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Use of molecularly imprinted polymers from a mixture of tetracycline and its degradation products to produce affinity membranes for the removal of tetracycline from water

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Abstract

The possibility of introducing multiple recognition in artificial receptors by imprinting polymers, using a mixture of tetracycline (TC) and its degradation products as templates, has been examined. The recognition ability of the resulting molecularly imprinted polymer (MIP), as evaluated by batch rebinding assay, was found to be group-specific to tetracyclines, while the single tetracycline imprinted polymer (MIP-2) prepared using TC free from degradation products as the print molecule showed considerably high selectivity for doxycycline (DC) and modest selectivity for TC and other TC derivatives, oxytetracycline (OTC) and chlortetracycline (CTC). Based on the recognition property of the multiple tetracycline imprinted polymer (MIP-1), the polymer was applied in affinity membrane extraction as a class-selective adsorption phase to remove tetracyclines residues from water. For this purpose, the ground MIP was incorporated in a plasticized poly(vinyl chloride)-membrane by casting method. Affinity separation of the obtained membrane was evaluated for the extraction of tetracycline and its analogs (CTC, OTC or DC) in aqueous solutions by a dialysis method. The membrane exhibited significantly stronger extraction ability towards tetracycline and structurally related compounds than a "blank" membrane having a non-printed polymer (NIP) as the adsorption phase. The result of these membrane extraction studies also indicates that the drug saturating at the receptor sites of MIP (deposited in membrane) faster will also be released into the receptor chamber faster. These affinity membranes were able to extract tetracyclines from water at all pHs, the highest selectivity being shown at pH 7 of the feed solution, which gives the lowest flux of the drug. Moreover, presence of salt in the feed solution increases the release of tetracycline bound in membrane. The results of the present study show that imprinting simultaneous with TC and TC degradation products formed in situ as a mixture template generates the group selectivity towards tetracyclines for the polymeric material. High affinity to a class of tetracycline of the membrane fabricated with this receptor, together with its fast and simple MIP fabrication, provides good possibilities for its application in separation processes of tetracycline antibiotics, which often contaminate the aqueous environment.

Keywords: Molecularly imprinted polymers; Affinity membrane; Tetracyclines; Poly(vinyl chloride)

1. Introduction

Tetracycline (TC) and analogs such as doxycycline (DC), chlortetracycline (CTC) and oxytetracycline (OTC) are antibiotic drugs that are still commonly used in veterinary medicine (Fig. 1). In recent years, concerns have been raised regarding the public health impact of the occurrence of these antibiotics in the aquatic environment [1–3]. There are indications of increased bacterial resistance in wastewater from hospitals and pharmaceutical industry [4–7], and this is also causing concern. Thus, it is necessary to develop reliable methods for the removal of these drugs from water supplies.

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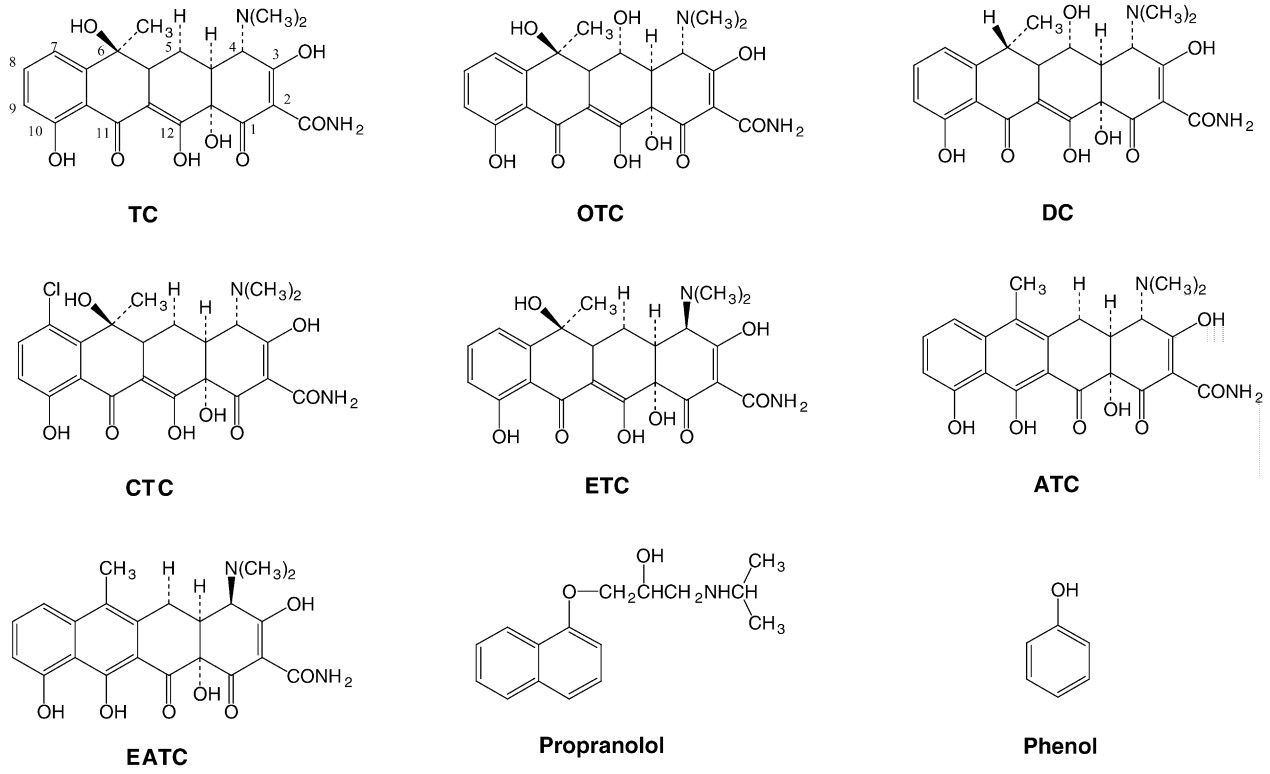


