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Exploration of The Selective Binding Property of The MIP-grafted Paper for Cochineal Dye

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Abstract

A molecularly imprinted polymer (MIP) is a synthetic polymer that provides specific cavities for its analyte. In this study, the MIP specific to carminic acid, an insect-derived pigment, has been synthesized using methacrylic acid (MAA) and 4-vinyl pyridine (4Vpy) as monomers and ethylene glycol dimethacrylate (EDGMA) as a cross-linker. The imprinted surface particles were characterized by Scanning Electron Microscope (SEM). The rough surface of the synthesized MIP represented the specific binding site for carminic acid. The paper-based MIP polymerization was performed by pre-treatment the cellulose paper with aminopropyltriethoxysilane (APTES) before polymerization with the MIP solution. The novel membrane-grafted MIP exhibits good performance for selective recognition with the target carminic acid, which can be demonstrated by the imprinted factor of 1.94 as compared to those of nonimprinted polymer. According to the Scatchard analysis, it was estimated that there are two types of binding strategy, including high and low affinity, which corresponded to the Ka of 1.24x103 mM and 0.10 x103 mM, respectively. It was thus preliminary concluded that the membrane-grafted MIP fabricated in this study has the potential to be implemented in many applications such as extraction and pre-concentration.

Keywords: *Molecularly imprinted polymer, carminic acid, halal, Cochineal red color, E120*

INTRODUCTION

Molecular Imprinting Technology (MIT) is a technique creating artificial recognition receptors with a predetermined selectivity and specificity for its analyte, which can be used as ideal materials in various application fields. The polymer created by this technology is known as molecularly imprinted polymer or MIP. This polymer is a robust molecular recognition element that is used mimically for natural recognition elements, such as antibodies and biological receptors (Vasapollo et al., 2011). The advantages of MIP compared with antibodies include a reduced molecular mass, enhanced stability, and cost-effective production methods. In addition, more efficient selection and screening procedures are offered in comparison with the screening

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procedure for the selection of aptamer, an alternative affinity receptor based on nucleic acids (Ruigrok et al., 2011).

Carminic acid is a natural red pigment derived from the Cochineal insect (E120). This dye is used as a colorant in various industries, including food, confectionery, drink and beverages, cosmetics, and the pharmaceutical industry. This dye is also used in the textile industry due to its relatively high chemical and biological stability. However, because this dye is extracted from insects, there are allergic cases of patients who have symptoms due to the consumption of food containing insect proteins (Takeo et al., 2018). In addition, for Muslim consumers, the use of this dye has some critical issues, including whether the use of dyes derived from insects is permitted. In addition, another issue has also risen because of the way they treat insects with strong conditions. To this point, the insects are boiled in hot water or heated at high temperatures for color extraction. This process is believed that the insect is treated improperly. Therefore, the color obtained from this process is an unlawful color for application in Halal products.

Because there are issues with using carminic acid in Halal industries, the sensitive detection method for insect-derived carminic acid is important for traceability and safety management. Previous studies for determination of this color are included using spectrophotometry (Samari et al., 2010, Ordoudi et al., 2018), high-performance liquid chromatography (HPLC) (Carvalho and Collins, 1997, Lancaster and Lawrence, 1996, Nishizaki et al., 2018, Ordoudi et al., 2018), FT-IR (Ordoudi et al., 2018) and electrochemistry (Yilmaz et al., 2014). Although these techniques are known as a sensitive procedure for determination in dilute samples, they require specific materials for selective binding or separation before analysis when detected in the sample with high complexity. Therefore, appropriate sample pre-treatment or sample manipulation is required before analysis.

Combined with the specific recognition of MIP, the synthesis of polymers with specific recognition for carminic acid is promising for selective extraction of carminic acid (Bibi et al., 2012). This polymer has the ability to apply for solid-phase extraction of carminic acid from dried body insects to obtain a highly effective yield similar to those previous reports (Bhawani et al., 2018, Karuehanon et al., 2018, Stevenson, 1999). In addition, in food and cosmetic products where the Cochineals color is added and appears at a very low content because of the FDA regulation (Harp and Barrows, 2015), this material could improve the selective absorption of the target carminic acid. Therefore, the better performance of the extraction could be obtained. This led to improvement of the sensitivity of the detection (Chianella et al., 2003, Andersson et al., 1997, Pichon and Combès, 2016, Mei et al., 2011).

