Detection of Mycotoxin in Foods Using an MIP-based Paper-fluidic Device

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Abstract

Mycotoxins are the toxic secondary metabolites produced by fungi and can cause adverse health effects. They can lead to acute poisoning, cancer and/or reproductive issues in livestock and humans so that development of detection tools is highly required. Traditional detection methods such as chromatographic-based techniques and immunoassays have certain disadvantages including high cost, complex instrumentation, massive amount of solvent, and low portability. Molecularly-imprinted polymers (MIPs) have been introduced as a cheaper, easier and more robust technique with high selectivity, high sensitivity and long shelf-life. MIPs are artificial polymers with cavities interacting with specific functional groups of the targeted compound. Paper is a cheap and commonly used laboratory material, and its 3-dimentional natural porous structure makes it suitable as a base for the fabrication of microfluidic "lab-on-a-chip" devices. In this thesis project, a Whatman #1 chromatographic paper-based device was developed to detect mycotoxin zearalenone by using MIPs and fluorescent quantum dots (QDs). CdSe/ZnS QDs could sensitively quench fluorescent signals when zearalenone was unbound from the MIPs. This microfluidic device could detect zearalenone in spiked corn samples with limit of detection of 1.085 mg/L within the linear range of 1-50 mg/L. MIP-based paper microfluidics has the potential for the detection of mycotoxin contamination in agri-food commodities in a cost effective, sensitive and user-friendly manner.

Résumé

Les mycotoxines sont les métabolites secondaires toxiques produits par les champignons et peuvent avoir des effets néfastes sur la santé. Ils peuvent entraîner des intoxications aiguës, des cancers et/ou des problèmes de reproduction chez le bétail et les humains, de sorte que le développement d'outils de détection est hautement nécessaire. Les méthodes de détection traditionnelles telles que les techniques basées sur la chromatographie et les immunodosages présentent certains inconvénients, notamment un coût élevé, une instrumentation complexe, une quantité massive de solvant et une faible portabilité. Les polymères à empreinte moléculaire (MIP) ont été introduits comme une technique moins chère, plus simple et plus robuste avec une sélectivité élevée, une sensibilité élevée et une longue durée de conservation. Les MIP sont des polymères artificiels avec des cavités interagissant avec des groupes fonctionnels spécifiques du composé ciblé. Le papier est un matériau de laboratoire bon marché et couramment utilisé, et sa structure poreuse naturelle tridimensionnelle le rend approprié comme base pour la fabrication de dispositifs microfluidiques «lab-on-a-chip». Dans ce projet de thèse, un dispositif chromatographique Whatman #1 à base de papier a été développé pour détecter la mycotoxine zéaralénone en utilisant des MIP et des points quantiques fluorescents (QD). Les QD CdSe/ZnS pouvaient éteindre de manière sensible les signaux fluorescents lorsque la zéaralénone n'était pas liée aux MIP. Ce dispositif microfluidique pourrait détecter la zéaralénone dans des échantillons de maïs dopés avec une limite de détection de 1,085 mg/L dans la plage linéaire de 1 à 50 mg/L. La microfluidique sur papier basée sur le MIP a le potentiel de détecter la contamination par les mycotoxines dans les produits agroalimentaires de manière rentable, sensible et conviviale.

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Preface

This thesis was prepared according to McGill University Thesis Preparation guidelines. This thesis is written in traditional monograph-style and contains five chapters; introduction, literature review, materials and methods, results and discussion, and conclusion. This work is original and wasn't published previously.

Contribution of Authors

The primary author, Zeqiao Wang, is responsible for all five chapters of this thesis. Dr. Xiaonan Lu is the thesis supervisor and had direct advisory on the research. Zeqiao Wang carried out all the experiments, including MIPs synthesis, QD@MIP@paper synthesis, adsorption tests and real sample testing, and data analysis. Staff David Liu performed the SEM tests for QDs@MIPs@paper and QDs@NIPs@paper. Dr. Xiaonan Lu and Marti Hua edited the thesis before submission.

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List of abbreviations

3-CCA	Coumarin-3-carboxylic acid
AIBN	Azobisisobutyronitrile
APTES	(3-Aminopropyl)triethoxysilane
CdSe	Cadmium selenide
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDMA	Ethylene glycol dimethylacrylate
FT-IR	Fourier-transform infrared spectroscopy
HAc	Acetic acid
HAP	Hydroxyapatite
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
LOB	Limit of blank
LOD	Limit of detection
LOQ	Limit of quantification
MAA	Methacrylic acid
MeOH	Methanol
MIP	Molecularly-imprinted polymer
NHS	N-Hydroxysuccinimide
NIP	Non-imprinted polymer
QD	Quantum dot
SEM	Scanning electron microscopy

SERS	Surface-enhanced Raman spectroscopy
UV	Ultraviolet
VIS	Visible light
ZEA	Zearalenone
ZnS	Zinc sulfide

1. INTRODUCTION

1.1 Background information

Mycotoxins are secondary metabolites of fungi and can cause adverse health effects to animals in low concentration. There are more than 300 known mycotoxins with diverse chemical structures, varying from small four-carbon molecules (e.g., moniliformin) to larger complex compounds (e.g., phomopsins) (Murphy et al., 2006; Zain, 2011). Some common mycotoxins that have raised most concern are ochratoxins (produced by *Aspergillus* spp. and *Penicillium* spp.), fumonisins (*Fusarium* spp.), aflatoxins (*Aspergillus flavus* and *A. parasiticus*) and zearalenone (*Fusarium* spp.) (Richard, 2007; Zain, 2011). *Aspergillus, Fusarium,* and *Penicillium* are the most common producers of mycotoxins (Murphy et al., 2006). Humans are exposed to mycotoxins by consuming contaminated plant-based food, meat, milk or eggs of animals fed with contaminated feed, and by contacts with air and dust containing mycotoxins (Rogowska et al., 2019; Zain, 2011).

In Canada, food mycotoxin contaminations are mainly found in cereal grains, nuts and fruits (Murphy et al., 2006). Stages of growth, harvest, drying and storage are all exposed to the risk of mycotoxin contamination (Winter & Pereg, 2019). Consumption of mycotoxin-contaminated foods can cause various adverse effects on humans and animals, including acute symptoms (e.g., gastroenteritis, borborygmi, vertigo, lethargy), chronic disease (e.g., cancer, immune suppression, necrosis of skin and oral mucosa) and even death (Zain, 2011). In addition, mycotoxin contamination in animal feeds is associated with reproductive disorders and the quality of animal products. Millions of dollars are lost every year due to contamination

of food and feed commodities caused by aflatoxins, ochratoxins, fumonisins, trichothecenes, zearalenone, tremorgenic toxins and ergot alkaloids (Zain, 2011). It is challenging to remove mycotoxins from contaminated food and animal feed without causing any waste. As a result, the best way to minimize the waste is to find traces of contamination as early as possible. Thus, detection of mycotoxin is critical to the food industry.

Zearalenone, a secondary metabolite of *Fusarium spp.*, is a widespread mycotoxin in agricultural products (Fu et al., 2020). Common plant-based sources of zearalenone are corn, wheat, barley, oats, cereals, sorghum, nuts and spices. This mycotoxin is found in foods and feeds in both natural and processed forms. Contamination can occur during growth, harvest, processing and storage stages (Fu et al., 2020; Huang et al., 2019; G. Li et al., 2017; Sergeyeva et al., 2020). Zearalenone can pass on to the animals via the consumption of contaminated feed. When people eat zearalenone-contaminated food or consume meat, eggs or milk from animals contaminated with zearalenone, zearalenone enters human body as well (Ropejko & Twarużek, 2021). Zearalenone is rapidly absorbed in human gastrointestinal tract and is metabolized into α- and β-zearalenol in the intestines, liver, lungs and kidneys. Zearalenone was reported to be found in human urine and serum (Ropejko & Twarużek, 2021) Zearalenone is a potential health hazard to both animals and humans. It is reported to be genotoxic, neurotoxic, hepatotoxic, hemotoxic, estrogenic and anabolic (G. Li et al., 2017; Sergeyeva et al., 2020; Urraca et al., 2006; Weiss et al., 2003; Y. Zhang et al., 2020) to different animals, and the toxicity is highly species specific in each toxin group. In humans, zearalenone has been associated with endocrine system disruption, premature puberty, lesion of female reproductive organs, oxidative stress and apoptosis of embryo stem cells. However, evidence of its carcinogenicity to humans is limited (IARC Group 3) (Ropejko & Twarużek, 2021). Zearalenone is heat resistant

and hard to destroy by regular cooking (Y. Zhang et al., 2020). Therefore, detecting the residue level of zearalenone in foods is highly important as a practical approach to prevent the consumption of contaminated food products.

Molecularly-imprinted polymers (MIPs) have been developed as an emerging technique of recognition. It is able to recognize small molecules (M. Jiang et al., 2015) and has been applied in the fields of food science, agricultural science and biomedicine (Hong et al., 2010; Kong et al., 2017; Saylan & Denizli, 2019). MIPs are artificially fabricated against the structure of a certain template molecule, thus being selective to this template (Turiel & Esteban, 2020). Novel biosensors integrated with MIPs have advanced simplicity, sensitivity and fast response (Hong et al., 2010). The template can be the target itself or another molecule with similar chemical structures and properties as called "dummy template". In this thesis project, coumarin-3-carboxylic acid (3-CCA) and naringenin were used together as the dummy template instead of the hazardous and expensive zearalenone for MIPs synthesis. Both 3-CCA (a derivative and reactant in pharmaceuticals) and naringenin (a flavonoid in grapefruit) are much safer to work with and hundreds to thousand times cheaper. Both 3-CCA and naringenin have similar carbonyl groups and benzene rings with two attached hydroxyl groups (Y. Zhang et al., 2020); therefore, they were able to have similar interactions (mainly hydrogen bonds) at similar positions in the molecule with the monomers as the target molecule would do during MIPs synthesis.

Microfluidics were initially developed as a more precise method of molecular separation, laboratory transfer, measurement or determination at small scales, thus having improved portability to combine multiple analytical steps onto one single device (Tian & Finehout, 2008). Traditional materials for fabricating microfluidic devices are silicon, glass and polymers such as

polydimethylsiloxane (PDMS; Tian & Finehout, 2008). Paper microfluidic devices have advantages of low cost, light weight, natural porous structure, comparably simple operation and compatibility with many reaction environments (X. Li et al., 2012). W. Li et al. (2021) credited that the first integration of MIPs on paper fluidics was developed by Ge et al. in 2013. Paper-based microfluidics have been mostly applied in medical field for point-of-care testing and diagnostics. Ge and coworkers (2012) designed a 3D origami paper-based electrochemiluminescence immunoassay platform for point-of-care detection of tumour markers and carcinoembryonic antigen. Lu et al. (2010) designed wax-printed paper-based nitrocellulose membranes for protein patterning, dot immunoassay and sample purification.

Quantum dots (QDs) are a type of novel fluorescent nanomaterials. Compared to the traditional fluorescent dyes, QDs have advantages such as the generation of broader adsorption spectra, narrower emission spectra, larger extinction coefficient, larger Stokes shift and higher photostability than the regular fluorescent dyes (Díaz-Álvarez & Martín-Esteban, 2021; Z. Zhang et al., 2018). MIPs were reported to be able to enhance the fluorescence sensitivity and selectivity of QD-based sensors (Z. Zhang et al., 2018). In this thesis project, CdSe/ZnS QDs were used. The fluorescence of these QDs was quenched with the nearby presence of zearalenone at 630 nm emission, making the fluorescence signals lower when the concentration of zearalenone in the sample was higher.

1.2 Rationale

Efficient detection of food mycotoxins can reduce food waste from mycotoxin contamination. Two main traditional detection methods (i.e., chromatography and immunoassays) are expensive, complex and require highly-trained personnel (Goud et al., 2018;

Guo et al., 2015). This thesis project presents an MIPs-based paper microfluidic approach to detect zearalenone. It shows the potential of a cost-effective, more portable and more rapidly progressed method (M. Jiang et al., 2015; Saylan & Denizli, 2019).

1.3 Research Hypothesis

The designed MIPs-integrated paper-based microfluidic device is able to detect the mycotoxin zearalenone.

1.4 Research Objectives

To test the research hypothesis, the following objectives will be completed.

1) We aim to develop MIPs fabricated with zearalenone dummies that can have selectivity to zearalenone.
2) We aim to integrate the MIPs into a paper-based microfluidic device.
3) We aim to apply this MIPs-based microfluidic device for the detection of zearalenone at ppm level.