Fig. 1. Chemical structures of the tetracyclines and other substrates used in this study.

Affinity membrane extraction has been used as a technique to isolate organic and inorganic contaminants from water, both in the field and in the laboratory. Several membrane polymers containing pre-designed recognition sites have been reported to show high selectivity and fast processing for the pre-concentration or removal of the specific molecules from natural liquid waste [8,9] and from air samples [10,11]. Molecularly imprinted polymers (MIPs) as new sorbents for affinity membrane extraction are receiving increased attention, due to their high selective recognition ability [12–14]. The production of this kind of selective receptor involves the pre-organisation of the polymerisable functional monomer molecule around the targeted template by covalent or non-covalent interactions, followed by cross-linking polymerisation to stabilise this arrangement in the three-dimensional polymeric matrix. Template removal then yields a polymer matrix containing randomly distributed, immobilised functional monomers with cavities that are complementary to the template, and this matrix can be used to selectively bind the template [15–18]. The incorporation of molecularly imprinted polymer in a membrane can be carried out in three main ways: in situ polymerisation of the MIP-based membrane [19,20]; grafting a layer of imprinted polymer on to the surface of a support membrane [21,22]; or casting the MIP into membrane [23]. The last method, ligand casting, can be advantageous in the preparation of affinity membranes as it allows very fast and simple MIP fabrication with polymer support. In addition, the synthesis protocol and the recognition ability of the MIP may be established prior to the membrane fabrication. Thus, this method was chosen in the present study for the preparation of affinity membranes having a MIP as selective receptor.

In molecular imprinting, specific recognition for an artificial receptor can be created either by a single-template approach [24] or a mixture-template approach [25–28]. In single-template approach, a MIP with pre-defined single activity is produced, and this activity can be shown with the template molecule or with other compounds having similar structures. However, with a mixture of such compounds, it is not possible for a single-template MIP to recognise them all, because it is not feasible to produce a MIP with such a broad range of activity. Likewise, the use of a mixture of single-template MIPs is labor intensive, since several MIPs have to be synthesised and their recognition abilities standardised. By contrast, the multiple-template imprinting approach offers a rapid and easy means capable of providing multiple selectivity for a MIP. When making a MIP with different combination of templates, a selectivity pattern can be generated, since the same imprinting mechanism is shared by all the templates. Hence, there is broad recognition of different compounds. For generating molecular selectivity towards the antibiotic tetracycline by molecular imprinting, tetracycline is required as single template. However, bulk supplies of TC often contain traces of such impurities as epitetracycline (ETC), epianhydrotetracycline (EATC) and anhydrote-

tracycline (ATC) (Fig. 1), arising from the degradation of tetracycline under non-neutral conditions, especially upon heating. Therefore, in the present work, we have studied the possibility of creating a multiple-recognition MIP by using a mixture of TC and its TC degradation products (formed in situ) as the multiple templates. This MIP has also been incorporated into affinity membranes for the selective extraction of TC and its analogs (DC, CTC and OTC) from aqueous solutions.

2. Experimental

2.1. Chemicals and apparatus

All solvents used were analytical grade and dried with molecular sieves before use. Working standard solutions were prepared daily. All solutions were stored at 4 °C in a refrigerator. Tetracycline hydrochloride, tetracycline oxytetracycline, doxycycline and chlortetracycline were purchased from Sigma (St. Louis, MO, USA). Ethylene glycol dimethacrylate (EDMA), methacrylic acid (MAA) and propranolol hydrochloride were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Epitetracycline, epianhydrotetracycline and anhydrotetracycline and 2,2′-azobis(isobutylonitrile) (AIBN) were obtained from Janssen (Geel, Belgium). MAA and EDMA were purified by extraction with a 10% aqueous $CaCO_3$ solution, washing with water, drying over anhydrous sodium sulfate and subsequent distillation under reduced pressure. The UV used was a Hewlett–Packard diode array spectrophotometer Series 8452A (Hewlett–Packard, CS, USA). Fluorescence measurements were made on a LS50B Perkin Elmer luminescence spectrometer equipped with a 150 W xenon lamp (Perkin Elmer, CT, USA). C, H, N analysis of polymers was performed on the CE Instrumentals Flash 1112 (Milan, Italy).

