KIMIKA Volume 23, Number 1, pp. 17-25 (2010)

ISSN 0115-2130

Molecular Recognition Properties of Artemisinin - Imprinted Polymer Microspheres

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The ability of molecularly-imprinted polymers (MIPs) to recognize and remember the shape of an imprinted molecule has expanded the application of these materials for separation and sample enrichment. This paper describes the binding efficiency and characteristics of a MIP prepared for the selective extraction of artemisinin, a potent anti-malaria compound present in *Artemisia annua*. Artemisinin is currently used in combination therapy for the treatment of severe cases of malaria.

The MIP microspheres were obtained *via* precipitation polymerization using 5:6:20 mmol artemisinin/methacrylic acid/ethylene glycol dimethacrylate in 5% toluene/acetonitrile (95% v/v) solvent. This material showed high selectivity for artemisinin by rebinding experiments. Scatchard analysis was also carried out to further understand the binding characteristics of this material towards artemisinin.

Keywords: molecularly imprinted polymer (MIP), molecular recognition, precipitation polymerization, artemisinin, Scatchard plot

INTRODUCTION

Molecularly-imprinted polymers are materials which contain recognition sites prepared by polymerizing functional monomers around a chosen template or molecule of interest. Subsequent removal of the template from the polymer matrix leaves minute cavities that are complimentary in size, shape and functionality, capable of binding the target molecule [1]. Because of the potential for high specificity and selectivity, MIPs have been used as sorbent materials in solid phase extraction and in the separation of complex mixtures [2].

MIPs are traditionally prepared by bulk polymerization where polymer blocks are

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obtained. Such polymer blocks require grinding and sieving to obtain the desired particle size. A disadvantage of this approach is that the formed polymer particles are highly irregular in size and shape, making them unsuitable for many separation purposes. More recently, precipitation polymerization has emerged as an attractive, simple, and general method in producing high-quality imprinted polymers. This method is a surfactant free process that can provide uniform spherically-shaped polymer beads possessing high binding efficiencies for the target compounds [5]. This method has been applied to various organic compounds from biological to environmental samples [6]. In this study, precipitation MIP selective for artemisinin, a potent anti-malaria compound, was prepared.

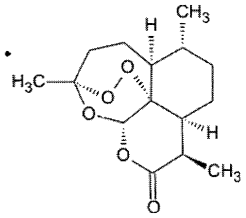


Fig. 1. *Structure of artemisinin (qinghaosu, QHS)*

Artemisinin (*QHS*, Figure 1), is a sesquiterpene, endo-peroxide lactone with an unusual 1,2,4-trioxane ring system. The endoperoxide ring is known to possess the anti-malarial activity [7]. Currently, artemisinin is being used in combination therapies (ACT) to treat severe cases of malaria worldwide [8].

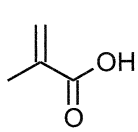
Artemisinin can be synthesized from natural precursors such as (R)-pulegone [9] and dihydroartemisinic acid [10]. But due to low reaction yields, these synthetic procedures have been shown to be inefficient and expensive. Various extraction methods for artemisinin from Artemisia annua have been developed [11], but because of the numerous purification steps required, the overall recovery of the compound is low [12]. Accordingly, an efficient purification method for artemisinin from the Artemisia annua extract is highly desirable and needed.

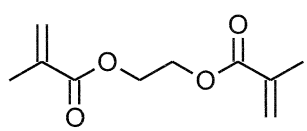
In this study, artemisinin-imprinted polymer microspheres were synthesized by precipitation polymerization using the functional monomer methacrylic acid (MAA) with an ethylene glycol dimethacrylate (EGDMA) crosslinker. The polymer obtained using a mole ratio of 5:6:20 for artemisinin/MAA/EGDMA showed high selectivity for artemisinin. The MIP morphology, its yield and studies on the binding efficiency and binding characteristics to artemisinin are discussed and delineated.