2. LITERATURE REVIEW

2.1 Traditional methods to detect food mycotoxins

Two major types of traditional analytical methods for the detection of food mycotoxins are chromatography-based methods and immunoassay-based methods. Examples of chromatographic methods are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) with UV, fluorescence or diode-array detectors, gas chromatography-mass spectrometry (GC-MS) and gas chromatography coupled with electron capture detector (GC-ECD). TLC is considered as one of the most popular methods (Bhat et al., 2010). It is fast, cheap but not very accurate or sensitive (Agriopoulou et al., 2020). GC is not often used in detecting mycotoxins because mycotoxins are non-volatile and needs a derivatization step before detection (Agriopoulou et al., 2020). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is more specific, sensitive and reliable than HPLC (Agriopoulou et al., 2020) and has been applied in the detection of multiple mycotoxins (Bhat et al., 2010).

Chromatography is used in sensitive and accurate determination, while immunoassays are mainly used for rapid, on-site detection and preferably for rapid screening of a large number of food commodities (Agriopoulou et al., 2020). Sometimes immunochemical-based methods are coupled with chromatography-based methods as reference methods (Anfossi et al., 2016). Major immunoassay methods are enzyme-linked immunosorbent assay (ELISA), immunoaffinity column-based analyses and immunochromatographic test (ICT), also known as lateral-flow devices (LFDs) (Agriopoulou et al., 2020; Anfossi et al., 2016; Sforza et al., 2004). Commercial ELISA kits are available for all regulated mycotoxins and provide an easy-to-operate fast

screening method for food mycotoxin detection. ICTs play the lead role in qualification and semi-quantification of mycotoxins in food samples (Anfossi et al., 2016).

Chromatographic methods require high cost in time, money, space, and the amount of solvents used (Guo et al., 2015). The extensive steps of sample preparation, system operation, system clean-up and data interpretation need to be monitored by highly-trained personnel. The large instrumentation only allows operations in the laboratory. On the other hand, immunoassays are smaller-scaled, cheaper, simpler and more portable. The sample preparation steps for immunoassays are also easier (Anfossi et al., 2016), but antibodies are generally also expensive (Guo et al., 2015). In addition, immunoassays are less accurate for quantitative analysis of toxins (Sforza et al., 2004). Therefore, my thesis project proposes a novel detection method for food mycotoxin that is cheaper and more user-friendly.

2.2 Molecularly-imprinted polymers (MIPs)

MIPs are a type of artificially synthesized polymers with molecular recognition properties. Its structure has specific cavities designed for a template molecule (Pichon, 2007). This polymer is prepared by first mixing the template with functional monomers, which bind with the template at multiple sites. With the help of cross-linkers and initiators, the monomers are polymerized around the template (Pichon, 2007). The recognition between the template and the binding sites is the determined factor to achieve the selectivity (Turiel & Esteban, 2020). MIPs technology has currently been used widely in biosensing, molecular separation, catalysis, solid-phase microextraction and drug delivery (Ashley et al., 2017; Barrey et al., 2014; Fu et al., 2020; Saylan & Denizli, 2019; Turiel & Esteban, 2020). Compared to traditional methods, MIPs-based devices are miniaturized and have high simplicity, sensitivity, selective recognition

property, specificity and stability, longer shelf-life, faster response and lower spatial and economical cost (Chauhan et al., 2016; Goud et al., 2018; Hong et al., 2010; Kong et al., 2017). On the contrary, the challenges of MIPs technique are the complexity of synthesis, the difficulty in complete template removal, the relatively lower affinity than natural receptors such as antibodies. Moreover, the specificity of binding brought limits to multi-target analyses (Baggiani et al., 2007; W. Li et al., 2021; Turiel & Esteban, 2020; Y. Wang et al., 2015).

2.2.1 Components of MIPs

Template

The template molecule binds with the monomers before being removed, leaving cavities with specific shape and electrochemical properties (M. Jiang et al., 2015; Pichon, 2007; Saylan & Denizli, 2019). The template can be the target itself or its dummy with resemblance in shape, electronic properties and hydrophobic properties. One example of the features of the dummy is that it has similar functional groups as the target, but its carbon skeleton chain has different lengths from the target (Baggiani et al., 2007); thus, the dummy could "deceive" MIPs recognition but not the detection system. One reason to use dummies instead of target molecules as the template is due to the lower cost. For example, the cost of zearalenone on Sigma-Aldrich is $169 per 10 mg, while its dummy naringenin costs $79 per gram, less than 1% of zearalenone cost. Two components to synthesize another dummy CDHB, named 1,1-carbonyldiimidazole and 2,4-dihydroxybenzoic acid, cost $32 and $34.10 per 5 grams, both of which are even cheaper than naringenin. The amount of the template needed is usually large so that it could provide more binding sites; therefore, using cheaper materials could significantly decrease the cost (Pichon, 2007). Another reason to use dummy is to reduce the error caused by template residues in the

MIPs. It has been highly challenging to remove all template molecules during washing. Template bleeding is the slow release of the remaining templates in the cavity of MIPs after washing process, which has been recognized as a challenge for MIPs to perform molecular separation (Baggiani et al., 2007). Since the template would still present in the polymer matrix after template removal, using a dummy would overcome the inaccuracy caused by the residue of the template for trace analyses (Pichon, 2007; Vasapollo et al., 2011). A third reason of replacing the toxic target analyte with a much less-toxic dummy is to provide more assurance for the health of the personnel. For example, an ochratoxin A dummy should have similar structure, chirality and planarity to the target toxin, while it should not be carcinogenic (Baggiani et al., 2007). Y. Zhang et al. (2020) chose coumarin-3-carboxylic acid and naringenin to be the double dummies of zearalenone because they all had similar benzene ring parent structures and hydroxyl groups. Urraca et al. (2006) synthesized a compound named cyclododecyl 2,4-dihydroxybenzoate (CDHB) to be a dummy of zearalenone. Both compounds have similar sizes, a benzene ring with two hydroxyl groups, an ester group and a large ring structure. Fu et al. (2020) used warfarin as the structural analog of zearalenone. These two molecules have similar sizes and an ester group connected with a benzene ring.

Solvent

At the beginning of the synthesis, monomers and template are mixed in the solvent. The solvent acts as a porogen and provides an environment for monomers and template to interact via intramolecular interactions, such as hydrogen bonding and electrostatic interactions (Pichon, 2007). Pichon (2007) pointed out that the physiochemical characteristics of the porogen determined the effectiveness of recognition between the template and the monomer. Common

solvents included polar molecules such as water, less polar molecules such as methanol and ethanol, non-polar or slightly polar molecules such as chloroform, dichloromethane and dimethyl sulfoxide (DMSO) and even protic reagents such as naphthalene sulfonate and chlorophenoxyacetic acid (Adumitrăchioaie et al., 2018; Pichon, 2007; Turiel & Esteban, 2020; Vasapollo et al., 2011).

Monomer

Monomers are the composition units of the MIPs. They form binding sites against the template with functional groups as they are polymerizable and can interact with the template via intramolecular covalent, semi-covalent or noncovalent chemical bonds or intermolecular forces such as Van der Walls force or hydrogen bonds (Adumitrăchioaie et al., 2018). Commonly used monomers are 1-allylpiperazine (1-ALPP) (Sergeyeva et al., 2020; Urraca et al., 2006), methacrylic acid (MAA) (Hu et al., 2015; Huang et al., 2019; Y. Zhang et al., 2020) and 4-vinylpyridine (4-VP) (Weiss et al., 2003). G. Li's group (2017) used MAA and 4-VP as double monomers to improve the selectivity of the MIPs for the template via stronger noncovalent interactions. Numerous studies used more than one monomer to induce stronger binding and better recognition. However, it was not always a good choice since more monomers could generate more complexity and sometimes induced disruptions (Saylan and Denizli, 2019).

Cross-linker and initiator

After the monomer is brought to a thorough dispersion with the template, the cross-linker and the initiator are added. The function of the cross-linker is to fix the position of the monomers surrounding the template, while the initiator is to trigger the polymerization process

(Adumitrăchioaie et al., 2018). The cross-linker and the initiator are mostly organic reagents. Common cross-linkers include trimethyl trimethacrylate (TRIM), ethylene glycol dimethacrylate (EGDMA) and tetraethyl orthosilicate (TEOS). Most common initiators are azobutyronitrile (AIBN) and azodiisopentanyl (AIHN) (Fu et al., 2020; Gao et al., 2020; Urraca et al., 2006; Weiss et al., 2003). The amount of cross-linker should maintain the stability of the binding sites and have some degree of flexibility to allow the sample to enter the recognition cavity (Y. Wang et al., 2015). The choice of the initiator should be based on the template and the principle of polymerization (Saylan and Denizli, 2019).

Other materials

Other supporting materials can also be added in the recipe of MIPs synthesis to exaggerate certain preferred properties of the polymer. Fu et al. (2020) combined MIPs with magnetic separation technology to increase the contact surface area between the polymer and the template, therefore improving the binding efficiency to the target molecule. Huang et al. (2019) supported their MIPs targeting zearalenone with metal-organic framework fabricated with metal ions to improve the accessibility of binding sites and to speed up diffusion. Y. Zhang et al. (2020) added hydroxyapatite in their MIPs because HAP was a good material for surface-imprinted carriers due to its good biocompatibility and activity. In literature, the supporting materials could either be purchased or synthesized beforehand and integrated into the procedures of MIPs synthesis. They were usually added at the beginning of synthesis together with the monomer and the template (Urraca et al., 2006; Y. Zhang et al., 2020).

2.2.2 Principles of polymerization

One way to distinguish methods to synthesize MIPs is based on their principle. Adumitrăchioaie et al. (2018) categorized the principles of polymerization into free radical polymerization, sol-gel polymerization, *in situ* polymerization and seed polymerization.

Free-radical polymerization was named by its way of initiating the chain reaction process. The reaction was triggered by free radicals of double bonds of the monomers (Gao et al., 2020). This polymerization method has been recognized as the most established technique and it can be further divided into bulk, precipitation and suspension polymerization methods (Adumitrăchioaie et al., 2018). Bulk polymerization has been popular as it has been recognized to be straightforward to synthesize and has a high stability over a wide range of pH (da Silva et al., 2019). On the other hand, the MIPs mixture needs grinding that may disrupt parts of the polymerized structure (Adumitrăchioaie et al., 2018). Based on experimental observations, the yield was relatively low due to the loss of micro-scaled particles during transfer between the containers (Urraca et al., 2006). Bulk polymerization also has disadvantage in the comparatively low site accessibility and adsorption capacity (da Silva et al., 2019).

Precipitation polymerization is another popular method, in which the polymers are formed as homogenized particles and thus grinding is not required. The binding sites are distributed more evenly than that of bulk polymerization (Gao et al, 2020). However, precipitation of the polymers only occurs when the size of the polymer is large enough to become insoluble (Adumitrăchioaie et al., 2018); therefore, it requires larger amounts of chemicals. Suspension method produces a monolithic MIPs film and requires a solvent with both aqueous and organic phases, while the monomer and the initiator should stay in the organic

phase. This method also requires a surfactant and a stabilizer (Adumitrăchioaie et al., 2018). One significant challenge of free radical polymerization method is that the polymerization reaction rarely occurs in the aqueous environment because the mixture has unsatisfactory stability in water, and the electrostatic interactions among each component are sensitive (Piletsky et al., 2000).

Sol-gel polymerization method is another popular method for MIPs synthesis (Gao et al., 2020). One unique feature of sol-gel polymerization is that it allows the reaction to occur in a mostly inorganic aqueous environment by acidic or basic hydrolysis and condensation (Cummins et al., 2005). This method is stable in physical, chemical, and thermal matters (Guoning et al., 2020). Common precursors of sol-gel polymerization are methyl orthosilicate and ethyl orthosilicate (Gao et al., 2020), while other precursors such as silane and siloxane were also reported in the previous works (Cummins et al., 2005; Moein et al, 2019). Sol-gel polymerization method has advantages in its simple preparation, controllable porosity, stable chemical properties, optical transparency and the potential to significantly improve the selectivity (Cummins et al., 2005; Gao et al., 2020). B. Li et al. (2014) compared one organic free radical MIPs and one inorganic sol-gel MIPs for their capacity of controlling the delivery of salicylic acid. The radical MIPs showed a better binding capacity in acetonitrile, while the sol-gel MIPs performed better in ethanol. El-Beqqali and Abdel-Rehim (2016) summarized that sol-gels were stable in different thermal and chemical environment, but their selectivity was not very satisfactory. MIPs fabricated by sol-gel polymerization technique could potentially solve this problem.