Although the technique for detection of Cochineals color is necessary, there are few studies focusing on the development of the detection method for this dye. This limitation is not only for on-site detection but also for in-house techniques using high instrumentation. With regarding this necessarily, the development of the facile absorption paper with the specific binding sites for

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carminic acid created by MIP polymerized on the cellulose-based membrane was fabricated. The binding performance of the developed material will be demonstrated in the text. This material shows the potential to be implemented for solid-phase extraction to gain higher recovery. Moreover, this material provided further opportunity to implement in a pre-concentration step which was combined prior to the analysis. In addition, this material also provided the potential for the paper-based colorimetric sensor. Therefore, the developed material has promised further development in various fields of study. The preliminary study provided in this study will thus have high benefits.

LITERATURE REVIEW

Scatchard Model

Scatchard analysis is a common model used to evaluate the binding behavior of MIP in the rebinding experiment. Typically, non-covalent binding between a template and the functional monomer gives two straight lines indicating heterogeneous affinity of the high and low-affinity binding site (Zhi et al., 2018, Royani and Abdullah, 2014).

The Scatchard equation is as follows;

$$\frac{B}{[CA]_0} = (B_{max} - B)K_a$$

Where [CA]0 is the initial concentration of carminic acid. B represented bound carminic acid to the MIP. Ka is the association constant, Bmax is the maximum number of binding sites. Ka and Bmax values could be obtained from the slope and interception, respectively.

Imprinted factor (IF)

It can be calculated from the following equation:

$$IF = \frac{Q_{MIP}}{Q_{NIP}}$$

Where QMIP and QNIP are the adsorption capacity of the template of carminic acid@-MIPs and carminic acid@-NIPs, respectively.

RESEARCH METHOD

Preparation of MIP of carminic acid

The standard grades of 4-vinyl pyridine (4Vpy) and methacrylic acid (MAA) were purchased from Sigma Aldrich (St. Louis, MO). The ethylene glycol dimethacrylate (EDGMA) was obtained from Merck & Co., Inc. 2,2-Azobis (2-isobutyronitrile) (AIBN) was from Fluka (Steinem, Germany).

ISSN 2828-1500 (Online) | 2828-1985(Print)

Dimethylsulfoxide (DMSO) was purchased was prepared from reagent grade chemicals from Sigma Aldrich (St. Louis, MO). Aminopropyltriethoxysilane (APTES) was obtained from the Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University. Deionized water was obtained using a Milli-Q system (Thermo Fisher Scientific). Whatman® filter paper, Grade 1004, was purchased from GE Healthcare UK Limited, UK. The MultiskanTM microplate reader (Thermo Fisher Scientific) was used for spectrophotometric studies. The immobilised membrane was employed from FT-IR Tensor ll (Bruker, Germany). A scanning electron microscope (SEM Quanta, FEI 250) was employed for the surface morphology study.

Preparation of MIP of carminic acid

The MIP of the carminic acid was firstly prepared by bulk synthesis according to previous studies (Bibi et al., 2012). The co-monomer, 4-vinyl pyridine (4Vpy) and methacrylic acid (MAA), were utilized in the presence of ethylene glycol dimethacrylate (EDGMA). For the polymer preparation, 0.081 mmol of carminic acid was mixed with the co-monomers (0.93 mmol each) for 1 hour at room temperature. Subsequently, EGDMA (4.66 mmol) was added to the reaction mixture to proceed with the reaction for additional 4 hours at 24°C. Finally, 10 mg of AIBN (an initiator) and 1.27 ml of dimethylsulfoxide (DMSO), a porogen, were added to the above solution mixture. The mixture was then purged by nitrogen gas for 10 minutes at room temperature. After purging, the tube was carefully sealed and was incubated at 60 °C for 16 hours to allow the polymerization. The solid polymer was crushed and ground. The template was removed by repeated washing with a mixture of 10 percent of acetic acid in methanol several times. The obtained polymer was then dried at 80 °C for 24 hours. The resultant polymer was further analyzed by FT-IR and SEM to demonstrate the physiological property of the synthesized polymer. The binding capacity of the polymer with the target carminic acid was also observed by HPLC. Furthermore, nonimprinted (NIP) was prepared in the same manner but without the addition of the template.