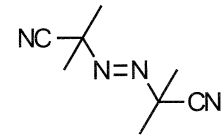
EXPERIMENTAL

Reagents

Artemisinin or QHS, 99% pure was purchased from Shanxi Sciphar Chemical Company (Shanxi, China) and used as received. Methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) were purchased from Merck. MAA was distilled under vacuum prior to use. The inhibitor was removed from EGDMA by washing with saturated NaCl solution and drying the organic layer over anhydrous sodium sulfate before use. The initiator, 2,2-azobis(iso-butyro)nitrile (AIBN) was obtained from Chem Service (West Chester, PA) and was recrystallized from methanol prior to use. Acetonitrile (CH_3CN), toluene ($PhCH_3$), methanol (MeOH), acetic acid (AcOH) and other chemicals were of analytical reagent grade and used as received. The chemical structure of







methacrylic acid, MAA ethylene glycol dimethacrylate, EGDMA 2, 2'-azobis-isobutyronitrile, AIBN

Fig. 2. *The chemical structures of MAA (monomer), EGDMA (crosslinker) and AIBN (initiator)*

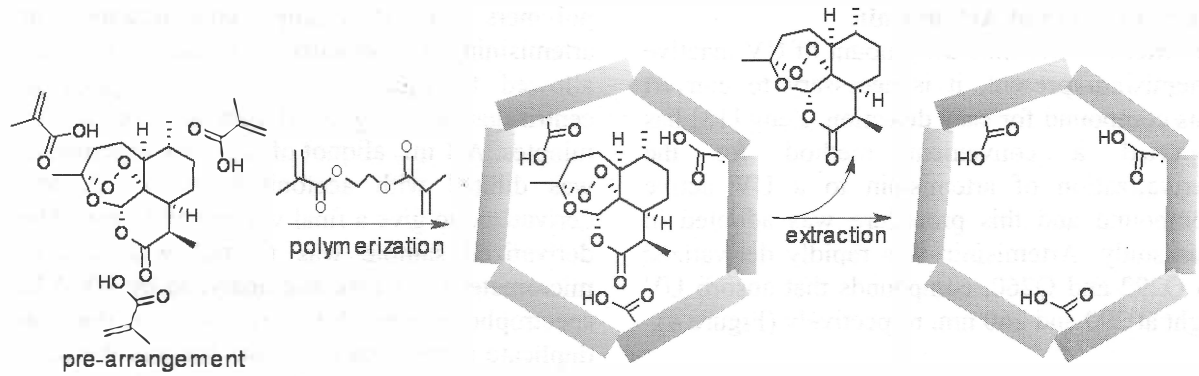


Fig. 3. *The reaction scheme for producing molecular imprinted polymer for artemisinin. Artemisinin was first allowed to pre-arrange with MAA monomers in toluene/acetonitrile solvent and polymerized with EGDMA crosslinker. Extraction of artemisinin using acetonitrile gave the artemisinin-imprinted polymer.*

the MIP precursors is shown below in Figure 2.

Instruments

Surface analysis was carried out using a JEOL JFC 1200 fine gold coater and a JEOL JSM 5310 Scanning Electron Microscope (SEM). Derivatized artemisinin solutions were analyzed using a Shimadzu 2401 PC UV-VIS absorption spectrophotometer at 260 nm.

Synthesis of MIP

Artemisinin (71 mg, 0.25 mmol), MAA (26 μL, 0.30 mmol), EGDMA (188 μL, 1.00 mmol) were dissolved in 5% toluene/acetonitrile (> 95% v/v). The mixture was sonicated for 5 minutes followed by the addition of AIBN (0.03 mmol). This mixture was purged with nitrogen for 5 minutes, and placed in an oil bath at 60 °C for 24 hours.

Polymer microspheres formed from the polymerization reaction were separated from the reaction medium by centrifugation. The beads were washed four times with 20% (v/v) acetic acid in methanol (5 mL). The presence of artemisin in the washing was monitored with UV-VIS spectrophotometer to ensure that all the compound was extracted from the beads. The polymers were subsequently washed with methanol and dried in vacuum oven overnight at 40 °C. As a control, a blank, non-imprinted polymer (NIP) was also prepared using the same experimental conditions except that artemisinin was not added to the polymerization mixture. All polymers were prepared in duplicate. The general imprinting and polymerization reaction scheme is shown in Figure 3.