MIPs prepared by *in situ* polymerization have been fast and simple for liquid separation and can be used to make HPLC polymeric monolithic columns (Amut et al., 2010). On the other

hand, the removal and rebinding of the template are challenging (Adumitrăchioaie et al., 2018). Piletsky et al. (2000) were the first to successfully perform this method in water upon a commercial polypropylene membrane. This research group used 2-acrylamido-2-methylpropanesulfonic acid as the monomer and *N*,*N*'-methylenebis as the crosslinker. The polypropylene (PP) membranes were soaked in the solvent (a mixture of methanol and benzophenone) and the reagents were then added. The treated membrane was placed in ultraviolet light to polymerize. The resultant MIP-coated PP membranes had a high performance affinity for the template desmetryn. Liu et al. (2006) developed a monolithic MIPs used as a monolith for HPLC stationary phase to separate racemic mixture of sulfamethoxazole (SMO). EDMA was used as the crosslinker and AIBN was used as the free-radical initiator, while the monomer with the best performance was 4-vinylpyridine. The template SMO was recognized by the binding sites due to hydrophobic interaction. The MIPs showed good selectivity and recognition ability in the aqueous environment. In another study, Y. Wang et al. (2015) synthesized an MIPs liquid film with a carbon electrode surface via *in situ* method to detect bovine serum albumin using 3-(3-aminopropyl)-1-vinylimidazole tetrafluoroborate as the monomer, methylenebisacrylamide as the crosslinker, and ammonium persulfate and tetramethylethylenediamine as the radical double initiators. This polymer achieved high binding site accessibility, high specificity and high sensitivity.

Seed polymerization was reported less in the literature. One main feature of seed polymerization is to make polymerization occur on the surface of sub-micron sized particles, thus creating a polymer with generally a homogeneous particle size and an easily controllable thickness (Adumitrăchioaie et al., 2018). These core particles were called cores or seeds. The step of fabricating the seeds is called seed emulsion or seed swelling. Kim et al. (2005) used

seed emulsion to develop an MIPs microgel containing polystyrene seed particles using 4-vinylpyridine (4-VP) as the monomer and EDGMA as the crosslinker. They summarized that the ratios of weights of monomer/seed and solvent/monomer together contributed to the particle size, pore size and adsorption capacity. The solvent that generally worked as porogen in MIPs synthesis also swelled the seed particles in seed polymerization. Except the seed emulsion step, seed polymerization could be otherwise similar to suspension polymerization. L. Zhang et al. (2002) combined seed emulsion and suspension polymerization to develop MIPs for detection of tyrosine with water and polyvinyl alcohol as dispersants. MAA and acrylamide were used as the monomers, while trimethacrylate and toluene were used as the crosslinkers. The seeds were also polystyrene seeds. This MIPs could distinguish between tyrosine and phenylalanine with similar structures. Moreover, Pang et al. (2005) synthesized polyacrylamide gel beads imprinted with bovine serum albumin with gel beads as seeds. These MIPs had good recognition against the target following the Langmuir adsorption rule.

2.2.3 Applications of MIPs

MIPs are widely applied in biomedical, chemical and microbiological fields due to its specific recognition capacity and relatively low cost. The applications of MIPs can be divided into the following categories: 1) detection, 2) molecular separation, and 3) delivery (e.g., drug release).

Detection

One major use of MIPs is to detect and quantify the target compound present in a sample. Hong et al. (2010) designed an MIPs-based plastic microfluidic chip to detect propofol. The

mixture of MIPs ingredients was injected into the desired place of the bottom layer of microfluidic device made of cyclic olefin copolymer (COC) before UV polymerization. After template removal, a PDMS layer with designed microchannels were topped via hot embossing. The sample was injected using a syringe pump. The voltage drop of the photodetector after sample injection was measured using laser diodes and photodetectors. The device was small-sized (80 mm × 80 mm × 70 mm including detection unit), fast (60 seconds of incubation), cheap and highly-selective (685% specific binding) and sensitive (0.25 ppm LOD). While only propofol solutions of different concentrations were tested, the authors stated that testing with human blood samples would be possible in future.

In another study, Guoning et al. (2020) proposed a surfactant-modified sol-gel MIPs-based biometric ELISA method to detect human serum albumin, whose content in urine is a significant indicator of kidney diseases. They used 3-(methacryloxy) propyltrimethoxysilane (MPS) as the "crosslinker-monomer", which acted both as monomer and crosslinker, and created a mild hydrolysis system with Tween-20 as solvent and surfactant. The MIPs was synthesized in a weak alkaline environment, grafting on the surface of Fe_3O_4 nanoparticles. In the ELISA step, ovalbumin was added to block non-specific adsorption. This method had a good recovery ranging between 85.4%-104.5%.

Sergeyeva et al. (2020) proposed a fast approach to detect and quantify zearalenone in maize, wheat and rye with a selective *in situ* MIPs membranes and smartphones. They used cyclododecyl-2,4-dihydroxybenzoate as the template, 1-allylpiperazine as the functional monomer, EDMA as the crosslinker and polyethylene glycol as the porogen. The reaction occurred under UV light, resulting in the generation of 60 μm-thick MIPs membranes. This MIPs device was applied for fluorescence-based detection. During testing, samples spiked with target

analyte were incubated for 1 hour with the membranes and treated with UV transilluminator for 1 min. A mobile phone camera was used to take a picture of the results. The images were processed with Spotxel microplate Reader®. The sensor showed a high specificity with LOD of 1 μg/mL. In addition, this MIPs membrane had a high intrinsic stability.

Y. Wang et al. (2015) developed an electrochemical sensing device composed of an MIPs ionic liquid film and a glassy carbon electrode for detection and quantification of bovine serum albumin in milk. They used 3-(3-aminopropyl)-1-vinylimidazolium tetrafluoroborate ionic liquid as monomer, *N*,*N*′-methylene-bis-acrylamide (MBA) as crosslinker and ammonium persulfate (APS) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) as initiators. The carbon nanotubes were modified on the electrode surface. Then, MIPs were synthesized on the surface of the nanotubes@electrode complex. During measurement, the oxidation peak current was measured using an electrochemical workstation before and after incubation with milk samples spiked with BSA. The device could detect BSA between 1.5×10^{-9} and 1.5×10^{-6} mol/L with LOD of 3.91×10^{-10} mol/L. The device was proved to have good selectivity to BSA, but not very high selectivity against human serum albumin possibly due to its similarities in amino acid sequencing with BSA. This method showed reliable reproducibility with a standard deviation of 4.37%.

W. Zhang et al. (2017) developed a MIPs-based electrochemiluminescence sensor to detect fumonisin B_1 in milk and maize samples. MIPs were imprinted onto the surface of a glassy carbon electrode, together with gold nanoparticles (AuNPs) and ruthenium-based silica nanoparticles ($Ru@SiO_2$ NPs) with chitosan composites. The selectivity of MIPs for fumonisin B_1 was based on its amino groups, and electrochemiluminescence intensity increased with binding to fumonisin B_1. AuNPs were coated on the glassy carbon electrode before $Ru@SiO_2$

NPs. Then, the MIPs mixture was casted onto the electrode and was polymerized under UV light. For testing, the finished device was soaked in the sample solution and the electrochemiluminescence response was measured. The device could effectively detect the target toxin from 0.001 to 100 ng/mL with LOD of 0.35 pg/mL.

Separation

Another major use of MIPs was for molecular separation, usually as a sample pre-treatment step before quantitative analyses. Over the recent years, molecularly imprinted solid phase extraction (MISPE) system has been widely applied as sample preparation method for the detection of food contaminants, aiming to avoid the lower selectivity of the traditional SPE and the higher cost of traditional immunoassays. The MIPs for MISPE has been mostly made by bulk polymerization. The prepared MIPs were loaded in either a regular SPE cartridge or applied on-line as a separate small column before HPLC analysis (Baggiani et al., 2007).

Y. Zhang et al. (2020) fabricated MISPE columns for HPLC to separate zearalenone from cereal samples. The MIPs were synthesized with hydroxyapatite as supporting center surrounded by a layer of polymers. Other materials used for this study were coumarin-3-carboxylic acid (3-CCA) and naringenin (double templates), MAA (functional monomer), EDMA (crosslinker) and AIBN (initiator). The MIPs were wetted with methanol before being loaded into SPE columns with a sieve plate on its top and bottom sides. The homemade MISPE column was conditioned, and the prepared sample was added. Millet, corn and coix lachryma were placed under extreme environmental conditions to allow them to grow zearalenone naturally. The LOD was 0.94 μg/kg in acetonitrile solution of zearalenone and 1.32 μg/kg in real sample matrices.

MIPs have been proved to separate a particular molecule not only from other compounds but also from its own enantiomers. Amut et al. (2009) designed the first chiral MIPs-based HPLC monolithic column (CMIPMC) for the separation of different enantiomers of amlodipine. Its *S*-(-)-enantiomer is a therapeutic drug for hypertension and angina pectoris, while other enantiomers cause adverse health effects. MIPs were synthesized by an *in situ* method of using *S*-(-)-amlodipine as template, MAA as functional monomer, EDMA as crosslinker and AIBN as initiator. The mixture was transferred to a stainless column before being polymerized and washed with HPLC mobile phase. The finished CMIPMC bonded the *S*-(-)-enantiomer, which had vasodilating effect with seemingly hydrogen bond or ionic force working in the recognition system. This column showed high specific affinity and selectivity to *S*-(-)-amlodipine from *R*-(-)-amlodipine and similarly-shaped compounds.

Drug delivery

A drug delivery system (DDS) manages and moderates the flow rate of a drug to be delivered to its target in order to optimize its therapeutic effects, as underdosing and overdosing might cause adverse effects or no effect (Zaidi et al., 2020). The drug was bound to a "targeting vector" such as a peptide or an antibody. The vector released the drug when triggered by specific stimuli such as a cell surface epitope. In some occasions, the drug-vector complex was moved to interior of the cell after binding to its surface. Then, the drug was released in the cell (Sellergren & Allender, 2005).

MIPs-based DDS showed many advantages compared to the traditional DDS. MIPs were easy to synthesize and integrate into a device (Kryscio & Peppas, 2009). With the ability of specific binding, MIPs-based DDS could be used both as diagnostic tool and therapeutic tool on

illnesses with specific biomarkers. When the targeted biomarker level is higher than normal, MIPs performs a swelling response and the drug is delivered. When the biomarker level falls back to normal, the swelling collapses and drug release stops (Kryscio & Peppas, 2009). This smart feature made MIPs-based DDS to maintain zero-order release of the drug over a long period of time, which was more stable than the conventional drug delivery methods with impulsive release (Alvarez-Lorenzo & Concheiro, 2004; Kryscio & Peppas, 2009; Liechty et al., 2010; Tieppo et al., 2012). The high selectivity of MIPs made it capable of recognizing a specific target even in a complex organic medium such as blood (Kryscio & Peppas, 2009), precisely controlled the release of the drug with a narrow therapeutic index, and selectively released a certain enantiomer in racemic drugs because pure enantiomers are expensive to produce (Alvarez-Lorenzo & Concheiro, 2004). Imprinted polymers as DDS also had a higher stability against light and moisture than the traditional DDS systems (Suedee et al., 2010; Zaidi et al., 2020).

MIPs-based drug delivery systems have yet been rapidly developed to be commercialized. Scientists have developed MIPs drug delivery devices synthesized using bulk, sol-gel, precipitation and *in situ* polymerization methods and applied via oral, dermal, injection and ocular routes in the forms of hydrogels, micelles, microcapsules, nanoparticles and other nanostructures, etc. (Luliński, 2017; Rostamizadeh et al., 2012)

Suedee et al. (2010) designed a MIPs-loaded hydrogel to selectively release esomeprazole [*(S)*-enantiomer of omeprazole] from racemic omeprazole in gastrointestinal route. The MIPs were synthesized via *in situ* polymerization into nanosized beads with selectivity to esomeprazole. The stereoselectivity might result from pi-stacking, hydrogen bonding and hydrophobic interactions between the template and the monomer. The MIPs particles were then

loaded with racemic omeprazole before being embedded into the hydrogel. Poly(2-hydroxyethyl methacrylate) (HEMA) and cellulose worked as structural materials of the hydrogel, while polycaprolactone-triol (PCL-T) was added to improve its enantioselectivity. The delivery tests were carried out by mixing the loaded MIPs-hydrogel device with a fluid stimulating gastric juice. This device released twice amount of *(S)*-omeprazole as *(R)*-omeprazole in the *in vitro* testing, showing the enantioselective potential of the MIPs-based drug delivery device.

Rostamizadeh et al. (2012) synthesized MIPs in the form of nanoparticles for delivery of the narcotic antagonist naltrexone. They used precipitation method with naltrexone as template, acrylic acid as monomer, EDMA as crosslinker and AIBN as initiator. The MIPs showed non-Fickian diffusion of the target drug. The group concluded that this combination showed the potential in the release of naltrexone in human body. Most research groups validated MIPs drug delivery capacities with experiments in a system mimicking a specific environment in human body. In this study, the diffusion capacity was calculated from data acquired during template removal using methanol/acetic acid (1:1, v/v), which was different from any environment in human body.

