia, diarrhea, and icterus (Bbosa et al., 2013; Tang, Guan, Ding, & Wang, 2007; Trebak et al., 2015). Moreover, the resulting liver damage triggers cells in the liver and gall channels to rapidly proliferate, leading to cramps, paralysis, balance disorders, and hemorrhaging; furthermore, the nervous system may also be affected (Trebak et al., 2015). Recently, the International Agency for Research on Cancer (IARC), a part of the World Health Organization, initiated many studies regarding the effects of aflatoxins on human health and defined aflatoxins as Group 1 Carcinogenic Substances in light of the results (Corcuera, Vettorazzi, Arbillaga, Gonzalez-Penas, & Lopez de Cerain, 2012; IARC, 2012; Frehse et al., 2015; Trebak et al., 2015). In Turkey and many other countries, authorities have imposed regulations to control aflatoxin levels (especially B1) in food and forage, as aflatoxin levels (especially M1) in milk to protect consumers, especially children, from contaminated products (TR Food Codex., 2011). Today, almost all countries have assigned maximum aflatoxin levels in food and forage, not only to protect themselves, but also to avoid economic loss from the retrocession of products during international trade (Akiyama, Goda, Tanaka, & Toyoda, 2001; Bryden, 2012).

The constant demand for new, fast, and efficient methods in environmental, medicine, and biotechnology fields has guided scientists to develop more selective and sensitive alternative detection methods. Among separation systems, molecular recognition-based carriers prepared via molecular imprinting have become more prominent due to their high selectivity for target molecules (Alexander et al., 2006; de Smet et al., 2010; Uzun & Turner, 2016). Molecular imprinting, a unique polymerization technique, is used for obtaining highly selective binding cavities through a three-dimensional (3D) orientation of functional groups around the target molecule (generally called the

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template) and solidifying them in synthetic polymers (Corman, Armutcu, Uzun, Say, & Denizli, 2014; Turner et al., 2004). Since molecularly imprinted polymers (MIPs) have attractive features such as easy tunability, chemical and physical stabilities, cost efficiency, and specific molecular recognition abilities, they have attracted considerable research attention for development as a new generation of adsorbent. In general, a single molecule of interest is imprinted into a single polymer (called a plastic antibody) to achieve highly efficient recognition capabilities (Ali et al., 2010; Kryscio & Peppas, 2012). Recently, multi-target imprinting strategies have been developed to produce MIPs, which could be termed synthetic multi-clonal plastic antibodies. These polymers are very useful for detecting a group of molecules that are structurally related and found in the same media, if there is no sharp restriction for high selectivity (Altintas, Pocock, Thompson, & Tothill, 2015; Baggiani, Anfossi, & Giovannoli, 2007; Corman, Armutcu, Ozkara, Uzun, & Denizli, 2015; Ozaydin Ince et al., 2013; Qu, Wang, Tong, & Wu, 2010). In this way, it is possible to remove or preconcentrate multiple analytes simultaneously, resulting in further decreased operation cost while conserving high binding and recognition capabilities. Moreover, the cost of the polymer production for each target analyte may decline while also avoiding batch-to-batch variation in product quality (Bereli et al., 2008).

Cryogels, a type of hydrogel matrix, are prepared via radical polymerization of monomers under semi-frozen conditions (Corman et al., 2014). Ice crystals formed in these conditions act as pore-makers and cause an extra increment in monomer concentration called cryo-concentration (Erturk & Mattiasson, 2014). Although the system appears to be a single ice mass, polymerization takes place in the unfrozen organic phase effectively. After polymerization, melting reveals the monolithic cryogel structure (cryo – means ice in Greek) (da Silva Junior et al., 2015). The resulting super-macroporous cryogels consist of a highly interconnected network of 5–200 μm pores, which allows examination at higher flowrates with much lower flow resistance and/or good flow dynamics, and also provides a unique spongy characteristic to the cryogel (Asliyuce et al., 2012). By altering polymerization conditions (especially temperature, freezing rate, feeding monomer concentration, crosslinking ratio), it is possible to adjust and control various structural properties of cryogels (Gunko, Savina, & Mikhalovsky, 2013). Depending on these structural features and flow dynamics, cryogels have been used as a stationary phase for rapid, easy, and cost-friendly separation, preconcentration, and extraction purposes at high capacity even when working with viscous mobile phases for analytical, environmental, medical, and biotechnological applications (Asliyuce et al., 2012; Bicen Unluer, Uzun, & Ozcan, 2014; Gunko et al., 2013; Yilmaz, Bereli, Yavuz, & Denizli, 2009).