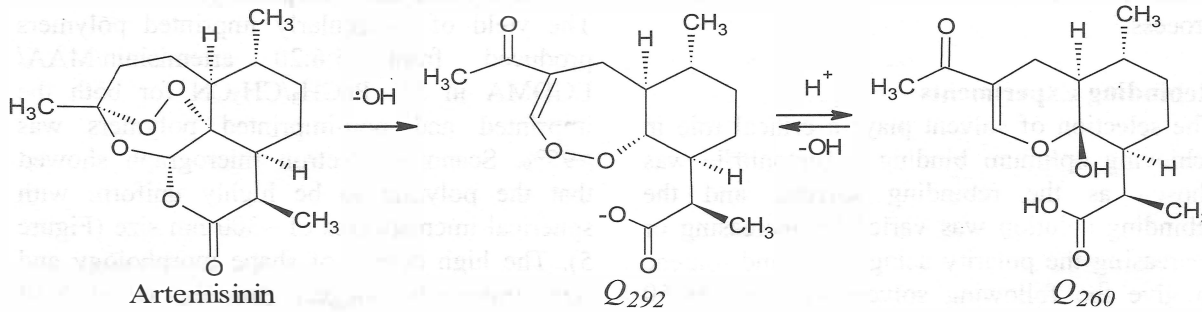


Fig. 4. *A reaction diagram depicting the conversion of Artemisinin to a UV-absorbing derivative,* Q_{260} [13].

Derivatization of Artemisinin

In order to determine the amount of UV inactive artemisinin present, it is necessary to convert this compound for easy detection. Zeng [13] has reported a convenient method for the derivatization of artemisinin to a UV active compound and this procedure was adopted in this study. Artemisinin was rapidly derivatized to Q292 and Q260, compounds that absorb UV light at 290 and 260 nm, respectively (Figure 4).

Artemisinin was derivatized by addition of NaOH (0.20 %, 20 mL) to 5 mL of artemisinin solution in acetonitrile and heated at 45 ^{o}C for 30 minutes. The solution was cooled to room temperature whereby acetic acid (0.08 M) was added to 50 mL volumetric mark to convert Q292 to Q260 . The derivatization process was monitored by UV-VIS spectrophotometer.

Calibration Plot

A calibration plot was constructed in order to quantify the amount of artemisinin present in a solution. From a 500 ppm artemisinin in acetonitrile stock solution, 0, 0.50, 1.0, 1.5, 2.0, 3.0 and 5.0 mL aliquots were transferred to 50 mL volumetric flask. Acetonitrile was added to aliquots less than 5 mL to give a total volume of 5 mL acetonitrile solution. The artemisinin in acetonitrile were derivatized separately to give 50 mL volume of 0, 5, 10, 15, 20, 30 and 50 ppm standard solutions. The standard calibration curve was reproducible and linear with a correlation coefficient of 0.9991. This is also indicative of the complete conversion of artemisinin to Q260 in the derivatization process.

Rebinding Experiments

The selection of solvent plays a critical role in achieving optimum binding. Acetonitrile was chosen as the rebinding solvent, and the rebinding solution was varied by increasing or decreasing the polarity using water and toluene to give the following solvent systems: 50/50 H_2O/CH_3CN, 25/75 H_2O/CH_3CN, CH_3CN, 25/75 $PhCH_3/CH_3CN$, and 50/50 $PhCH_3/CH_3CN$.

Equilibrium binding experiments were carried out by suspending equal amount (25 mg) of the polymers in increasing concentration of artemisinin in acetonitrile (3 mL). This was allowed to equilibrate for 1 hour prior to centrifugation using 5000 rpm at 5 ^{o}C for 15 minutes. A 1 mL aliquot of the clear supernatant was diluted with acetonitrile to 5 mL and derivatized to give a final volume of 50 mL. The derivatized sample was filtered with a 0.45 micrometer filter disk and analyzed by UV-VIS spectrophotometer. All analyses were done in duplicate using both the imprinted and the non-imprinted polymers. The measured concentration in ppm of the supernatant was reported as the unbound artemisinin, [QHS]. The concentration of the bound artemisinin (Bbound) to the MIP was calculated by getting the difference of the concentration of the unbound from the starting concentration of the artemisinin in acetonitrile suspending solution.