B. Li et al. (2014) reported one of the earliest studies on MIPs as drug delivery carrier synthesized in a polar environment. The sol-gel MIPs were prepared with salicylic acid, an anti-inflammatory drug with variable uses, with 3-(aminopropyl)triethoxysilane (APTES) and trimethoxyphenylsilane (PMOS) as double monomers and tetraethyl orthosilicate (TEOS) as the crosslinker. The release of salicylic acid controlled by MIPs in aqueous-based environments was predominantly diffusion-controlled and fit well with Fick's law of diffusion. Its rebinding capacity was determined by fluorescence spectroscopy. This polymer shows a remarkably binding specificity of 11.6 mg salicylic acid per gram of polymer.

Tieppo et al. (2012) developed an MIPs-based, daily disposable hydrogel soft contact lens to steadily release diclofenac sodium for more than a day. The MIPs were synthesized with 2-hydroxyethylmethacrylate (HEMA) as backbone monomer, Diethylaminoethyl methacrylate (DEAEM) as functional monomer and polyethylene glycol (200) dimethacrylate (PEG200DMA) as crosslinker. The mixture was added to polypropylene contact lens molds and free-radical UV polymerization was applied. To test the drug release properties of the MIPs contact lens, A PDMS-based microfluidic device was fabricated to mimic the human eye flowing tears. These MIPs lenses allowed slow and steady zero-order release of the target drug for two days in an experimental environment mimicking human eye.

Challenges have existed for the commercialization of MIPs drug delivery products. The main challenge is that most studies relied on *in vitro* tests that mimic the physiochemical environment of human organs, but *in vivo* tests were rare (Zaidi et al., 2020). MIPs-based DDS could sometimes delay the normal release rate of the drug. The target drug could move within the porous MIPs structure from one binding site to another. This process could repeat several times before being released. In other situations, the template and surrounding molecules might compete for limited binding sites, thus abnormally accelerating the release or causing initial burst of the drug (Luliński, 2017; Zaidi et al., 2020). Other challenges occurred during the transfer of the drug in the route. For example, diffusion through the surface of the skin and lacrimal fluid could wash away the drug (Kryscio & Peppas, 2009). Economic affordability is another factor to slow down the commercialization of MIPs-based DDS (Luliński, 2017).

2.3 Microfluidics

2.3.1 General introduction of microfluidics

Microfluidics is the science featuring behaviors of small amounts of fluids (10^{-18} to 10^{-9} L) in small chambers or channels (< 10^{-3} m). Initially being developed for more precise molecular separation, microfluidic techniques were applied in the forms of lab-on-a-chip, organ-on-a-chip, miniaturized total analysis systems (μTAS) and microelectromechanical systems (MEMS) (Dietzel, 2016; Tian & Finehout, 2008). Commercial microfluidic devices are available and produced by different companies, such as Agilent, Caliper, Microfluidic Chip Shop and Waters (Nge et al., 2013). The major aim of microfluidic applications is to manipulate cells and/or molecules in a miniaturized style to save time and money (Dietzel, 2016; Saylan & Denizli, 2019).

Compared to the fluids of macroscopic volumes, the flow of fluids in very small chambers or capillary tubes have very different properties. First, the flow rate is much smaller than fluids in larger channels. Since Reynolds number is directly proportional to the flow rate and channel diameter, microfluidic flows usually have very small Reynolds numbers, typically <1 (Tian & Finehout, 2008). Besides, the small channels make microfluidic systems to have a higher surface area-to-volume ratio and higher electronic fields than the larger systems (Tian & Finehout, 2008). Moreover, the flow is dominated by capillary forces, as gravity has minor influences on the flow compared to molecular interactions within the fluid or between the fluid and the channel (Dietzel, 2016; Saylan and Denizli, 2019). Channel geometry and hydrophobicity are the main factors of accelerating or decelerating the microfluidic flow (Tian & Finehout, 2008).

Compared to the traditional methods, microfluidic devices have several advantages. They simplify multiple laboratory steps of using large instruments required into a single miniaturized chip, which saves a lot of processing time and space and enables on-site test. The small volumes of the sample and solvents or buffers required results in lower cost, especially for samples with high price, such as toxins and antibodies (Tian & Finehout, 2008). The laminar flow prevents turbulence, making the movement of molecules more predictable. The high surface area-volume ratio and higher electronic fields can improve the detection sensitivity (Tian & Finehout, 2008).

On the other hand, microfluidic techniques have faced challenges and concerns. First, the small channels of microfluidic devices decline efficiency drastically when sample mixing is preferred to separation, since mixing in laminar flows relies on diffusion and diffusive mixing is often too slow. Additional geometric designs for the channels are required for mixing, and the designs seen in the literature were often complicated (Tian & Finehout, 2008; Dietzel, 2016). Second, saving time and space was often claimed in review papers as a main advantage of microfluidic techniques, but the amount saved could be very limited. For example, if the fluid inside the chip or outside the outlet needed to be observed by FT-IR spectroscopy or HPLC, it still required large instrumentation that did not save extensive amount of money or space. Despite the microfluidic device is simple, the labor may also be intense during the sample preparation step (Hua et al., 2018).

2.3.2 Materials of microfluidic devices

Common materials of microfluidic devices are glass, ceramics, polymers, silicon, and paper. Among these materials, silicon was the first to be used (Tian & Finehout, 2008). Later, silicon was over-popularized by glass and polymers since they were easier to fabricate and more

compatible with other fluidic components (e.g., valves and pumps) and had high transparency and higher compatibility with other detection modes (Nge et al., 2013). Ceramics are another category of inert inorganic material, which is less popular than glass and silicon. The main type of ceramics used was low temperature co-fired ceramic (LTCC) (Nge et al., 2013). Ceramics were also used as an ingredient of polymer-based microfluidic devices. Hybrid ceramic polymers and preceramic polymers were reported in the previous studies (Sikanen et al., 2010; Ye et al., 2010).

2.4 Paper microfluidics

2.4.1 Paper as a material

Paper is a flexible cellulose material for the fabrication of microfluidic devices. Microfluidic paper-based analytical devices are defined as "μPADs". The technique using μPADs is also called "lab-on-paper" (Hua et al., 2018; X. Li et al., 2012). Among the microfluidic devices fabricated by using different materials, μPADs is an emerging technique (Nge et al., 2013). Paper has multiple advantages as a material. Paper is cheaper compared to glass, silicon, ceramics and polymers. Paper is also readily available, lightweight and easily modified (W. Li et al., 2021). Moreover, paper is compatible with many applications in the chemical, biochemical and biomedical fields. The surface of paper can be chemically modified (Nge et al., 2013). Besides, paper has a natural fibrous structure, making fluids move via only capillary forces and without the need of a pump (Guo et al. 2015; Kong et al., 2018; X. Li et al., 2012). This fibrous structure makes paper a good candidate to deposit MIPs (W. Li et al., 2021). Moreover, paper is environmentally friendly. It can be disposed by natural degradation or

burning (Nge et al., 2013). Common paper used are filter paper (especially Whatman #1) and chromatography paper.

2.4.2 Patterning of the channels

One step of creating a paper-based microfluidic device is to add microscale channels to the paper so that the reagents may flow along the channels without diffusing to exceed the edge. One approach is to create a hydrophilic-hydrophobic contrast to form microscale channels. The techniques used are photolithography, plotting with PDMS, printing, and laser treatment. The most popular methods are wax printing and inkjet printing. Examples of materials used in hydrophobization of paper are SU-8, PDMS, wax, polystyrene, and alkyl ketene dimer (AKD). Sometimes a heating step is required after deposition of the hydrophobization material to allow it melt and penetrate through the paper (X. Li et al., 2012). The work conducted by Lu et al. (2010) demonstrated high quality photos showing the penetration of wax through paper before and after heat treatment. The other approach is to physically change the shape of paper. One example is cutting the paper into designed pattern and encase the paper with sticky tape (X. Li et al., 2012). Another way is to emboss paper using molds. Thuo et al. (2014) stacked paper between two reusable plastic molds with the channel design, making the channels to concave up from side view. They then treated paper with a fluorinated alkyltrichlorosilane vapor to make the paper omniphobic. To prevent vaporization, the paper was covered with transparent adhesive tape except fluid inlet and outlet, where tubes were connected. Among the methods of adding channels to paper, printing is the most convenient. Many copies of the design can be printed on one large sheet of paper before cutting them.

2.4.3 Applications of paper microfluidics

A variety of 2D and 3D origami paper-based microfluidic devices have been used in health diagnostics, environmental monitoring and food quality testing (X. Li et al., 2012). In the field of food science, multiple research groups have designed µPADs to detect different regulated compounds in foods such as pesticides, toxins, illegal dyes, illegal preservation agents, heavy-metal ions and allergens (Hua et al., 2018). Ge et al. (2015) developed a wax-printed folding-paper µPAD to perform an electrochemiluminescence (ECL) immunoassay aiming to detect tumor markers. The device was composed of a piece of paper-A with loading zones and working electrodes and a piece of paper-B with contacting zones. After loading samples and signal antibodies on paper-A, paper-B was placed on top of it. The device was connected to a conductive wire and ECL reaction was triggered. After the test, paper-B could be washed with water for recycling. This device showed promising results and high reproducibility. It could be used for 25 cycles and could be stored for 3 weeks under refrigeration.

Q. Jiang et al. (2019) designed a µPAD chip to detect and quantify deoxynivalenol (DON) in grain samples. The design was wax-printed, composed of two wells connected with two channels. On each channel, there were two test areas spotted with DON-BSA and secondary antibody. The two wells were assembled with a conjugate pad and an absorbent pad, both of which were treated with different reagents. Colorimetric signals were read using a custom-made portable imaging system. The results were not significantly different from ELISA. This device proved a low-cost and rapid method for mycotoxin detection.

2.5 Detection of food hazards using MIPs and paper-based devices

2.5.1 Detection modes

Result readout is an essential step in analytical testing. For MIPs-based paper fluidic devices, various detection modes have been used. Currently, colorimetric detection and fluorescence detection were the most used. Colorimetric is one of the most renowned detection modes of MIPs-PADs (W. Li et al., 2021). Colorimetric method revealed the results very intuitively. By observing the color change of the signal indicator on the usual white background of the paper, one can briefly tell if the result is positive or negative. To achieve more precise results, the observation is coupled with a colorimeter or an image processing software. Signals could be expressed as hue (Hong et al., 2010), color intensity (Guo et al., 2015; Q. Jiang et al., 2019) or grey scale (Kong et al., 2017). On the other hand, colorimetric detection was less sensitive, and many relevant studies only achieved semi-quantitative results. To overcome this limitation, researchers have tried to amplify signals to increase the sensitivity, such as using a metal-organic framework (MOF)-based MIPs strategy (W. Li et al., 2021). Fluorescence detection is highly sensitive and its operation is also simple. The typical principle is to measure the enhancement or quenching of fluorophores caused by the presence of the analyte. MIPs work as an enhancer of selectivity and sensitivity in fluorescence-based assays (W. Li et al., 2021). Among the fluorescence materials, quantum dots (QDs) have been a popular one to be used with MIPs. Other detection methods such as chemiluminescence, electrochemical, photoelectrochemical, SERS and thermal detection have been used to sense the results (Guo et al., 2015; W. Li et al., 2021).

2.5.2 Detecting pesticides

Currently, the studies on MIPs-grafted paper-based devices to detect food hazards focus on the detection of pesticides. S. Wang et al. (2013) designed a paper-based multi-disk micro-disk plate for the detection of 2,4-dichlorophenoxyacetic acid (2,4-D) from tap water and lake water samples. A silane coupling method was used to graft C=C groups on the surface of paper. MIPs were synthesized using the *in situ* method. The detection was based on a luminol-TOP-H_2O_2 chemiluminescence (CL) system. An immobilized 2,4-D-tobacco peroxidase (TOP) conjugate filled the binding sites in the MIPs layer. During the assay, the MIPs paper disk was exposed to the samples containing free 2,4-D and incubated till equilibrium binding. Then, luminol and H_2O_2 were injected to trigger the CL reaction. The more free 2,4-D in the sample, the less 2,4-D-TOP in the MIPs cavities, and the lower the CL signal. This method generated satisfactory results and showed the potential of detecting pesticides with chemiluminescence MIPs-grafted paper-based devices.