Physiological characterization of polymer

1. Fourier Transform Infrared Spectroscopy (FT-IR)

FTIR analysis of the synthesized MIP and NIP obtained from the bulk polymerization method was carried out using FT-IR Tensor ll. The transmission region was studied in wavenumbers ranging from 400 cm−1 to 4000 cm−1. A number of 32 scans were operated. The data was analyzed by OPUS software.

2. Scanning Electron Microscope (SEM)

The morphology of the synthesized MIP and NIP was studied using SEM. The ground MIP and NIP were subjected to critical point drying using liquid CO2 substitution before analysis. Dehydrated samples were adhered to the stubs with clear nail polish, coated with gold-palladium, and analyzed under SEM. The MIP grafted membrane was analyzed in the same manner.

Preparation of MIP grafted on cellulose membrane

1. Activation of cellulose membrane by coupling agent

ISSN 2828-1500 (Online) | 2828-1985(Print)

Surface modification of the cellulose membrane was prepared according to the previous reported with slight modification (Howarter and Youngblood, 2006). Briefly, the cellulose membrane was cut into 0.5 x 1 cm and dried at 110 °C in the oven for 2 hours. Dried cellulose membrane was then submersed in 5% of aminopropyltriethoxysilane (APTES) in ethanol for 15 minutes at room temperature. Washing was performed carefully by rinsing with ethanol several times. The modified membrane paper was dried in the oven at 110°C overnight. The modified paper was kept in the desiccator before modification and polymerization.

2. Membrane grafting with MIP

The solution mixture MIP (2.0 mL) was dropped onto the silanized membrane, which was placed on a cleaned glass slide. The cover slit was placed onto the paper soaked with the polymerization solution to form a thin film on the cellulose membrane. Once the air was removed from the MIP-treated membrane, the polymerization was allowed to perform at 60°C for 4 hours. The grafted cellulose membrane was rinsed several times to remove carminic acid with 10% of acetic acid in methanol, as mentioned previously. The cellulose membrane grafted with NIP was prepared in the same manner using the solution mixture of NIP in which the target carminic acid was not included.

3. Rebinding analysis

The rebinding analysis was performed by incubation of carminic acid with the developed MIP and NIP. The reduction of the carminic acid after the binding was determined with ultra-high performance liquid chromatography (UHPLC). In this study, the carminic acid at the concentration ranged from 0 – 2.0 mM in 50% methanol. The reduction of the carminic acid concentration was calculated from the calibration curve of carminic acid prepared in the same manner. The rebinding parameter was calculated from the equation 1, and 2 explained in the above session.

FINDINGS AND DISCUSSION

Physiological characteristic of the synthesized carminic acid@-MIP

Recently, the MIP prepared by polymerization technique was interested in being used in solid-phase extraction to improve its recovery and yield of the target analyte. The bulk polymer synthesized with the co-monomer prepared in this study was solid. The resulting solid polymer was ground in a mortar and sieved to obtain the defined cut of particle size of 25 – 100 μm. Considering the solid MIP with FT-IR, the spectra are depicted in Figure 1. According to the ATR-FTIR spectra of carminic acid@-MIP (pink), the bands are dominant, including the band at 2960 cm-1 of the –CH stretch, the band at 1640 cm-1, which is the combination of –OH bend and intermolecular hydrogen bond between –COOH groups, the band at 1150 cm-1 which referred the stretch of the –C-O-C in hexose ring of carminic acid. These characteristics are in accordance with a previous report (Silverstein and Bassler, 1962). In addition, the bands typically for carminic acid are obviously visible at 1712 cm-1, which referred to the –C=O stretch, and at 1620 cm-1 –CC stretch in aromatics, 1245 cm-1 – assigned to catechol functions (Guillermin et al., 2019), except