RESULTS AND DISCUSSION

In this study, the functional monomer MAA was chosen because of its capability to interact with the artemisinin molecule *via* hydrogen bonding. MAA has been commonly used in molecular imprinting which requires H-bonding interaction with the template [1]. This interaction allows the functional monomers to assemble around the imprint molecule prior to polymerization in order to incorporate the size, shape and the functional interaction of the imprint molecule to the imprinted sites.

Polymer Yield and Morphology

The yield of molecularly imprinted polymers produced from 5:6:20 artemisinin/MAA/ EGDMA in 5% $PhCH_3/CH_3CN$ for both the imprinted and non-imprinted polymers was >95%. Scanning electron micrograph showed that the polymer to be highly uniform with spherical microspheres of ~300 nm size (Figure 5). The high degree of shape morphology and size uniformity suggest that the novel MIP particles may be suitable for use as sorbent materials in chromatographic separations compared to materials obtained from traditional bulk polymerization method [3].

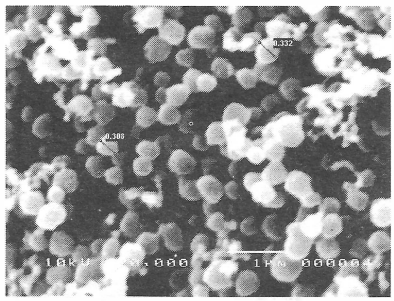


Fig. 5. *A SEM image of the artemisinin-imprinted MAA/EGDMA polymer obtained from 95% v/v solvent (5% $PhCH_3/CH_3CN$).*

Rebinding Analysis in Different Solvents

The selection and choice of a solvent plays a critical role in achieving optimum binding. The adsorption of any compound to a given substrate is highly affected by the suspending solvent. The solvent may enhance or inhibit the adsorption of artemisinin in the imprinted polymer substrate. were investigated. As shown in Figure 6, 50/50 $PhCH_3/CH_3CN$ and 50/50 H_2O/CH_3CN gave the highest concentration of artemisinin bound in the MIP. However, the same binding effect can also be observed for the corresponding non-imprinted polymer.

The increase in the amount of artemisinin bound does not equate to increase in selectivity (artemisinin bound to the MIP/artemisinin bound to the NIP). The lower ratio obtained with the use of 50/50 H2O/CH3CN and 50/50 PhCH3/CH3CN is due to the decrease in solubility of the artemisinin in the suspending solution, thus, pushing the artemisinin to bind more to both the MIP and NIP. Therefore, an ideal rebinding solvent should allow good binding of the artemisinin to the MIP where selective binding in specific binding sites should occur rather than non-specific surface adsorption