Kong et al. (2017) developed a novel MIPs-paper microfluidic colorimetric sensor to detect bisphenol A (BPA), a substance used in plastic food packaging and a potential food contaminant. Paper fibers were coated with $ZnFe_2O_4$ magnetic nanoparticles ($ZnFe_2O_4$ MNPs) and MIPs membranes. To achieve this result, $ZnFe_2O_4$ MNPs and paper were added to the pre-polymerized MIPs mixture. Another sheet of paper was treated with 3,3′,5,5′-tetramethylbenzidine (TMB) and stacked with the MIPs@paper. TMB allowed color change of $ZnFe_2O_4$ MNPs with the presence of the analyte. This method provided a low detection limit, high selectivity, sensitivity and reproducibility. One limitation of this study was that the MIPs were prepared by bulk polymerization. The pre-polymerization mixture for bulk polymerization is far more concentrated than the mixture for precipitation or sol-gel polymerization.

Z. Zhang et al. (2018) used a MIPs@QD@paper method to detect the pesticide 2,4-dichlorophenoxyacetic acid (2,4-D) in soy and mung bean sprouts. Paper was cut into small pieces and treated with APTES to graft amino groups on its surface. CdTe QDs were combined with EDC and 2-*N*-morpholinoethanesulfonic acid (MES) solutions and then soaked with paper to bind QDs and paper together. Then, the MIPs were synthesized on the treated QD@paper substrate. The pattern of the microfluidic chip was designed with Adobe Illustrator and wax-printed. The samples were juiced, filtered, diluted, spiked with 2,4-D and brought to the test. The results showed good consistency with the results obtained by HPLC.

Sawetwong et al. (2013) fabricated an MIPs-grafted paper microfluidic device for the detection of glyphosate in different grain samples. The sensing mode was colorimetric and was processed using ImageJ, while they used fluorescent material Mn-doped ZnS QDs. QDs did not act as a signal indicator, but as a reactant to generate a color change. Mn-ZnS QD-MIPs reacted with H_2O_2 to produce a radical $^{\bullet}OH$, which caused catalytic oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), thus producing dark green $ABTS^{\bullet+}$. When the target glyphosate was present in the sample, it bound with Mn-ZnS QD-MIPs and inhibited $^{\bullet}OH$ production, reducing the intensity of green color. This device produced satisfying results for inspecting grains such as peanuts, sweet corns and pumpkin seeds.

2.5.3 Detecting natural toxins

The concept of natural toxins is very broad. There were a few studies covering the detection of natural toxins in food using MIPs paper-based devices. L. Wang et al. (2022) tried to design a molecularly imprinted paper-based fluorescence sensor to detect domoic acid. A mixture for sol-gel MIPs synthesis were added to blue-emitting carbon quantum dots (B-CDs) to

form a B-CDs@MIPs complex. This group succeeded in detecting spiked domoic acid in shellfish meat and lake water by incubating B-CDs@MIPs solution with prepared samples in a plastic tube and achieved 95.5-103.4% recovery for shellfish and 103.4-105.5% recovery for lake water. The authors then coated B-CDs@MIPs solution on the surface of filter paper. They obtained acceptable results in testing the standard solutions, but not applied the testing for real samples. Many studies have focused on the detection of food mycotoxins with MIPs-based device or with paper-based device. Yet very few publications about food mycotoxin detection have combined these techniques together. Most studies showed MIPs-based SPE columns, MIPs membranes or paper microfluidics without MIPs.

2.5.4 Detecting other compounds

B. Li et al. (2017) developed a 3D origami paper microfluidic device imprinted with MIPs and fluorescent quantum dots (QDs) to detect phycocyanin, a pigment protein that may promote algae bloom in drinking water. Whatman No. 1 filter paper was cut into small pieces and treated with APTES in order to add amino groups to the surface of the paper. QDs in a solution was coated on paper, and MIPs were synthesized on QD@paper. The 3D μPAD had three layers with wax-printed channels. The sample was added to the inlet on the top layer, flow through the middle layer and reach the QD@paper piece at the bottom layer. This μPAD device was able to detect phycocyanin in the range of 10-50 mg/L with a limit of detection of 2 mg/L and a relative standard deviation from 3.3% to 5.7%.

Xiao et al. (2017) designed an MIP-grafted paper-based device that could detect 17β-estradiol (17β-E_2) in both cow milk and human urine after different sample preparation steps. For milk, it was treated with acetonitrile, centrifuged and the supernatant was taken to analysis. The

paper was treated with 3-(trimethoxsilyl)propylmethacrylate to add C=C groups to the paper surface. The MIPs were synthesized on the treated paper surface via *in situ* polymerization method. After template removal, 17β-E_2-horseradish peroxidase (HRP) conjugate was grafted in MIPs cavities. The 17β-E_2 in the sample would react with 17β-E_2-HRP, causing a change in color intensity. The limits of detection by visual judgement for milk and urine were both 0.25 µg/L.

Research has shown multiple attempts to combine MIPs with paper microfluidics. The fabrication of MIP-PADs included preparing MIPs, making PADs and the combination of the two (W. Li et al., 2021). Grafting MIPs onto microfluidic chips or immunoassay platforms are specific per experiment, unstable and cause much non-specific binding (Guoning et al., 2020) One method to integrate these two components was to synthesize MIPs to let it grow on paper. The stiffness of the paper might be affected since paper was soaked in the solvent during polymerization. *In situ* polymerization was the most frequently used polymerization method (W. Li et al., 2021), while other polymerization methods were also reported. Kong et al. (2017) developed a paper-based MIPs colorimetric sensor highly selective for bisphenol A. Wax printing was applied on paper before MIPs membrane was synthesized via bulk polymerization method on the surface of the paper device. The excess MIPs attached on the paper were washed away using ethanol, while the template was removed with methanol and acetic acid. Prior to each use, tetramethylbenzidine was added to the paper device as an indicator. The color change could be observed with naked eye, and the target could be quantified using colorimetric readers. The second way to integrate MIPs into paper was to synthesize the MIPs beforehand and then added to the paper. This method caused less damage to paper, but it required more MIPs to make the

device. Common polymerization methods combined with this technique were free-radical polymerization and sol-gel polymerization (W. Li et al., 2021).

Fungi such as *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. have been sources of food mycotoxin contamination. These fungi produced secondary metabolites that are toxic and could be carcinogenic to humans, such as aflatoxins, ochratoxin A, zearalenone, fumonisin B, deoxynivalenol and citrinin (Guo et al., 2015). Therefore, detection of mycotoxin using a simple, portable MIP-imprinted paper microfluidic device has been interested by researchers. MIPs electrochemiluminescence sensor incorporated with nanomaterials was used in the detection of ochratoxin A and fumonisin B_1 (W. Zhang et al., 2017). The nanomaterials were added to the surface of electrodes to improve electrocatalytic properties of the electrodes of the sensor (Goud et al., 2018). Q. Jiang et al. (2019) focused on the detection of deoxynivalenol in food and animal feed with a paper-based microfluidic chip. A density-based sampling was used due to the heterogeneous distribution of deoxynivalenol in foods. MIPs were proved to be powerful in selectivity and efficiency in the measurement of targeting mycotoxins (Hatamabadi et al., 2020).

3. MATERIALS AND METHODS

3.1 Reagents and Materials

Zearalenone was purchased from Toronto Research Chemicals (North York, ON, Canada). Hydroxyapatite nanopowder (d = 200 nm), methacrylic acid, acetonitrile, ethylene glycol dimethacrylate (EDMA), 2,2'-azobis(2-methylpropionitrile) (AIBN), *N*-(3-himethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), hydrochloric acid and Whatman No.1 cellulose chromatography paper were purchased from Sigma-Aldrich (St. Louis, MO, USA). Coumarin-3-carboxylic acid, naringenin, *N*-hydroxysuccinimide (NHS), methanol and glacial acetic acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cadmium selenide zinc sulfide quantum dots (CdSe/ZnS QDs) was purchased from Cedarlane Labs (Burlington, ON, Canada). It was dispersed in alkyl solvent (toluene) and had a maximum emission wavelength at 630 nm. Ultrapure water was freshly collected from a Milli-Q purification system. Dried corn samples were purchased from local grocery stores in Montreal, Canada.

3.2 Synthesis of MIPs

The process of the synthesis of MIPs is shown in **Figure 3.1**. The MIPs were synthesized using a precipitation polymerization method adapted from a previous study reported by Y. Zhang et al. (2020) with modifications. First, 0.75 g hydroxyapatite (HAP) was mixed with 5 mL of ethanol and sonicated to full dispersion. Then, 3-CCA (0.25 mmol, 0.0476 g), naringenin (0.25 mmol, 0.0681 g), and MAA (1.5 mmol, 0.125 mL) were dissolved in 15 mL of ethanol by sonication. The two mixtures were transferred to the same container, followed by sonication for

5 min. Then, EDMA (7.5 mmol, 1.41 mL) and AIBN (0.2 mmol, 0.0323 g) were added and sonicated thoroughly. The mixture was mechanically stirred with refluxing at room temperature for 30 minutes and at 80°C for 5 hours. After the reaction, excessive solvent and unpolymerized materials were removed by suction filtration. The obtained MIPs were repeatedly sonicated in methanol/acetic acid (4:1, v/v) and then with methanol to remove the templates as monitored by UV-VIS spectrometry. The washed MIPs were dried in a vacuum oven at 65°C for 10 hours. The NIPs were synthesized in the same manner except the template molecule was not added.

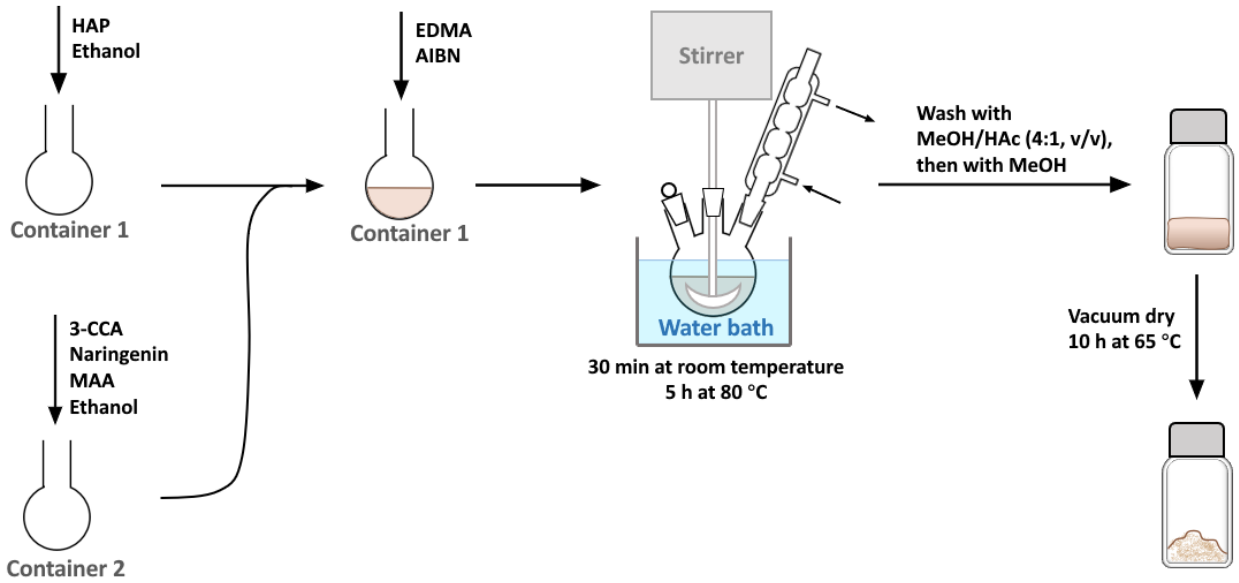

Figure 3.1. The fabrication process of the MIPs powder.

3.3 Static and dynamic adsorption tests of MIPs and NIPs

The adsorption ability of the polymers was evaluated with the target zearalenone. In the static test, 10 mg MIPs or NIPs were added to 4 mL of 10, 50 and 100 ng/mL zearalenone solutions. All solutions were dissolved in 50% methanol in a 5-mL plastic vial. One vial of 50% methanol worked as control. The mixture was vortexed for 5 seconds and rotated for one hour to

reach to the adsorption equilibrium. The mixture was centrifuged at 15000 rpm for 15 minutes at room temperature, and the supernatant was analyzed by using a Waters (USA) HPLC with a fluorescence detector and a C_{18} column (isocratic, water: acetonitrile: methanol = 35:10:5, v/v/v). The adsorption capacity (Q) was calculated by

$$Q = (C_0 - C_1)\ V / m$$

, where C_0 is the initial concentration (mg/L) of zearalenone in the solution, C_1 is the concentration (mg/L) of the remaining zearalenone in the solution after adsorption, V is volume of the solution (mL), and m is the mass of MIPs or NIPs (mg).