ISSN 2828-1500 (Online) | 2828-1985(Print)

for the bands centered at 1570 cm-1 (–CC stretch in aromatics) and 1040 cm-1 (–CC stretch in glucose unit). These bands were not observed in carminic acid@-NIP (black). Therefore, it was thus confirmed that there were some interactions between the target template carminic acid and the polymerization solvent, including monomers, linkers, and initiators.

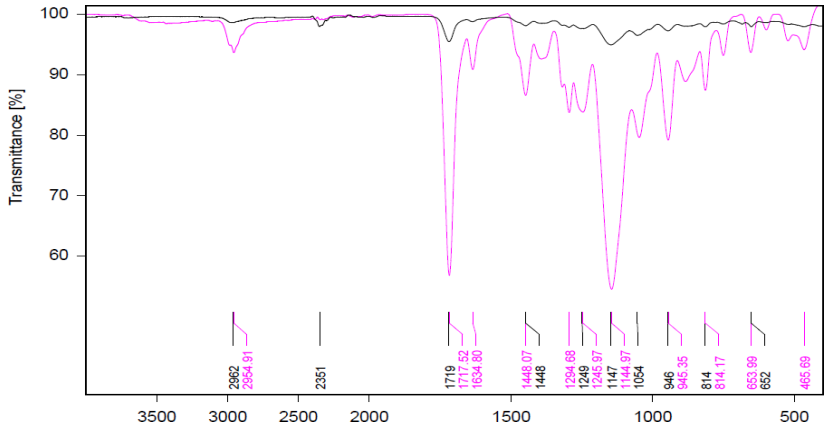

Figure 1. FTIR spectrum of NIP (black) and MIP (pink)

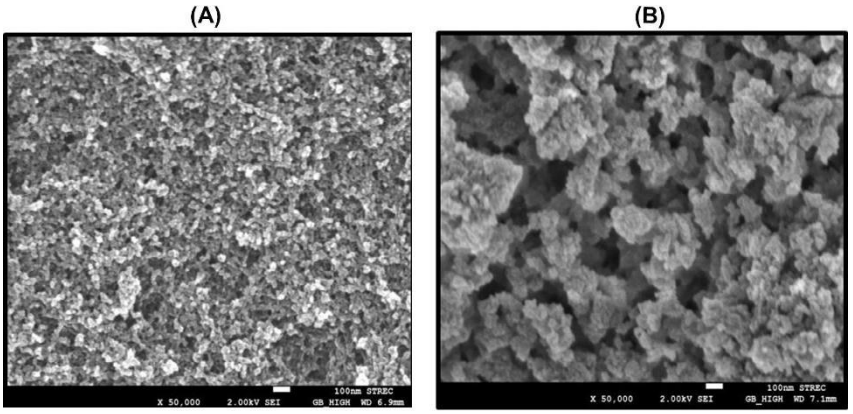

Figure 2. SEM micrographs of polymer (A) nonimprinted and (B) imprinted with carminic acid as the molecule template (the magnify of 50,000x).

The scanning electron micrograph shows the morphology, and the structure of the carminic acid imprinted MIP and its nonimprinted analog. Figure 2 (A) corresponds to the NIP, which was synthesized exactly by the same method but excluding the template. The SEM image shows an appreciable difference in the surface morphology. The NIP had a more uniform and smooth shape than that of the MIP, in which the irregular and rough morphology was distinguished under SEM. It could be described by the fact that the regular morphology attributed to that there is no specific binding for the target carminic acid in NIP. On the contrary, the cavity in the MIP was probably caused by the structure of the template carminic acid.