2.2. Preparation of the imprinted polymer

Two types of imprinted polymer, the multiple tetracycline imprinted polymer (MIP-1) and the single tetracycline imprinted polymer (MIP-2), were prepared and validated with regard to their recognition. The template exploited in preparing the multiple tetracycline imprinted polymer was an artificial mixture of TC and its TC degradation products and used as free base. This was prepared by heating a solution of tetracycline in 10% aqueous sodium hydroxide at 60 °C for 15 min and subsequently purified by the extraction with chloroform washing with a concentrated sodium chloride solution and drying with anhydrous sodium sulfate before removing chloroform. The component of the template mixture was then analysed by HPLC, as described previously [29]. Moreover, the quantity of TC and its TC degradation products in the template mixture was determined by reference to a calibration curve using the standard solutions. The composition was found to be TC 60%, ETC 34.2%, EATC 4.3% and ATC 1.1%. For the single tetracycline imprinted polymer, tetracycline free from degradation products was used, as free base. Due to the low solubility of the templates in non-polar solvents, a mixture of acetonitrile and benzyl alcohol was used as the porogen.

In a typical preparation of the imprinted polymer, 9.95 g (0.05 mol) EDMA, 1.05 g (12.2 mmol) MAA, 1.24 g (2.8 mmol as equivalent to TC) template and 0.12 g (0.7 mmole) AIBN were dissolved in a mixture of acetonitrile and benzoyl alcohol 25 ml (3:2, v/v) in a glass vial (60 ml). The mixture was then degassed under vacuum, sonicated for 5 min, purged with nitrogen for 5 min, and polymerised at 40 °C for 18 h. The resulting solid polymer was ground in a mechanical mortar and passed through a 100 μm mesh sieve. The powdered polymer was extracted in a Soxhlet apparatus with 10% acetic acid in acetonitrile (300 ml) for 5 days, and then with acetonitrile (300 ml) until no tetracycline was no longer detectable by UV (360 nm). The polymer was then dried in vacuum overnight. The amount of the template remained in polymer after washing was analysed by elemental analysis. The non-printed polymer (NIP), used as the control, was obtained by the same procedure as for the MIP but omitting the template mixture.

An approximation of the double bond conversion of polymerisation was obtained by estimating the elemental content of hydrogen atom in the monomers that reduces after polymerisation. The gravimetric yield of polymerisation (G) was calculated according to the following relationship from the results of elemental analyses: G (%) = total of polymer (g)/total of monomer charged (g) × 100.

2.3. Determination of the rebinding ability of the imprinted polymers under equilibrium conditions

The recognition ability of the two imprinted polymers was examined by batch rebinding, using NIP for the parallel control experiments. In a typical binding assay, the powder polymer (100 mg) was added to 5 ml of an acetonitrile or aqueous solution containing 5 mM of each analyte of interest or to acetonitrile or distilled water (blank), and stirred overnight at 25 °C. The polymer particles were then filtered off and the filtrate was analysed for phenol, propranolol or tetracyclines by UV spectroscopy at 270, 290 and 360 nm, respectively, and the quantity of the drug in solution was determined by reference to a calibration curve. The amount of bound drug was calculated from the difference between the concentrations of the original stock and the final filtrate. Additionally, the imprinting factor (α), representing the effect of the imprinting process, was the ratio of amount of substrate bound by MIP to that bound by NIP. Partition coefficient (K) was calculated as following: $K = C_p/C_s$, where C_p is the concentration of the analyte inside

the polymer, and C_s is the concentration of the analyte in the solution, according to the procedure described elsewhere [30].

2.4. Determination of the binding affinity of the multiple imprinted polymer by fluorescence measurements

The binding affinities of the multiple tetracycline imprinted polymer and control polymer were evaluated on 100 mg samples of polymer with either aqueous or acetonitrile TC solutions ranging in concentration from 0.1 to 1000 μM. A typical fluorescence experiment is described below. The polymer samples were shaken in a vial with 5 ml of water overnight for cleaning purpose, separated by centrifugation, and then resuspended in 1.25 ml of water in a 1 cm quartz fluorescence cell which was then sealed with Parafilm. Afterwards, the water was discarded and 1.25 ml of TC aqueous (or acetonitrile) solution was added and mixed in cuvette. In order to ensure that equilibrium binding has been reached, the fluorescence emission of the samples was followed over 1 h; a steady state was observed after 30 min. After the polymer–drug suspension was stirred and the solid particles were set at the bottom of cuvette, the fluorescence emission spectrum of the solution was measured using an excitation wavelength of 340 nm and an emission wavelength of 420 nm. The amount bounds of TC on the MIPs were quantified from the non-bound fractions. The values of amount bound were plotted against the concentrations of TC. Scatchard analysis was used to estimate the binding parameters of the polymers. Scatchard equation is as follows. $B/[TC] = (B_{max} - B)/K_D$, where B is the amount of TC bound to the polymer, B_{max} is the apparent maximum number of binding sites, K_D is the equilibrium dissociation constant, and [TC] represents the equilibrium concentration of TC. K_D and B_{max} were determined from the slope of the straight line and the intercept of the Scatchard plot, and the association constant (K_a) value was obtained from reciprocating of K_D value, according to the procedure described elsewhere [31]. The average data of triplicate independent results were recorded.