In the dynamic test, 50 mg MIPs or NIPs were added to 20 mL of 50 ppb zearalenone dissolved in 50% methanol. The mixture was vortexed for 5 seconds and kept rotary shaking. At designated time points (i.e., 0, 5, 10, 20, 30, 60 min), 1.8 mL of the mixture was transferred into a separate tube, followed by centrifugation at 15000 rpm for 15 minutes at room temperature. The supernatant was analyzed in the same manner as the static test.

3.4 Fabrication of QDs@MIPs@paper

The process of the synthesis of QDs@MIPs@paper is shown in **Figure 3.2**. A reactant mixture was prepared by mixing 0.0119 g 3-CCA, 0.0170 g naringenin, 20 mL of ethanol, 0.031 mL of MAA, 0.352 mL of EDMA and 0.0081 g AIBN. The mixture was sonicated to dissolve and left at room temperature for 30 minutes. Whatman No.1 chromatography paper was cut into circles (d = 6 mm) with a hole puncher. The paper circles were immersed in 0.2 M HCl for 30 minutes and then rinsed with ultrapure water to remove excess acid. The paper was soaked in 50% ethanol containing 1% APTES for 3.5 hours, followed by washing with ultrapure water and then ethanol to remove the moisture. One layer of the treated paper was placed on the bottom of

flat-bottomed glass vials with screwcaps. Then, 1 mL of the reactant mixture and 4 mL of ethanol were slowly pipetted into each vial. The caps were screwed tightly and were heated at 80°C for 5 hours. The obtained MIPs@paper was washed with methanol for multiple times to remove the templates and rinsed with ultrapure water. In a separate container, 5 mL of EDC solution (40 mM), 5 mL of NHS solution (40 mM) and 6 mL CdSe/ZnS QD (2.5 mg/mL) were mixed, homogenized by sonication, and shaked using the reciprocating mode of the tube rotator in the dark for 15 minutes. Twenty pieces of MIPs@paper were added to the container. The container was reciprocated in the dark for 2 hours to bind QDs on the surface of MIPs@paper. The excess reagents were removed by washing with DI water for 5 times. QDs@NIPs@paper was prepared in the same manner without adding the template molecules.

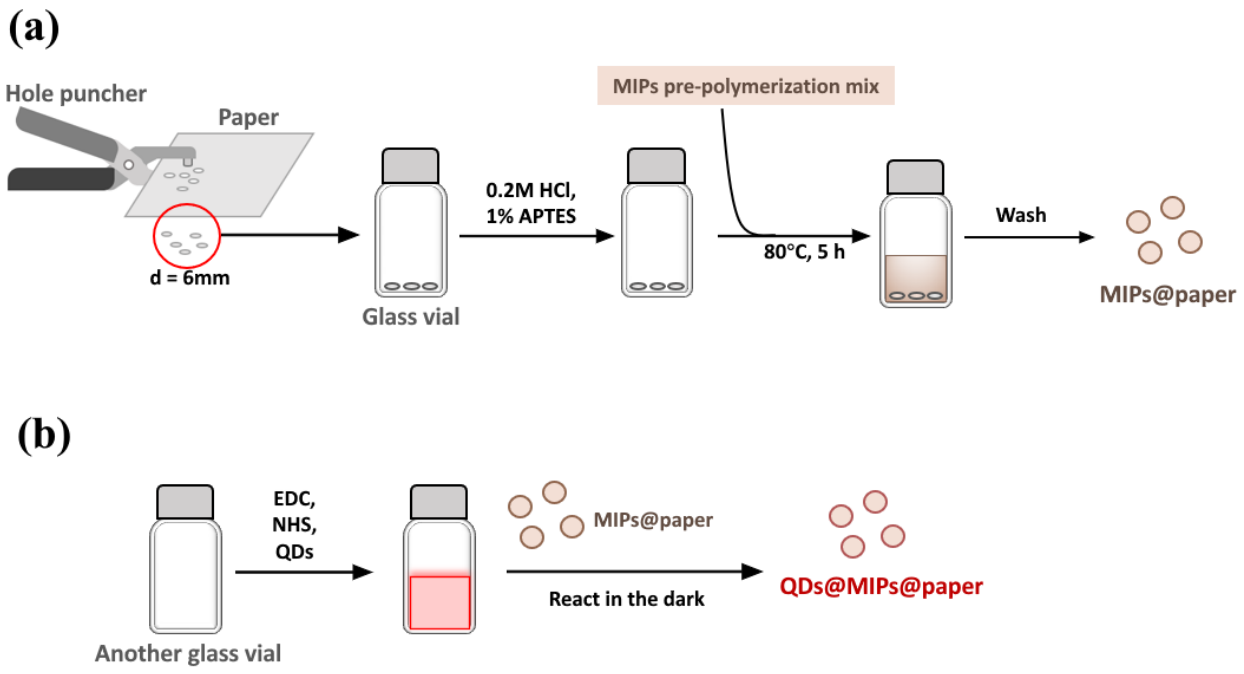

Figure 3.2. The fabrication process of QDs@MIPs@paper.

3.5 Adsorption tests of QDs@MIPs@paper

The adsorption ability of MIPs@QDs@paper was evaluated via measuring the fluorescence signal change upon rebinding to zearalenone. Standard toxin solutions of different concentrations (i.e., 1, 2, 5, 10, 30, and 50 mg/L) were prepared by dissolving zearalenone in 90% acetonitrile. For each test, one piece of QDs@MIPs@paper or QDs@NIPs@paper was placed onto the bottom of a well of a black flat-bottom 96 well plate. In each well with paper, 10 µL of 90% acetonitrile was added, followed by measuring the initial fluorescence intensity (FI) using a microplate reader. Afterwards, the solvent in each well was removed and then 100 µL of the standard solution was added. The plate was sealed and incubated in the dark for 1 hour to reach to the binding equilibrium. Then, 90 µL of the standard solution was removed from each well and the final FI was measured using a microplate reader. A standard curve was obtained by establishing the relationship between FI change and zearalenone concentration.

3.6 Real sample testing

Dried corn sample was homogenized using a laboratory mill (Tube Mill 100 control, IKA, USA). Then, corn powder (4 g per sample) was spiked with different amounts of zearalenone, reaching to the final concentrations of 1, 2, 5, 10, 30, and 50 mg/kg. For each sample, 20 mL of 90% acetonitrile was added to extract the toxin via vortex for 3 minutes. The mixture was then centrifuged at 6000 rpm for 15 minutes. The supernatants were collected and tested using the prepared QDs@MIPs@paper following the same procedure as the standard solution.

The limit of blank (LOB) was calculated by

$$LOB = 1.645\sigma + \bar{x}$$

, where σ is the standard deviation of the lowest concentration of zearalenone that can be detected and $\bar{x}$ is the mean of the lowest concentration of zearalenone that can be detected (Armbruster & Pry, 2008).

The limit of detection (LOD) was calculated by

$$LOD = LOB + 1.645\sigma + \bar{x}$$

The limit of quantification (LOQ) was calculated by (Bargańska et al., 2014)

$$LOQ = 3 \times LOD$$

4. RESULTS

4.1 Synthesis and testing of MIPs

4.1.1 Synthesis of MIPs

MIPs were synthesized via precipitation polymerization. Precipitation polymerization is a simple and widely used method to synthesize MIPs because it is a simple one-pot reaction, can control the particle sizes of the end product, and does not require milling or sieving after polymerization. Template molecules, instead of the target molecule zearalenone, participated in polymerization due to their significantly lower cost and toxicity. The choices of template molecules were based-on their similarity in structure to zearalenone and the interactions among their functional groups with functional monomers. The hydroxyl groups of methacrylic acid (MAA) could form hydrogen bonds with the hydroxyl groups of coumarin-3-carboxylic acid (3-CCA) and the hydroxyls and ketones of naringenin. Hydroxyapatite (HAP) was used as a carrier material for MIPs particles to imprint on. Hot water bath was selected over direct heating to provide a more uniform heating environment and reduce the chance of cracking. The heating temperature (80°C) was higher than the boiling point of ethanol (78°C); therefore, a condensing tube was attached to reduce solvent loss. The templates were removed to leave the binding sites. Their specific recognition property would be tested in the adsorption tests. The prepared and dried MIPs were in the form of small and white beads. MIPs were then sealed and stored in a cool and dry place at room temperature for further use.

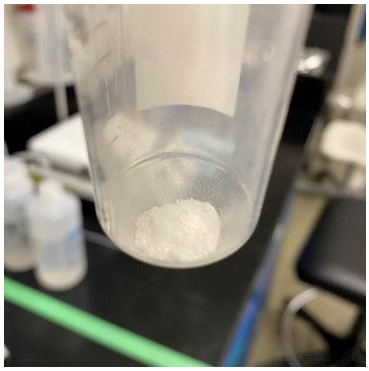

Figure 4.1. Photograph of the synthesized MIPs in a plastic tube.

4.1.2. Adsorption tests of MIPs

Adsorption tests were critical in evaluating the performance of MIPs as the selective adsorbent for the target molecules. These tests could provide a systematic view of determining the capacity and speed of MIPs to re-bind to the target. The target zearalenone was used in the adsorption tests, as the templates 3-CCA and naringenin were not the analytes for the real sample tests. The static adsorption tests showed the capacity of MIPs to form specific bonds with the targeted molecule, while the dynamic adsorption tests showed the speed of MIPs to form specific bonds with the targeted molecule.

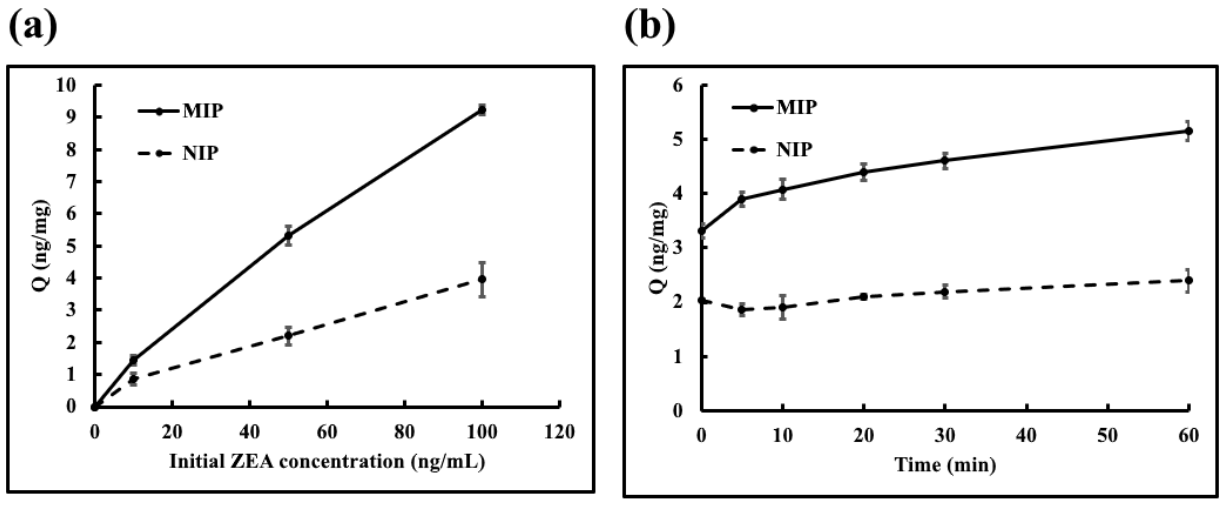

Figure 4.2. (a) Static and (b) dynamic tests on the adsorption performance of MIPs and NIPs towards zearalenone.

The differences between MIPs and NIPs and the calculation of adsorption capacity (Q) could be referred in the Methods section. The Q value demonstrates the amount of target per mg of MIPs/NIPs that could bind in each situation. In static adsorption tests (**Figure 4.2a**), Q values of MIPs were higher than that of NIPs regardless of the initial concentration of the target. The Q values of MIPs and NIPs were 9.23 ng/mg and 3.95 ng/mg for the maximize initial concentration (100 mg/mL). In dynamic adsorption tests (**Figure 4.2b**), most rebinding of the analyte occurred within 5 min for both MIPs and NIPs. The growth trend of Q turned steadier after 5 min. One hour after the beginning of re-binding, the Q values of MIPs and NIPs reached to 5.16 ng/mg and 2.40 ng/mg. In both static and dynamic tests, Q values of MIPs were always higher than that of NIPs, indicating the presence of the imprinted cavities that could lead to specific binding. Non-specific binding by NIPs was also observed.