Preparation of MIP grafted on cellulose membrane

ISSN 2828-1500 (Online) | 2828-1985(Print)

MIP-grafted cellulose membranes were obtained by in situ polymerizations of a MIP layer on the surfaces of the cellulose membrane. For covalent grafting on a cellulose membrane, the membrane was silanized with ATPES. Figure 3 showed the FTIR spectrum of the bare cellulose membrane (black) and C=C modified cellulose membrane (pink), which can be indicated from the stretching peak at 1711 cm-1. PVDF membrane was also used for silanization, but the membrane was degraded after the silanization procedure. Therefore, it was not suggested to be used as a scaffold (data not shown). The thin film formed on the cellulose membrane could be clearly observed for the MIP-grafted cellulose membrane. Then, removal of the template was performed by soaking it with 10% acetic acid in methanol. The rebinding properties of MIP- grafted cellulose membranes were analyzed.

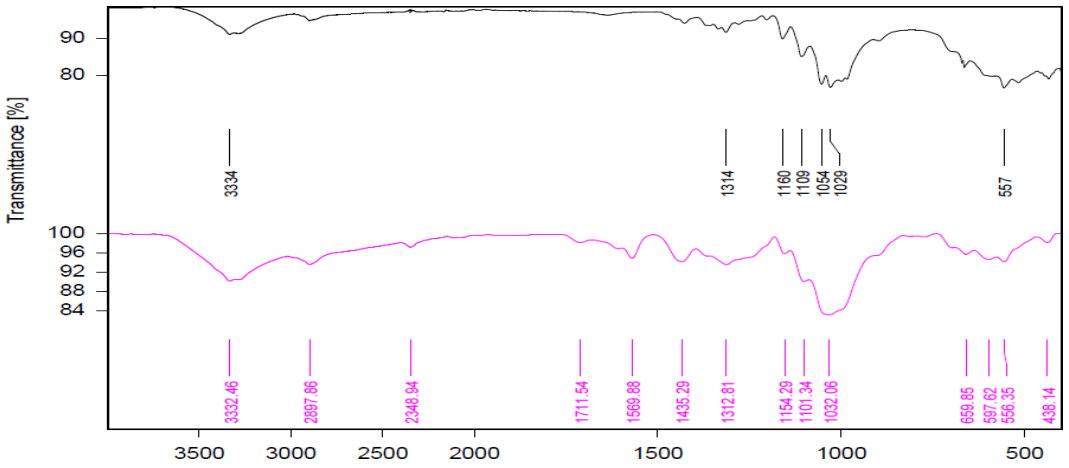

Figure 3. the FTIR spectrum of the bare cellulose membrane (black) and the cellulose membrane after silanization (pink)

Rebinding analysis

Because the MIP was made with the proposed to selectively bind a target, the absorption of the carminic acid at the binding sites was thus analyzed. The molecular recognition of the target at the binding sites or binding cavities is mostly attributed to the non-covalent interaction such as hydrogen bond, ion pairing, and dispersive forces. In addition, steric exclusion of the other interfering compounds from the cavities was also expected (Dorkó et al., 2015). This property is very promising for MIP as similar to the specific binding property of antibody, a biological recognition element. The binding interaction could be explained by the dissociation constant (Kd) and association constant (Ka) of a similar magnitude to antibodies when binding with the proteins such as melting (Vaidya et al., 2001) and trypsin (Hoshino et al., 2008). Besides, MIP has more potential for recognition and binding to the small molecule. It could be explained in the same manner. Therefore, in this study, the binding property of the synthesized solid polymer was characterized by the dissociation constant to explain the specific binding of the binding cavities of the MIP to be potentially used as a sorbent in the solid-phase extraction.