2.5. MIP fabrication with polymer membrane support

In this experiment, the multiple tetracycline imprinted polymer was applied in affinity membrane extraction as a class-selective adsorption phase. The membranes were prepared from a THF solution containing the required components. A poly(vinyl chloride) with dibutyl phthalate plasticizer (5%, w/w) was adopted as the membrane matrix and MIP or NIP particles were incorporated as a solid adsorption phase in that membrane. In a typical membrane preparation, 500 mg of PVC powder was dissolved in 6 ml THF, and 1 ml of dibutyl phthalate and 500 mg of polymer particles (80–100 μM) were added. The mixture solution was poured into a petridish (9.0 cm diameter). The organic solvent was allowed to slowly evaporate at room temperature for 3 days before use. With this method, membranes with thickness of 0.23–0.25 mm were obtained. The "blank" membrane was prepared by using NIP in place of a MIP.

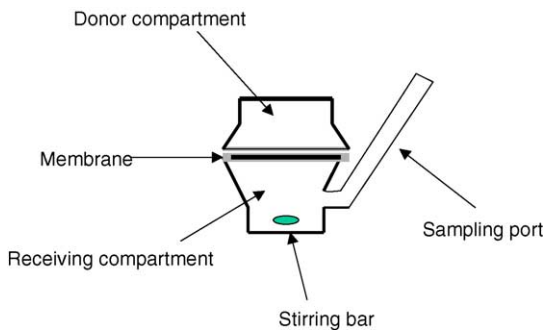
2.6. Affinity membrane extraction

Affinity extraction of MIP-based membrane for TC and its analogs was performed by a dialysis method using a vertical Franz-type diffusion cell (see Scheme 1). The initial concentration of TC and its analogs was 1 mM. A membrane (exposed area 2.17 cm^2) was mounted between two chambers of the Franz diffusion cell. The volume of the donor chamber was 1.0 ml and the volume of the receiving chamber was 2.5 ml. The solution of the drugs in deionized water was placed in the donor chamber. Either pH 7.4 phosphate buffered saline (ionic strength = 0.27) or distilled water (when the effect of buffer pH was being studied) was placed in the receiving chamber. The drugs diffuse into the receiving chamber through the membrane due to the concentration gradient between the chambers. Drug release was measured by taking samples from the receiving chamber at appropriate time intervals for 48 h. The volume of the sample withdrawn was replaced by the same volume of the medium. All the tests were performed in triplicate.

The drug concentrations in the samples were determined by UV spectroscopy at 360 nm. The amounts of drug diffused were calculated from the concentrations measured in the receiving chambers. The cumulatively permeated amounts (millimoles) were calculated and plotted against time. The flux J (μmol m^{-2} h^{-1}) is defined by

$$J = QA^{-1}t^{-1}$$

where Q (mmol) is the amount of analyte permeated, A (m^2) is the effective membrane area, and t (h) is the time. The selectivity of the extractions is defined as the ratio of permeation flux through MIP-based membrane to that through the blank membrane. It is noteworthy that since the binding of TC by the membrane is reversible, the membranes could be reused by simply washing with distilled water after each experiment, giving reproducible and consistent results with the reused membrane.



Scheme 1. An illustration of Franz-type diffusion cell used in this study.

3. Results and discussion

3.1. The recognition ability of the imprinted polymers

In this investigation, the multiple tetracycline imprinted polymer was prepared by thermal polymerisation using a mixture of TC and its TC degradation products formed in situ as the multiple template, by a procedure reported previously [32]. Methacrylic acid was employed as a functional monomer such that the acid group of the monomer interacted with the hydroxyl, amine and amide groups of the print molecule. The most commonly used bi-functional monomer, EDMA was used as a cross-linking monomer and AIBN was an initiator. The polymerisation was carried out at 40 °C, which is lower than that (60 °C) described by Vlatakis et al. [32], in order to reduce the degradation of the print molecules during polymerisation. Since AIBN generally provides a good reaction rate above 60 °C, polymerising at 40 °C may result in a low double bond conversion of MAA/EDMA in the bulk free radical polymerisation due to the slow decomposition of AIBN. From the result obtained, 20% of double bond conversion of the monomers and 88% of *G* were obtained at the chosen polymerising temperature.

Recognition mechanisms based on electrostatic interactions such as hydrogen bonding are obtained only in non-aqueous and weakly polar organic solvents. Here, the interactions between the functional monomer and the template molecules during polymerisation were medium in strength in aqueous medium used for the investigation of the rebinding capability of the MIPs, as the polymer would be applied in this type of medium. Commonly, in aqueous medium, the hydrophobic effects of the MIPs get more dominant, leading to an increase in non-specific adsorption of polymer [33]. Therefore, the rebinding capability of the multiple tetracycline imprinted polymer under aqueous conditions was verified by the batch binding experiment. To clarify the recognition ability of this polymer, the equilibrium binding of the polymer prepared with tetracycline free from degradation products as the template was also determined in this experiment. Table 1 lists the amounts of TC and its analogs bound on MIP-1 or MIP-2 in aqueous media under equilibrium conditions. It is evident that the amounts of TCs bound by the imprinted polymers in aqueous medium are much higher than amounts bound by the control polymer (NIP). This indicates that the presence of the template in the imprinting process does impart recognition ability to the MIPs. The small amount of binding by the NIP in aqueous medium indicates non-specific sorption by this polymer. Also, the binding efficiency of TC and TC analogs with the MIPs or control polymer was evaluated in acetonitrile. The amounts of TC or TC analogs bound on the MIPs or the NIP decreased, but the selectivity of binding to both MIPs improved (see Table 2). This result indicates that in acetonitrile, the specific interaction of the MIPs with TC or analogs is stronger than in aqueous medium. Also, the value of partition coefficient of the MIPs and NIP in water was higher than that in acetonitrile for all substrates, as shown in Tables 1 and 2. This may be explained that the hydrophobic repulsion between TC or analogs and solvent will be stronger with more polar solvent; hence, more TC or analogs is adsorbed by polymer [30]. The results obtained demonstrate that the selectivity of the MIP is maintained in an aqueous medium, although there is non-specific sorption onto the polymer. Thus, the application of polymer under aqueous condition remains possible.