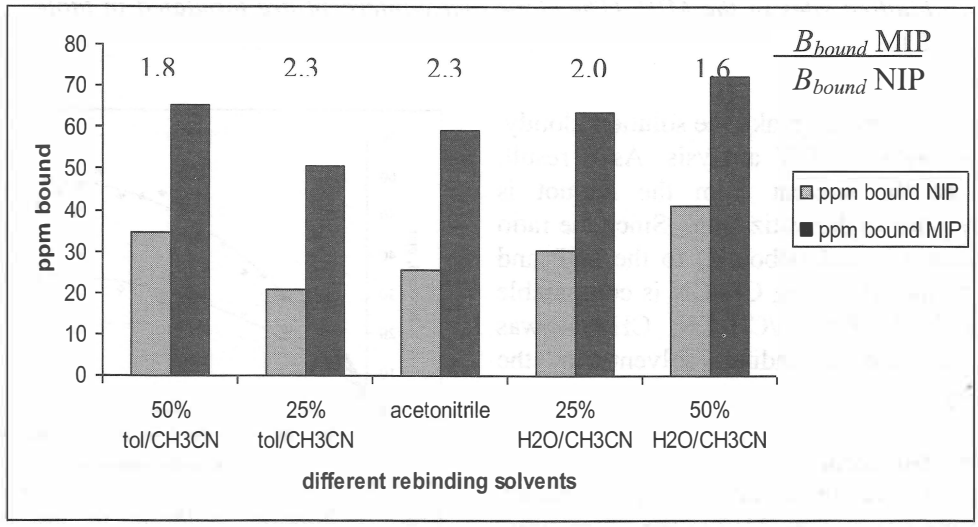


Fig. 6. *Rebinding data of artemisinin for MIP and NIP using different rebinding solvents. Rebinding study was done in 1-hour suspension. The B_{bound} is the amount of artemisinin (in ppm) bound to the corresponding polymer. The ratio of the artemisinin bound by the MIP over the NIP determines the selectivity of the*

In this study, equilibrium rebinding experiments were carried out to investigate the selectivity and binding capacity of the imprinted and non-imprinted polymer to artemisinin. Suspending solvents with different polarities such as 50/50 H_2O/CH_3CN, 25/75 H_2O/CH_3CN, CH_3CN, 25/75 $PhCH_3/CH_3CN$, and 50/50 $PhCH_3/CH_3CN$ due to decreased solubility of artemisinin in the rebinding solvent.

The data suggest that a solvent comprised of 25/75 $PhCH_3/CH_3CN$ and 100% CH_3CN are superior compared to the other solvent systems for the rebinding of artemisinin to the MIP. However, the presence of the toluene in the

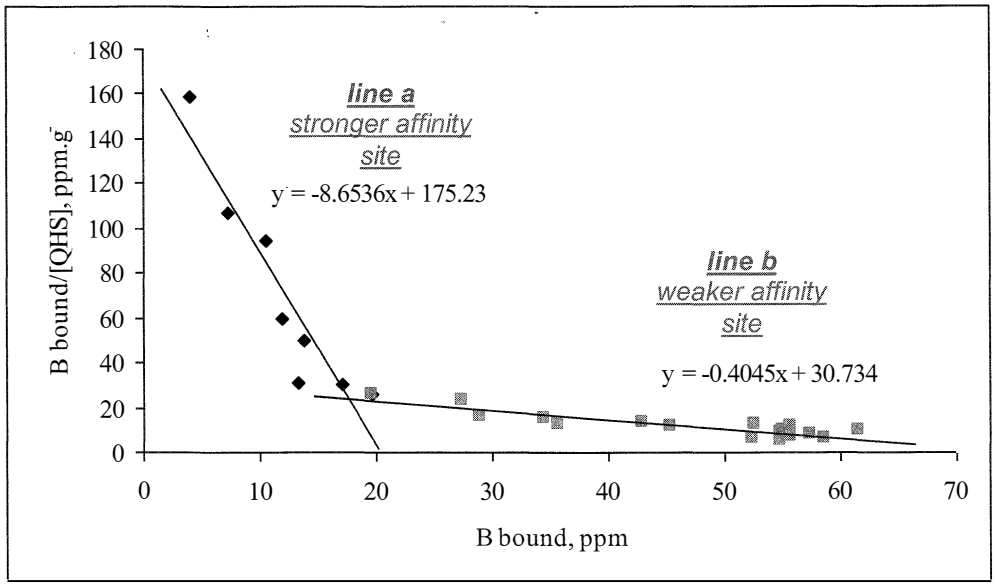


Fig. 8. *Scatchard plot of the rebinding data from artemisinin-imprinted polymer. Dashed lines indicate the regions that correspond to the type of binding sites in the MIP. (The slope and y-intercept are tabulated in table 1.)*

rebinding solution can make the solution cloudy, interfering with the UV analysis. As a result, removal of the solvent from the aliquot is necessary prior to derivatization. Since the ratio of artemisinin bound (Bbound) to the MIP and Bbound to the NIP using CH_3CN is comparable with the 25/75 $PhCH_3/CH_3CN$, CH_3CN was adopted as the rebinding solvent in the succeeding studies.