In this study, we developed multiclonal plastic antibodies for an alternative aflatoxin extraction column by combining molecular imprinting with cryogels. Specifically, we produced aflatoxin (B1, B2, G1, and G2)-imprinted cryogel-based poly(2-hydroxyethyl methacrylate) columns. After characterizing the prepared multiclonal plastic antibodies, extraction columns were optimized for extracting aflatoxins from aqueous solutions while considering factors such as aflatoxin concentration, flowrate, temperature, pH, and particle amount embedded into the polymeric structure. Then, aflatoxins were extracted from four real food samples including hazelnuts, peanuts, figs, and red pepper flakes using our multiclonal plastic antibodies alongside commercial aflatoxin immunoaffinity (AflaPrep) columns to compare performance. Finally, selectivity, recovery, reusability, and intra- and inter-column repeatability tests were applied to assess the economic and marketing feasibility of our developed product.

2. Materials and methods

2.1. Chemicals

2-Hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA), L-tryptophan methyl ester hydrochloride and methacryloyl chloride, N,N′-methylenebis(acrylamide) (MBAAm), ammonium persulfate (APS), 2,2′-azobisisobutyronitrile (AIBN), and N,N,N′,N′-tetramethylethylenediamine (TEMED) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Methanol (LC Grade), acetonitrile (LC Grade), potassium bromide (LC Grade), sodium chloride, nitric acid, dichloromethane, triethylamine, and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Deionized water was used during experiments, which was produced by a Barnstead ion-exchange ultra-pure water system (Dubuque, IA), a ROpure LP reverse osmosis cellulose acetate membrane (Barnstead D2731), and Barnstead D3804 NANOpure organic/colloid filters. Before use, all glassware was rinsed in 5% (w/v) aqueous nitric acid solution overnight and then dried in dust-free conditions after washing with deionized water.

2.2. Synthesis and characterization of multiclonal plastic antibodies

Herein, we performed a four-step approach to produce multiclonal plastic antibodies: (i) synthesis of functional monomers, (ii) optimization of monomer:template ratio, (iii) synthesis of MIPs, and (iv) synthesis of cryogel-based multiclonal plastic antibodies. Before molecular imprinting, functional N-methacryloyl-L-tryptophan methyl ester (MATrp) monomers were synthesized and characterized according to our previous methods (Yilmaz et al., 2009) (details given in Supplementary Material, SM). We optimized the proper monomer:template ratio by characterizing the pre-polymerization complex of functional monomers and template molecules using spectrofluorimetry. To accomplish this, we prepared pre-polymerization complexes with different molar ratios before spectrofluorimetric measurements (Shimadzu RF-5301 PC, Kyoto, Japan) at wavelengths of 350–600 nm (details given in SM). In the process of preparing aflatoxin-imprinted polymers, bulk polymerization method was performed. At this stage, three different polymers were produced: (a) MATrp-free poly(HEMA) (polymer code: Plain); (b) non-imprinted polymers (polymer code: NIP); and (c) aflatoxin-imprinted polymers (polymer code: MIP). Briefly, we applied the bulk polymerization method as follows: 2-Hydroxyethyl methacrylate was used as the monomer phase (HEMA, 1 mL), ethylene glycol dimethacrylate (EGDMA, 2 mL), and the pre-polymerization complex (0.5 mL) were mixed and sonicated until a homogeneous solution was achieved. Toluene (2 mL) was added to the solution as a pore maker and diluent while magnetically mixing at 150 rpm at room temperature for 30 min. Polymerization was initiated by the addition of 25 mg AIBN and kept at constant temperature (70 °C) for six hours. The polymers obtained were crushed into fine particles using a ball-miller type Retsch MM200 (Dusseldorf, Germany). A Nano Zetasizer (NanoS, Malvern Inst., London, England) was used to characterize the ground polymers, and the results and detailed methods are given in the SM. For the synthesis of multiclonal plastic antibodies, cryogel-based imprinted columns were prepared with seven different features (SM, Table SM-1) in the form of monolithic columns (abbreviated as Afla MIP; Fig. 1). During the preparation of Afla MIP multiclonal plastic antibodies, cryogelation was conducted as follows: N,N′-methylene bisacrylamide (0.283 g) was dissolved in water (10 mL) by sonication at 45 °C for 30 min while HEMA (1.30 mL) was separately dissolved in water (5 mL) before mixing both solutions. Then, polymeric fine powders containing particles of approximately 3 μm were added