A series of TC analogs (CTC, OTC, DC) and non-related compounds (phenol, propranolol) were used to establish the recognition range of the polymer prepared by using a mixture of TC and its degradation products as template. The results reveal that the MIPs bind more effectively to closely related template compounds than simple aromatic compounds (phenol and propranolol), as shown from the values of binding amounts in Table 1. This suggests the selectivity of polymer for TC compounds under aqueous condition. Generally, the amount of TC and analogs bound on the polymer imprinted with the multiple TC template compounds was relatively lower than that on the polymer imprinted with the single TC template, indicating the lower affinity of the former polymer to TC and analogs.

Moreover, the obtained data in Table 1 were transformed to cross-reactivity (CR) values by using following equation: CR for MIP-1 = α-$1_{analog}/\alpha$-1_{TC} and CR for MIP-2 = α-$2_{analog}/\alpha$-2_{TC} for MIP-2, where α-1_{TC} is the imprinting factor of MIP-1 for TC (template) and α-2_{TC} is the imprinting factor of MIP-2 for TC (template), respectively, and α-1_{analog} is the imprinting factor of MIP-1 for the analogs and α-2_{analog} is the imprinting factor of MIP-2 for the analogs. As seen in Fig. 2, the values of cross-reactivity of MIP-1 for adsorption of TCs were similar. This indicates that molecular imprinting

Table 1
Equilibrium binding and partition coefficient of the MIPs and NIP in water

Substrate	Partition coefficient			Amount bound (μmol/g polymer)			Imprinting factor (α)	
	MIP-1	MIP-2	NIP	MIP-1	MIP-2	NIP	MIP-1	MIP-2
Tetracycline	31.4	72.8	13.2	2.96	6.15	1.49	1.74	4.13
Doxycycline	36.2	283.3	16.2	3.73	22.79	1.82	2.05	12.52
Chlortetracycline	42.5	113.8	14.5	3.79	7.89	1.41	2.71	5.60
Oxytetracycline	25.7	80.9	10.1	2.38	6.21	1.02	2.33	6.08
Propranolol	0.4	7.7	0.4	0.06	0.13	0.06	1.03	2.12
Phenol	0.4	0.5	0.4	0.21	0.23	0.22	0.94	1.03

Table 2
Equilibrium binding and partition coefficient of the MIPs and NIP in acetonitrile

Substrate	Partition coefficient			Amount bound (μmol/g polymer)			Imprinting factor	
	MIP-1	MIP-2	NIP	MIP-1	MIP-2	NIP	MIP-1	MIP-2
Tetracycline	8.7	23.7	4.5	0.98	2.45	0.51	1.92	4.80
Doxycycline	15.4	49.8	5.0	1.62	4.89	0.49	3.31	9.98
Chlortetracycline	6.1	12.5	1.9	0.59	1.21	0.18	3.28	6.72
Oxytetracycline	11.02	14.5	3.6	1.11	1.46	0.36	3.08	4.06
Propranolol	ND	ND	ND	ND	ND	ND	–	–
Phenol	ND	ND	ND	ND	ND	ND	–	–

ND is not detectable.

with the mixture template provides the MIP with selectivity to TC analogs. The polymer prepared using TC free from degradation products (MIP-2) as the print molecule showed considerable high cross-reactivity for DC and modest selectivity for other TC derivatives, as shown in Fig. 2, indicating the recognition ability of polymer to the specific molecule, although this specificity was achieved for the structural analog instead of the imprinted species. Also, Cai and Gupta [30] have recently studied the binding of TC to a TC-imprinted polymer at 30 °C, and report a value of 2.23 mg/g polymer (6.61 μmol/g), a value very similar to that obtained in the present study.