Rebinding Isotherm

In order to investigate the binding efficiency and characteristics of the MIP to the artemisinin, equilibrium binding experiments and Scatchard analysis were carried out. The plot of the amount of artemisinin bound (Bbound) to the 25 mg of the MIP beads is shown in Figure 7. The MIP beads were suspended in varying concentrations of artemisinin in acetonitrile (5, 10, 15, 20, 25, 30, 40, 50, 75, 100, 125, 150, 175, 200, 225, 250, 375, 300, 325, 350, 375, 400, 450, 500 ppm) for 1-hour equilibration time. In order to compare the ability of the MIP to bind artemisinin, the rebinding of artemisinin in the blank polymer (NIP) was also determined.

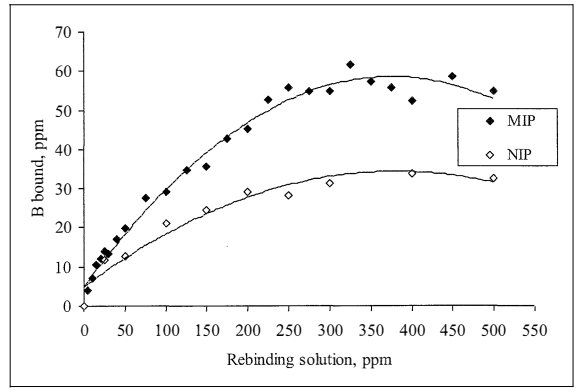


Fig. 7. *Binding isotherm of the artemisinin-imprinted polymer and its NIP using 1-hour equilibration time. B_{bound} is the concentration of artemisinin bound to the 25.0 mg polymer. The amount of artemisinin bound to MIP is significantly higher that its corresponding NIP.*

The plot of the binding isotherm using 1-hour equilibration time showed that the saturation point occurs at ~250 ppm artemisinin concentration. It can also be seen that the amount of artemisinin bound to MIP is significantly higher that its corresponding NIP at any point using different concentration of the binding solution. A 24-hour equilibration

rebinding study was also carried out giving a saturation profile similar to the one-hour rebinding.

Scatchard Analysis

The rebinding data for the imprinted polymer were recast using the Scatchard equation [16]:

$$\frac{B\ bound}{[QHS]} = \frac{B\ max - B\ bound}{Kd} \qquad (1)$$

where K_d is the equilibrium dissociation constant and Bmax is the maximum number of binding sites. The Scatchard plot was obtained by plotting the concentration of artemisinin bound to the MIP over the unbound artemisinin, Bbound/[QHS] versus Bbound. The Scatchard plot for the binding data of the artemisinin-imprinted polymer is shown in figure 8.

A linear Scatchard plot represents a homogeneous binding property of the polymer [1,16]. However, in this experiment, two distinct lines were obtained. This suggests that the binding of artemisinin to the MIP is heterogenous. There are two types of binding sites with different binding properties.

These two types of binding sites can be best defined by looking at the binding strength (represented by the K_d) and binding capacity (represented by the Bmax). From the Scatchard plot, Kd and Bmax values were calculated from the slope and the intercept of the equation, respectively.The values of the Kd and Bmax are summarized in Table 1.

Table 1. K_d and B_{max} values of the lines generated from the Scatchard plot.

Line	Recognition type	Linear regression equation	K_d	B_{max} (mg QHS/g MIP)
a	*strong affinity*	$y_1 = -8.6536x_1 + 175.23$	0.116	2.43
b	*weak affinity*	$y_2 = -0.4045x_2 + 30.734$	2.47	9.12

The binding strength represented by the K_d value for line *a* is 20 times smaller than the K_d value from line *b*. The smaller the equilibrium dissociation constant indicates that the binding affinity of the artemisinin to the binding sites represented by line *a* is stronger than line *b*.

The presence of strong and weak binding sites of the MIP for artemisinin is illustrated in Figure 9. The strong affinity is demonstrated by the binding of artemisinin in specific binding sites (Fig 9A) at low analyte concentration, as depicted in line *a*. At a higher artemisinin concentration (>250 ppm) the specific binding sites are saturated, as a result, further increase of artemisinin in the rebinding solution leads to non-specific binding. These non-specific recognition can be due to simple adsorption of artemisinin on the MIP surface (Fig 9B) and possible binding to the non-specific cavities (without functional monomers) in the MIP as shown in Fig 9C.