4.2 Synthesis and characterization of QDs@MIPs@paper

4.2.1 Synthesis of QDs@MIPs@paper

The preparation of QDs@MIPs@paper involved three key steps. The first step involved the modification of paper with APTES to graft amino groups onto the paper, which was to serve as the basis of further synthesis on the paper surface. The second step was to synthesize MIPs on the surface of the modified paper using precipitation polymerization method. During this step, specific binding sites capable of recognizing the target were fixed onto the paper. The third step involved coating of QD (-COOH) onto MIPs@paper (-NH_3) via EDC-NHS coupling, which combined the selective properties of MIPs and the fluorescence properties of QDs onto the same device. The overall process is shown in **Figure 4.3**.

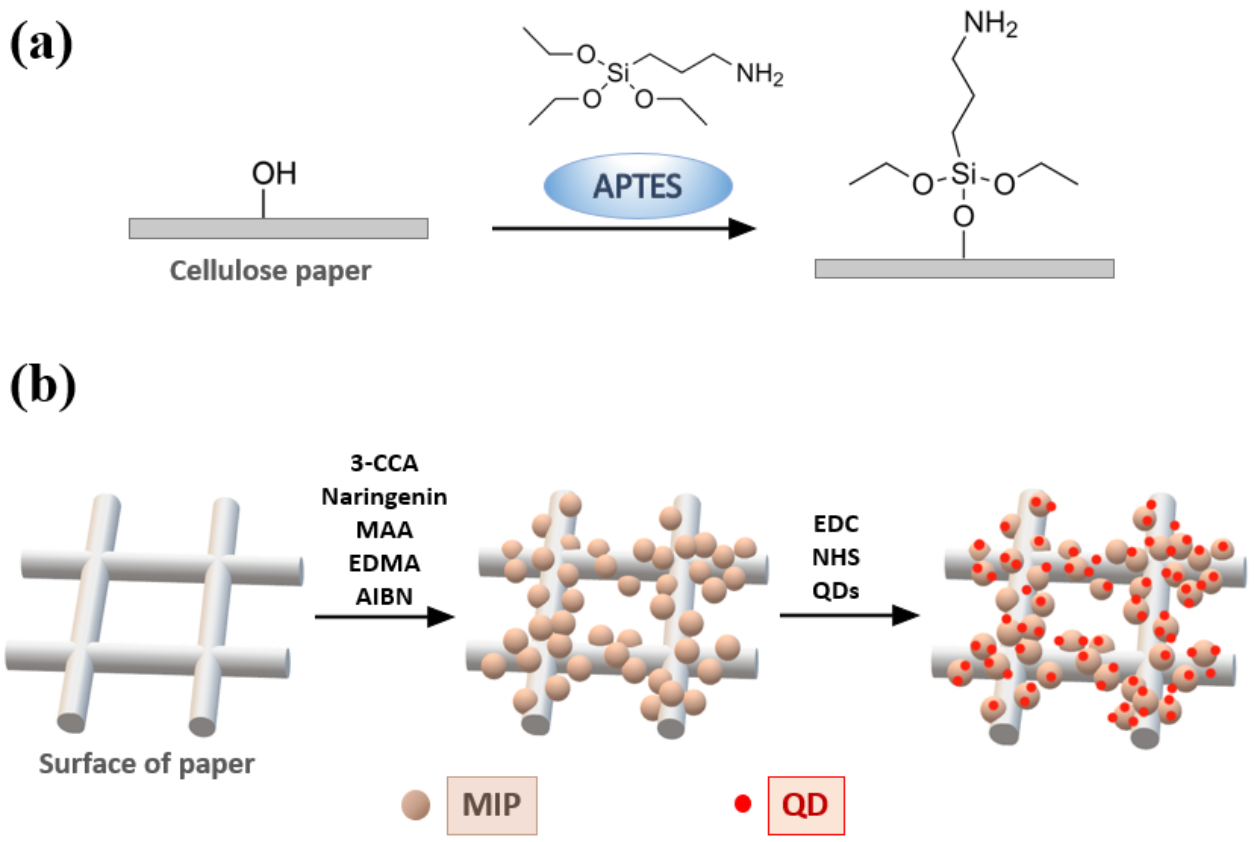

Figure 4.3. (a) The schematic illustration of the modification of paper with APTES. (b) The schematic illustration of coating MIPs and QDs on the surface of the paper.

Paper is a cheap, widely used and flexible material that can be easily cut into different shapes for many purposes. Its light weight makes it a good material as the basis for fabricating novel portable and on-site testing devices. Besides, paper has a natural 3D porous structure of cellulose fibers with hydroxyl groups on its surface, making it a promising place to attach MIPs. Therefore, paper was used as the supporting material for the development of this QDs@MIPs sensor. The modification of paper involved treating paper with HCl to add protons and enhance the hydrophilicity of the surface of the paper. Then, APTES was added to attach amino groups to the hydroxyl groups on paper surface. Both steps promoted the coating of MIPs and QDs onto the paper.

The synthesis of MIPs@paper involved some modifications to the recipe compared to the synthesis of MIPs powder. One modification was in the reagents used. In the synthesis of MIPs powder, hydroxyapatite (HAP) was used as a carrier material. It provided a surface for the MIPs particles to imprint on. In contrast, the synthesis of MIPs@paper did not involve the use of HAP as the carrier material. Instead, chromatography paper was used to serve the same purpose. Another difference between the synthesis of MIPs powder and that of MIPs@paper was in the proportion of the reagents. The proportion of the solvent ethanol was increased, and the proportions of the functional monomer MAA, the crosslinker EDMA and the initiator AIBN were lowered. This modification aimed to synthesize a very thin layer of MIPs on paper, which reduces excessive MIPs formation and reduces reagents waste.

Finding a balance between the amount of the solvent ethanol and that of other reagents was crucial to MIPs@paper synthesis. Excessive dilution of the pre-polymerization mixture was observed to limit the contact and interaction among molecules and hinder the polymerization reaction. On the other hand, an insufficiently diluted mixture would lead to the formation of

lumps that are difficult to wash. Overall, the dilution factor impacts the success of polymerization. In this part of the experiment, we used the term “dilution factor” to refer to the ratio of ethanol to other reagents. To determine the optimized dilution factor, a series of trials were performed using dilution factors of 1/10, 1/20, 1/30, 1/40, and 1/50. While the ratio of ethanol to other reagents changed, the ratio among MAA, EDMA and AIBN remained constant. The results are shown in **Table 4.1.** The dilution factor of 1/10 had the highest polymerization success rate of 100%. The success rate declined along with an increased dilution factor. Therefore, 1/10 dilution was the most favorable dilution factor to be used for further synthesis.

Table 4.1. Dilutions for MIPs@paper synthesis

Dilution factor	Number of trials	Number of polymerized trials	Polymerization success rate
1/10	6	6	1.00
1/20	6	5	0.83
1/30	6	2	0.33
1/40	6	2	0.33
1/50	6	1	0.17

To remove the template and excessive polymers that were physically attached after polymerization and drying, the MIPs@paper was soaked in methanol. Methanol was replaced frequently for more effective removal of templates and non-polymerized reagents. To avoid potential damage to paper structure, a milder washing method of simply soaking, shaking and replacing the washing solvent was used instead of more destructive methods such as sonication. The aim was to effectively remove unwanted materials and at the same time preserve the microstructure of the paper. The MIPs@paper was sealed in a capped vial for storage to prevent

undesirable impact on the microfiber structure of paper caused by excessive drying. A photograph of MIPs@paper is shown in **Figure 4.4.**

Figure 4.4. Photograph of the synthesized MIPs@paper.

After incorporating MIPs on the surface of the paper, water-soluble CdSe/ZnS QDs with the maximum fluorescence emission at 630 nm was coated on the MIPs@paper. QDs were coated after MIPs synthesis since the fluorescence signals would decline after polymerization steps probably caused by MAA and AIBN. The details are included in **Table 4.2.** The process of embedding QDs onto the paper involved a reaction called EDC-NHS coupling. During this step, the carboxyl group on QDs reacted with EDC and NHS to form a reactive ester. The ester further reacted with the amino group of APTES, which was attached on paper in advance to form an amide bond. Therefore, a stable covalent bond was formed between QDs and paper.

Table 4.2. The presence of fluorescence after MIPs mixed with QDs and stayed at 80°C for 5 hours. For MAA, EDMA and AIBN, the solvent used was ethanol.

Material	Fluorescence (yes or no)
Ethanol	Y
MAA	N
EDMA	Y
AIBN	N

4.2.2. Characterization of QDs@MIPs@paper

To review the surface morphological structure, QDs@MIPs@paper and QDs@NIPs@paper were examined using a scanning electron microscope (FEI ESEM Quanta 450, USA). In **Figure 4.5**, the fibrous structure of paper is clearly shown. The sizes of these fibres vary over a wide range, from nanometer scale to tens of micrometers. This feature of paper is most clearly viewed in **Figure 4.5 (a)**, the SEM image of plain paper. **Figure 4.5 (b)(c)** show that MIPs and NIPs are spherical particles with different sizes. The particle size of MIPs (d = 1–2 µm) was generally larger than that of NIPs (d = 0.5–1 µm). The thickness of the MIPs/NIPs layer was not consistent. One layer of MIPs/NIPs particles covered most of the paper, while the remaining parts of the paper were covered with clusters of MIPs/NIPs or not covered at all. The clusters were most frequently observed in the gaps on the paper surface, while voids were more common in flatter areas.

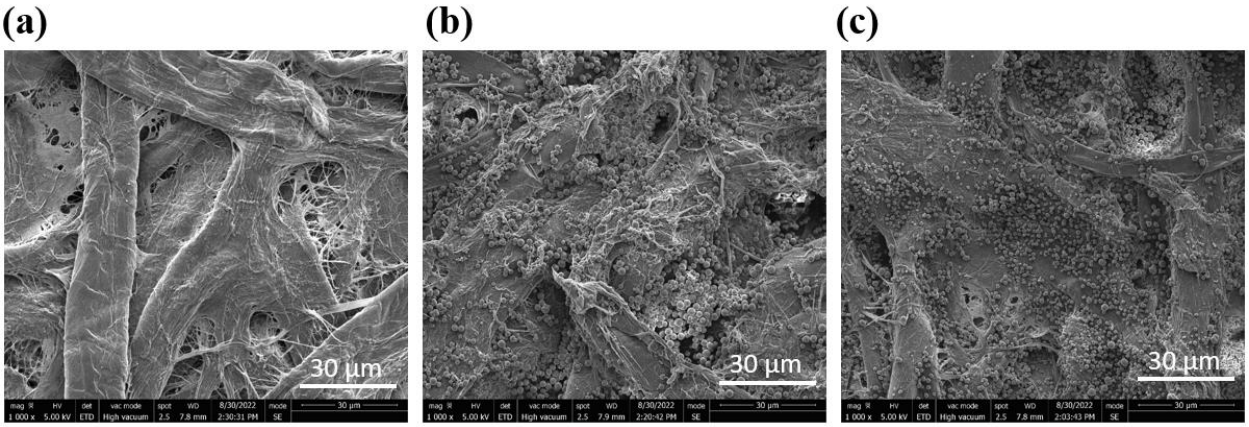

Figure 4.5. SEM images (n = 50) of (a) paper, (b) MIPs@paper and (c) NIPs@paper.

After characterization of the morphological structure of QDs@MIPs@paper and QDs@NIPs@paper, the fluorescence properties were evaluated. Under the excitation light of the UV lamp at 405 nm, the chromatography paper emitted fluorescence of light violet hue, whereas the CdSe/ZnS QDs emitted fluorescence of red colour (max. at 630 nm). With only a very thin layer of QDs, the QDs@MIPs@paper presented pinkish light under the UV lamp. The comparison of fluorescence colors of QDs and paper coated with QDs under UV light can be seen in **Figure 4.6**, showing successful coating of a thin layer of QDs onto MIPs@paper.

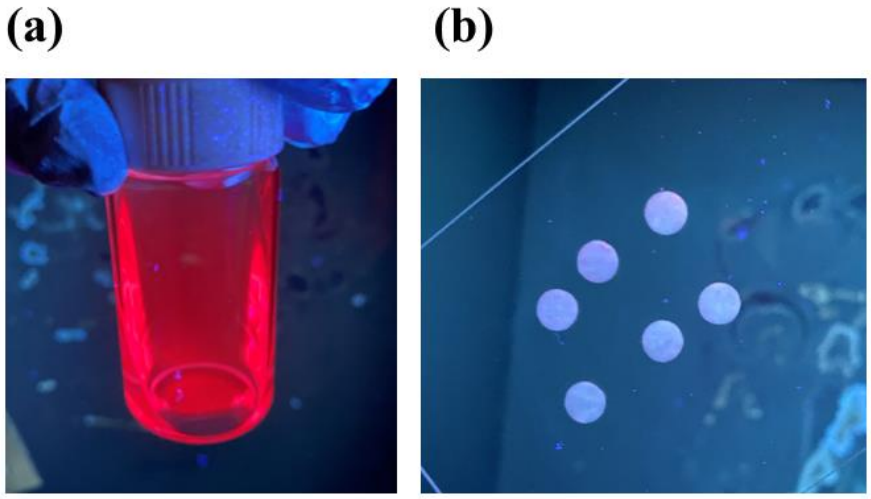

Figure 4.6. Representative photographs under UV light: (a) mixture of QDs, EDC and NHS in a glass vial; (b) pieces of MIPs@paper on a glass panel coated with a suitable amount of QDs.