Interestingly, the selectivity shown by the single tetracycline imprinted polymer indicates that the absence of the hydroxyl group at C-6 of TCs is of great importance for recognition, as shown in the large differences in the binding selectivity values between DC and other TC derivatives (CTC and OTC), where the template species contains a hydroxyl group at C-6. The multiple tetracycline imprinted polymer, which was prepared identically, also gave substantially high selectivity for DC (118% cross-reactivity). With respect to the stability of TC, the hydroxyl of TC at C-6 rapidly dehydrates at high temperature or in acidic pH [34]. Since the polymerisation procedure of the imprinting was carried out with thermal polymerisation, even at low temperature (40 °C), it may be that the TC used for imprinting dehydrates (and/or epimerizes) at this temperature during the prolonged polymerisation process. Hence, the hydroxyl group at C-6 of the template cannot position its pendant inside the polymer, hence a better fit of DC in the polymer than the original TC template. The recovery of template after polymerisation of both MIPs was investigated and this was found to be about 99.0%. The identity of the template after polymerisation was also determined using HPLC–UV. It was found that at least three degradation products were detected either from MIP-1 or MIP-2 sample. Degradation products of TC such as ATC (56%), EATC (28%) and one unknown component (∼15%) were found with MIP-1 sample, but TC itself was not detected. For MIP-2 sample, ETC (67%), ATC (21%), TC (7%) and EATC (5%) were found. These results indicate the possibility of changing component of the templates during polymerisation and the reaction of epimerization or dehydration possibly involves with the decomposition.

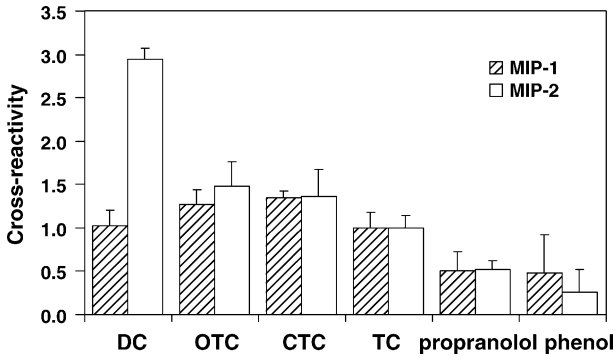


Fig. 2. The cross-reactivity of the multiple tetracycline imprinted polymer (MIP-1) and the single tetracycline imprinted polymer (MIP-2) for adsorption of TC derivatives and non-related TC in aqueous medium. Each value represents the average of three independent measurements for all compounds except DC, for which an average of six independent measurements were used.

When the chemical structures of the template compounds of the multiple-template imprinting are considered (Fig. 1), two major compounds in the mixture template, TC and the ETC degraded product (total amount = 94.2%), have a tetracyclic ring and binding groups such as –OH groups at C-3, C-10 and C-12, the same as TC analogs. Two impurities ATC and EATC, which are minor components in the mixture template (total amount = 5.8%), possess a different aromatic ring system and functionalities at C-6 and C-12. This demonstrates that the selectivity generated with mixture of TC and its degradation products refers to similarity of the shape complementary cavities for the compounds composed in the mixture template. In fact, the selectivity pattern given by imprinting with a mixture of similar compounds cannot be pre-defined and also it is difficult to explain the relationship between structures of TCs with selectivity profile obtained. This is because the decomposition of the template molecules during the imprinting process may be occurring.

The results of the present study show that it is possible to produce an artificial receptor with multiple-activity towards TC compounds by template imprinting using a mixture of TC and its degradation products as templates. The recognition property shown by the multiple tetracycline imprinted polymer may be of use as class selective adsorption phase in affinity membrane extraction of TCs, and this possible use was investigated in further experiments (see Section 3.3).

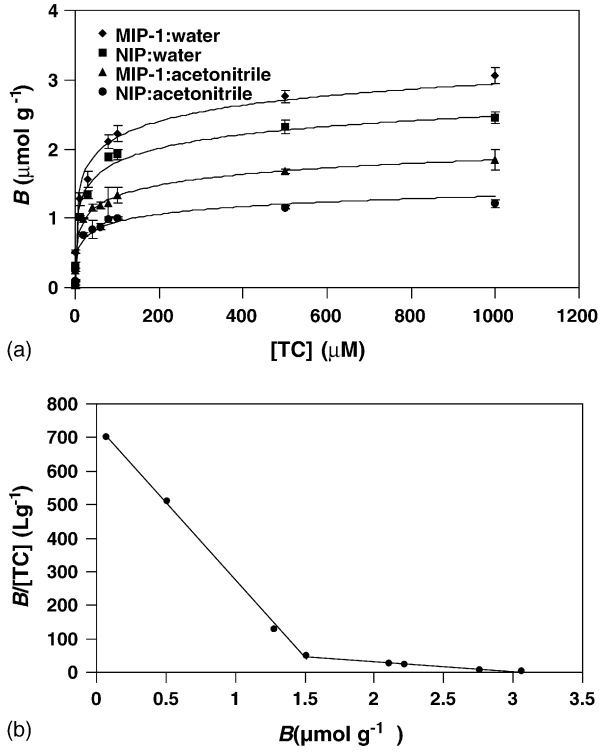


Fig. 3. (a) Binding isotherm for TC on MIP-1 in aqueous and acetonitrile media at room temperature. Each value represents the average of three independent measurements. (b) Scatchard plot of MIP-1 for TC.