The non-specific binding of artemisinin is also evident from the values of binding capacity (Bmax). From Table 1, the B_{max} value from line *b* is higher than the value obtained from line *a*. This indicates that additional binding of artemisinin occurred after the specific binding sites were saturated, resulting to non-specific recognition of artemisinin to the MIP. From these, it can be drawn that the artemisinin imprinted polymer prepared binds to artemisinin with high capacity and specificity at concentrations not more than 250 ppm artemisinin in acetonitrile.

SUMMARY

The artemisinin-imprinted polymer produced from 5:6:20 mmol ratio of artemisinin/MAA/EGDMA in 5% solvent (PhCH3/CH3CN) gave high yields (<95%) and uniformly spherical microspheres of ~300 nm

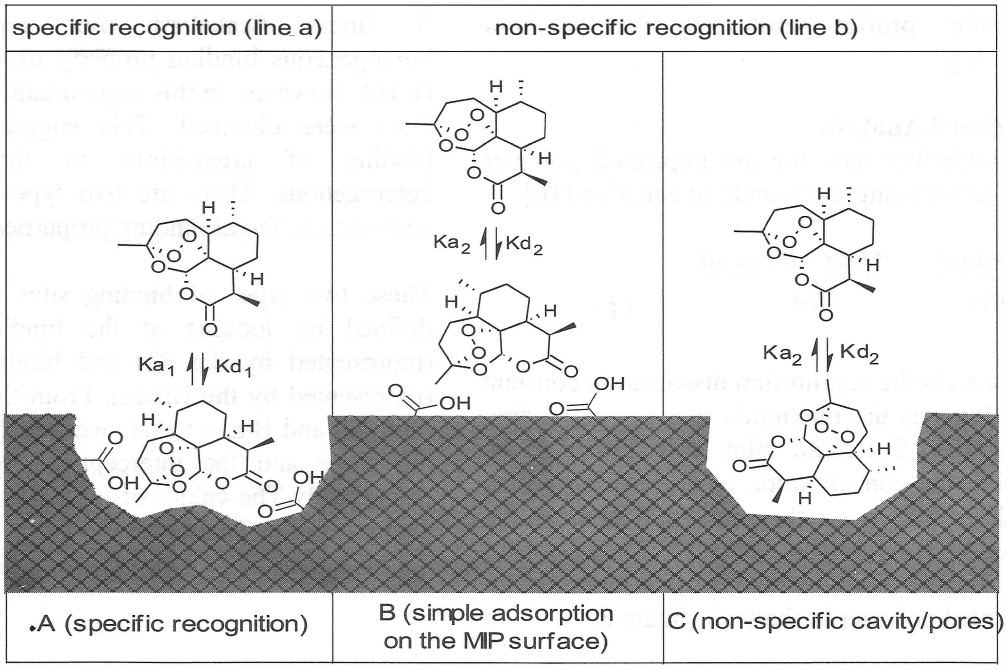


Fig. 9. *Binding scheme showing artemisinin being adsorbed on the MIP. Binding of artemisinin is due to both specific and non-specific recognition, where specific recognition is demonstrated as the binding of artemisinin in the specific cavity (A) and non-specific recognition is the binding of artemisinin by simple adsorption on the surface (B) and non-specific cavity or pores in the MIP (C).*

size. Studies on the binding characteristics of this polymer for artemisinin in acetonitrile solution indicate that the binding saturation profile is 250 ppm for a one and 24-hour equilibration time. The MIP produced exhibited high binding affinity and selectivity towards artemisinin. Although, at high artemisinin concentration, limited non-selective binding was observed. The high degree of shape morphology, size uniformity and selective binding suggests that the novel MIP particles may be suitable for use as sorbent materials in chromatographic separation of artemisinin in plant extract.

ACKNOWLEDGEMENT

The authors would like to thank the Ateneo de Manila University Loyola Schools Faculty Grant to RCS, DOST-PCASTRD scholarship to CAM, De La Salle University Physics laboratory for the SEM analysis, Dr. Erwin P. Enriquez and Dr. Richard B. Gross for the helpful discussions, and the Ateneo de Manila University Chemistry department.

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