4.2.3. Detection of zearalenone

The process of the decline of fluorescence intensity is referred as quenching. When a compound is brought close to the QDs, the amount of light emitted by the QDs decreases. This effect can be used as a signal indicator to determine specific chemical reactions. QDs have been combined with MIPs in studies on the detection of various analytes in different sample matrices (Díaz-Álvarez & Martín-Esteban, 2021; B. Li et al., 2017; Z. Zhang et al., 2018). In the current study, the fluorescence of QDs could be quenched in contact with the zearalenone solution, and the presence of a higher concentration of zearalenone was associated with a more intensive decrease in the fluorescence intensity.

We then further evaluated the fluorescence properties of QDs after being coated on MIPs@paper. QDs@MIPs@paper was loaded with zearalenone solutions at different concentrations. To reduce the influence of solvent on the quenching effect, 50% acetonitrile was

selected as no quenching was observed with 50% methanol. Additionally, flat bottom plates were selected over round bottom plates to keep all pieces of paper at the same depth in the wells. This step aimed to ensure the fluorescence response of QDs@MIPs@paper to the target concentration.

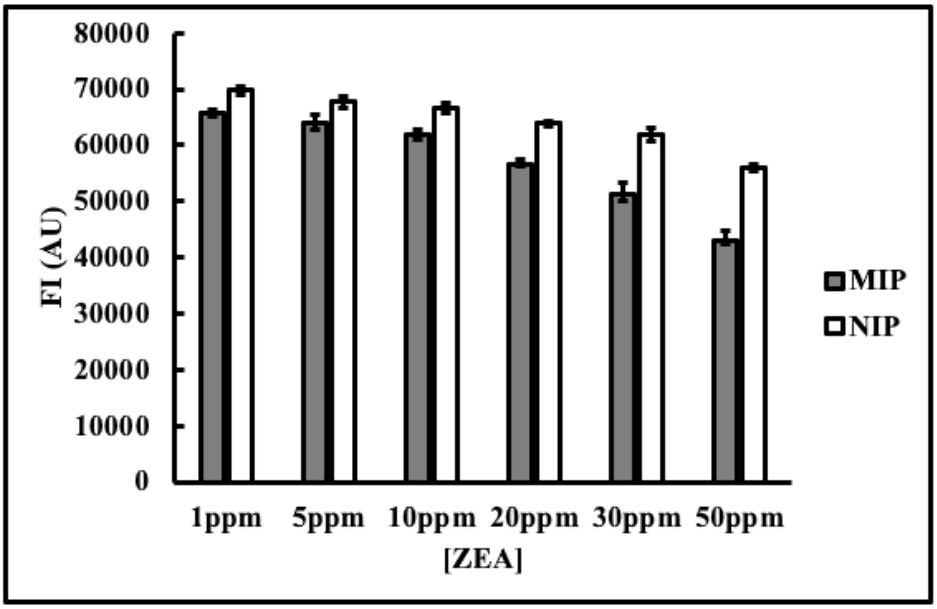

Figure 4.7. Fluorescence intensity readings of QDs@MIPs@paper and QDs@NIPs@paper in the presence of zearalenone. The y-axis represents the fluorescence intensity at 630 nm.

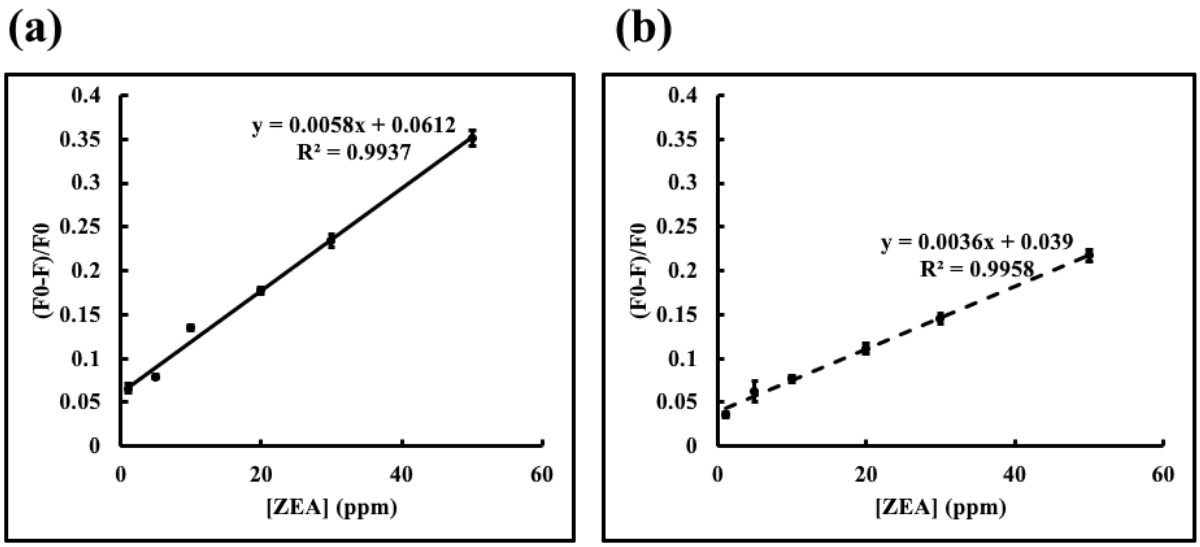

Figure 4.8. The linear relationship of the change in fluorescence intensity and zearalenone concentration of (a) QDs@MIPs@paper and (b) QDs@NIPs@paper.

The changes in fluorescence intensity were evaluated and the results are shown in **Figure 4.7**. Along with the increase in zearalenone concentration, the fluorescence intensity decreased for both MIPs and NIPs due to the specific and non-specific binding of zearalenone. The decrease trend for QDs@MIPs@paper was more intensive than that of QDs@NIPs@paper, indicating that the fluorescence of QDs was more significantly quenched by zearalenone when coated with MIPs than that coated with NIPs. This was likely due to the presence of specific binding sites in MIPs, allowing for more interactions between zearalenone molecules and QDs. In the meanwhile, NIPs did not have specific binding sites. There were less interactions between zearalenone and QDs, resulting in less significant fluorescence quenching effects. However, nonspecific binding was present between zearalenone by MIPs and NIPs. Therefore, although NIPs had no specific binding towards the target, there were still a degree of fluorescence quenching observed.

The relationship between the change in fluorescence intensity and the increase in zearalenone concentration is shown in **Figure 4.8**. Within the range of concentrations from 0 to 50 ppm, the relationship remained linear for both QDs@MIPs@paper and QDs@NIPs@paper.

4.3 Actual sample tests

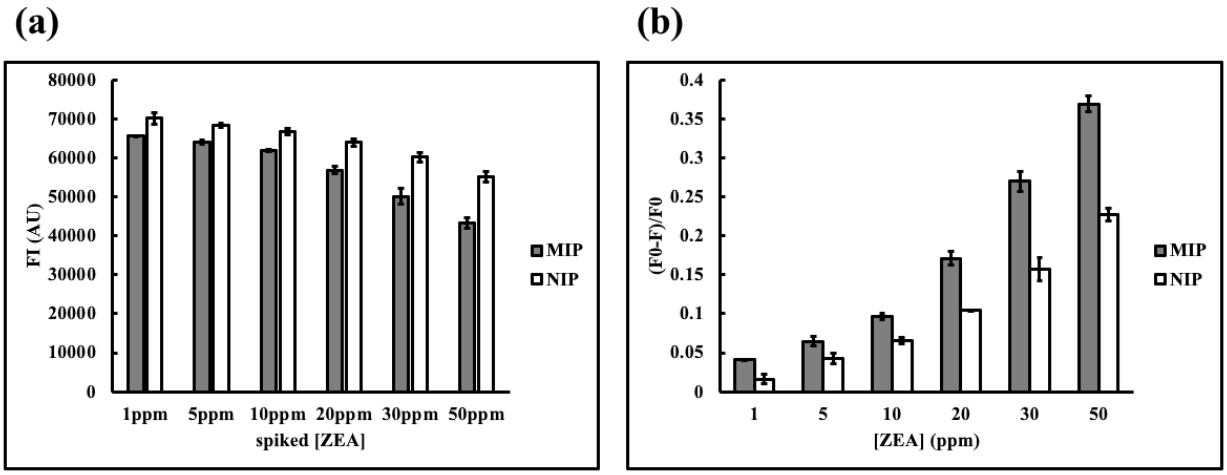

Figure 4.9. (a) Fluorescence intensity readings of QDs@MIPs@paper and QDs@NIPs@paper in the actual sample tests. (b) The proportions of fluorescence quenched by zearalenone in the samples.

The toxin in the spiked corn samples were extracted with acetonitrile/water (90:10, v/v). The sample recovery rate could be within 90.56–99.96% (Fu et al., 2020). **Figure 4.9** shows the relationship between fluorescence intensity and zearalenone concentration in real samples. A strict maximum residue level of zearalenone in food for human consumption has not been established in Canada, but Health Canada recommends the tolerance levels of zearalenone in animal feed (**Table 4.3**). The calculated LOD and LOQ of the QDs@MIPs@paper device were 1.085 mg/kg and 3.254 mg/kg, respectively. The calculations can be referred in the methods

section. As compared to the values shown in **Table 4.3**, the QDs@MIPs@paper device can detect zearalenone in swine diets and cow feed within the respective recommended tolerance level. However, the QDs@MIPs@paper is not capable of detecting zearalenone in gilt feed or cow feed within the respective recommended tolerance level. Therefore, further improvement is needed to improve the performance of QDs@MIPs@paper in detecting zearalenone in a wide variety of agri-food sources.

Table 4.3. Recommended tolerance levels by the government of Canada (unit: mg/kg).

Mycotoxin	Canada: Recommended tolerance levels	United States Guidelines
Zearalenone	Gilt diets < 1 - 3 Cow diets 10 (1.5 if other toxins present) Swine industry has voiced concern over levels of 0.25 - 5 in diets for sheep and pigs.	N/A

Note. From "RG-8 Regulatory Guidance: Contaminants in Feed (formerly RG-1, Chapter 7) Section 1: Mycotoxins in Livestock Feed" by Canadian Food Inspection Agency, 2017.

5. CONCLUSION

In this thesis project, molecularly-imprinted polymers (MIPs) aiming to selectively bind zearalenone were synthesized by precipitation polymerization method with 3-CCA and naringenin as templates, MAA as monomer, EDMA as crosslinker and AIBN as initiator. Corresponding non-imprinted polymers (NIPs) without template molecules were also synthesized for comparisons in tests. MIPs had specific binding capacity and fast binding speed (< 5 min) towards the target compound. One layer of MIPs was successfully coated on small pieces of filter paper. Red-colored CdSe/ZnS quantum dots (QDs) were grafted onto paper for fluorescence signaling via EDC-NHS coupling. QDs@MIPs@paper could recognize different levels of zearalenone in standard solutions. QDs@MIPs@paper was tested with spiked corn samples and reached desirable results with an LOD of 1.085 mg/kg and an LOQ of 3.254 mg/kg.

Future studies may focus on improving the convenience and applicability of QDs@MIPs@paper devices. In the current study, the fluorescence signal was measured by placing the QDs@MIPs@paper in a microwell plate, followed by using a fluorescence microplate reader. To simplify the process, QDs and MIPs can be grafted on a paper-based platform and the measurement can be conducted using a handheld fluorescence reader. Wells and channels can be designed and added to the paper-based platform using methods such as wax printing and inkjet printing. Therefore, the QDs@MIPs@paper can be organized into another format of paper-based microfluidic chip. Sample preparation step followed by dilution or concentration has the potential to be miniaturized and incorporated into the microfluidic chip. This assembly can minimize the instrumentation required for testing. Moreover, the currently developed MIPs could only specifically recognize and bind to zearalenone. Other MIPs targeting

multiple mycotoxins could be synthesized in developing novel sensing platform to achieve multiplex detection of mycotoxins in food samples.

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