ISSN 2828-1500 (Online) | 2828-1985(Print)

The rebinding property of MIP towards carminic acid template was performed by the Binding isotherm and the Scatchard analysis as a demonstration in Figures 4 and 5, respectively. The calibration curve of carminic acid in 50% methanol was achieved with the linear equation of y = 5x106 x with the R2 = 0.999. The linear range was obtained at the carminic acid concentration between 0 – 2.0 mM. In Figure 4, the saturated curve of NIP was observed when carminic acid concentration was exceeded 2.5 mM. On the contrary, for MIP, the saturated curve was observed when increasing carminic acid to as higher as 4.5 mM. This is due to the increasing contribution of the specific and non-specific binding site of MIP (Alenazi et al., 2016). Furthermore, the imprinted factor (IF), a parameter that usually used to characterize the molecular recognition abilities of the imprinted polymer, was calculated as 1.94. This value indicated a satisfactory imprinting factor (1.5 – 3) (Zahedi et al., 2016).

The association constant (Ka) and the maximal biding site (Bmax) of MIP can be archived from the Scatchard plot, as was shown in Figure 4. Two straight lines of the high and low-affinity binding site of the synthesized polymer were obtained. The Ka and Bmax calculated from the slope and intercept were summarized in Table 1. The two straight lines fitting the Scatchard equation indicated the affinity of the binding site in the synthesized MIP are heterogeneous, and thus the two association constants have corresponded to the high and low affinity (Hervy and Bicout, 2019).

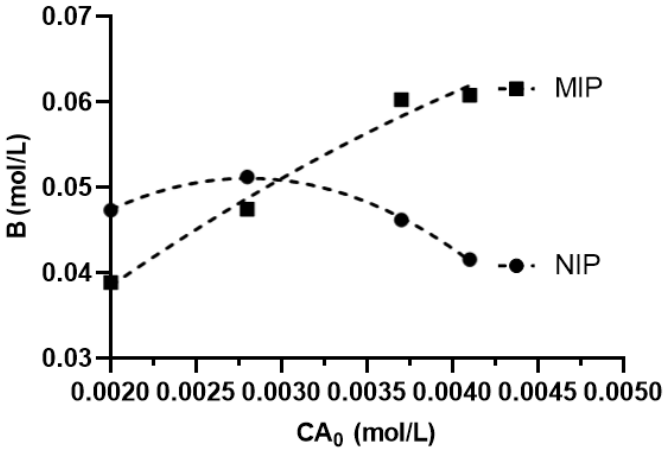

Figure 4. Binding isotherm study for the synthesized MIP and NIP.

ISSN 2828-1500 (Online) | 2828-1985(Print)

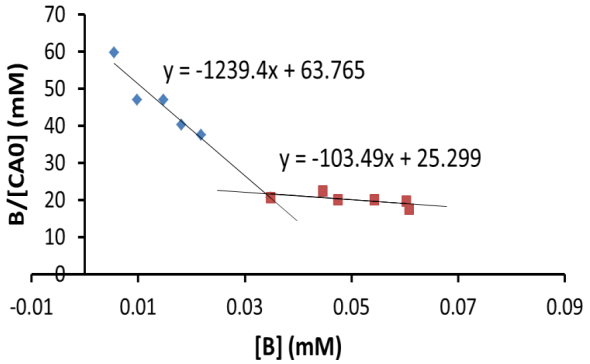

Figure 5. The Scatchard plot of the synthesized MIP. The two straight lines exhibit high and low-affinity binding.

Table 1. Ka and Bmax of high and low-affinity binding of MIP towards the carminic acid template

Polymer	Ka (103 mM)	Bmax (mmol/g of polymer)
MIP (high affinity)	1.24	0.051
MIP (low affinity)	0.10	0.244

CONCLUSION AND FUTURE RESEARCH

In this study, the selective molecularly imprinted polymer to carminic acid was obtained from the co-monomer polymerization technique. The imprinted factor of 1.95 indicated the higher specific binding compared to those that of nonimprinted polymer. The novelty of the immobilized MIP on the modified cellulose membrane was demonstrated using cellulose membrane as a substrate for forming polymerization. The membrane treated with silane derivative provided the functional moiety to react with the MIP. The obtained MIP grafted membrane could thus exhibit superior advantages because of its specificity, mobility, and facility. Nonetheless, it shows the potential to be further applied not limited to the application for the on-site extraction but on-site pre-concentration and sensor.

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