3.2. Binding affinity of the MIP by fluorescence measurements

The binding affinity of the multiple tetracycline imprinted polymer was evaluated in binding experiments using fluorescence spectroscopy as an analysis method. Fig. 3a shows the binding isotherm of adsorption of TC on MIP-1 or the control polymer in aqueous and acetonitrile media. As seen, the binding amount increases gradually with the increase of the concentration of TC in the initial solution for polymers. The Scatchard plot reveals two lines with different slopes within the plot corresponding to two classes of binding sites of MIP-1 (see Fig. 3b). Table 3 shows K_a and B_{max} of the higher and lower affinity binding sites of MIP-1 and the control polymer in aqueous and acetonitrile media. The results obtained indicate that the binding affinity of the polymer for TC was higher in water than that in acetonitrile, but the binding selectivity shown towards the TC was higher in acetonitrile. This is due to lower non-specific adsorption of TC onto MIP-1 in acetonitrile. Although any non-specific interactions can be reduced in acetonitrile, the MIP is required for use in aqueous media for the proposed application.

3.3. Selective extraction capability of MIP casting PVC-membrane

Polymer PVC is one of the materials most commonly used as membranes for solid-phase extraction [35]. Due to its low polarity, it is suitable for attracting organic compounds of relative low polarity from aqueous solutions. It also possesses low compactness, which enables it to attract analyte molecules at high speed. Additionally, PVC has good stability in aqueous solutions. With the advantages of PVC for membrane extraction, though it has limitation of polarity, we choose it as a polymer membrane substance for our affinity membrane extraction system. To facilitate the transportation of the TC molecule in the membrane, dibutyl phthalate was included to plasticize the PVC membranes, which additionally gave them a better mechanical strength.

For this part of the study, the affinity membrane extraction based on co-applying a MIP adsorption phase with the plasticized PVC-membrane was investigated for removal of TC and its analogs from aqueous solutions. The MIP powder is casted into the pores of membrane and a co-polymerisation of a polyvinyl chloride is performed. The proposed MIP membrane can be considered as membrane adsorbers whose separation efficiency mainly relates to MIP binding capacity, as selectivity is caused by specific adsorption [36]. The template binding to MIP sites in the membrane can be coupled with selective transport through the membrane, hence enabling the facile and selective extraction of TC and its analogs from the water sample into a receiving chamber. Therefore, the interaction of TC and its analogs with a MIP, whose recognition ability was already established, would be shown by the permeability of these compounds in the membrane. The recognition properties of the fabricated membranes were tested by measuring their capability to extract tetracycline or its analogs from aqueous solutions using dialysis experiments. The permeation of the drugs into the receiving chamber of Franz-type diffusion cell was therefore measured, when the feed solution contains either TC or one of its analogs as single compounds. The result reveals that the TCs permeated through the MIP-based membrane at a considerably greater rate than through the NIP-membrane, as shown in Fig. 4.

Table 3
Binding parameters of polymers

Polymer	Solvent	High binding site		Low binding site	
		K_a (mM^{-1})	B_{max} (μmol/g)	K_a (mM^{-1})	B_{max} (μmol/g)
MIP-1	Water	4.49×10^5	1.59	2.56×10^4	3.07
NIP	Water	2.73×10^5	1.44	3.63×10^4	2.48
MIP-1	Acetonitrile	2.48×10^5	1.24	2.32×10^4	1.87
NIP	Acetonitrile	1.02×10^5	1.05	4.89×10^4	1.22

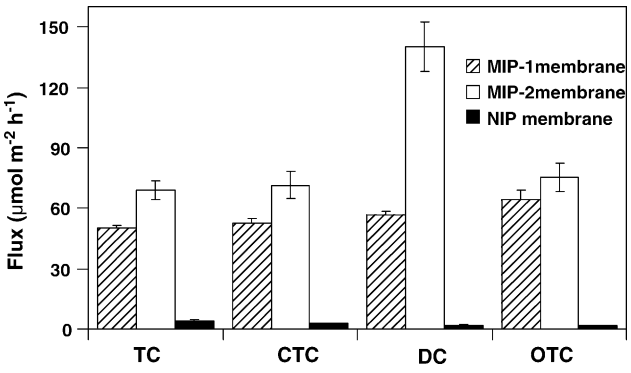


Fig. 4. The flux of extraction of TC and TC analogs in water by a MIP membrane or a blank membrane into a receiving chamber containing pH 7.4 phosphate buffer at 25 °C. Each value represents the average of three independent measurements.

The background release through the blank (NIP) membrane may be caused by weak non-specific sorption by the polymer materials (MIP and PVC). Moreover, the desired selectivity patterns of extraction for the individual TCs were demonstrated.

PVC can attract *TC* molecules, since hydrogen bonds can be formed between polar groups of the drug and the PVC. PVC is known to bind alcohols well [37]. As shown from flux values of diffusion in Fig. 4, MIP-2 membranes exhibit higher separation efficiency for all TC compounds than MIP-1 membranes. MIP-1 membrane shows slightly higher selectivity to TC analogs rather than TC itself, while MIP-2 membrane demonstrates compound-specific rather than group-specific. This observation is similar to that seen with MIP alone. Although the differences in flux between the different compounds are not in all cases significant, the recognition selectivity of MIP-1-based membrane for TC compounds can be compared as OTC > DC > CTC > TC. This pattern is different from that of the powdered MIP as shown by batch rebinding assays (Table 1). This difference may be interpreted as arising from different conditions used in the two experiments, such as concentration or non-equilibrium conditions versus equilibrium conditions. The lower extraction obtained for TC compared to TC analogs in the case of MIP-1 membrane may be due to the nature of TC itself, perhaps having a lower diffusion ability into the plasticized PVC-membrane. In the literature [12,36], some of mechanisms are proposed for conferring selectivity of transport to MIP membrane pores and/or selective channels. According to that, the typical behavior of a MIP membrane can be explained either by facilitated or retarded permeation. In this case, the permeability of the MIP membrane prepared by a casting method was found to be facilitated transport mechanism, involving preferential sorption of the template together with the availability of a better diffusion path. The preferential release of the template molecule is attributed to the saturation of the template binding in the MIP membrane, as this can occur with membrane extraction having slow filtration rate. However, it is not easy to explain the precise mechanism of permeability of the MIP membrane. Further investigation in membrane structure as well as membrane porosity may be necessary to the deeper understanding in selective affinity and permeability of the MIP membrane.

The present study demonstrates that the selective extraction of TC and analogs from water sample is feasible with the use of the membrane co-applied with the MIP as a selective receptor. MIP-1 membrane gives the flux of extraction of TCs about 50 μmol m^{-2} h^{-1} while the flux obtained from MIP-2 membrane for the extraction of DC is as high as 140 μmol m^{-2} h^{-1}. Furthermore, the use of these polymer membranes in affinity separation has several advantages, such as consistency of membrane properties and physical stability of membrane in aqueous media. In further experiments, the effect of some working parameters on the affinity membrane extraction of TC with the use of MIP-1 as selective ligand was investigated (see next section).

3.4. Influence of pH on the affinity extraction of TC

Fig. 5 shows that the amounts of drug permeated through MIP-1 based membrane and blank membrane are sensitive to the pH of feed solution. Besides, it shows that TC flux of both MIP-1-based membrane and blank membrane is the largest at a pH value of 5.0 and was lower stepwise at pH values of 4.0, 6.0 and 7.0. TC used in this study as TC hydrochloride which has several ionizable groups such as acidic hydroxyl groups at C-3 (pK_a = 3.30) and C-12 (pK_a = 7.68) and dimethylamino group at C-4 (pK_a = 9.69) [38]. Therefore, TC may exist in solution as positively and/or negatively charged species as a function of pH. In the buffer pH 5.0 solution, TC is predominantly neutral with internal zwitter ion of dimethylamino group protonated and the hydroxyl group at C-3 ionized. TC occurs either as a zwitter ion or a cation in the buffer pH 4 solution or as anionic ion in the buffer pH 6 and 7 solutions. It is feasible that the lower flux of TC at higher pH is due to repulsion, since the MAA residues are present in a negatively charged form in alkaline solution. The results suggest that the drug in zwitter ion form can transport across the plasticized PVC-based membrane better than the

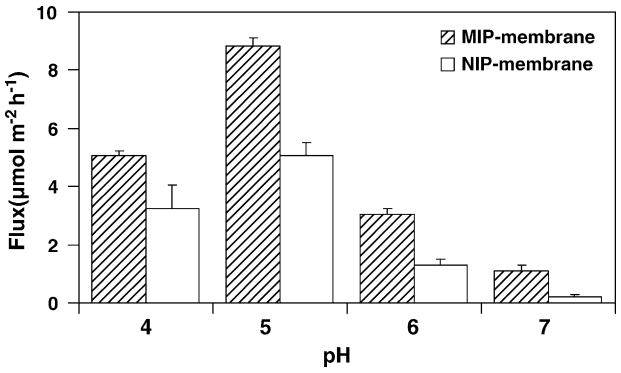


Fig. 5. The effect of pHs on the permeation of TC through MIP-1 or NIP membranes into a receiving chamber containing distilled water.

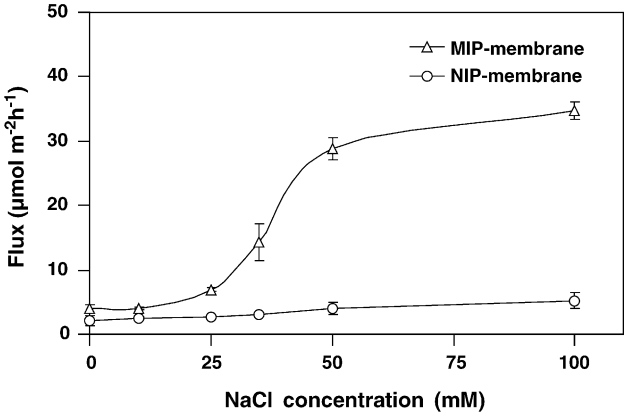


Fig. 6. The effect of the ionic strengths on the permeation of TC through MIP-1 or NIP membranes into a receiving chamber containing distilled water, using 25 mM phosphate buffer (pH 7.4) varying concentrations of NaCl as feed solution.

drug in cationic or anionic form. However, there was significant difference in TC flux obtained from MIP membrane and that from blank membrane, suggesting the selective releasing of the MIP membrane to TC, at every pH studied. Moreover, the selectivity of extraction that is presented as the flux ratio of MIP-membrane to blank membrane was slightly different for all the pHs studied, except at pH 7.0 of feed solution, which gives the lowest flux of the drug.

3.5. Influence of ionic strength of feed solution on the permeation of TC

The effect of the ionic strength of the feed solution on the affinity extraction of MIP-based membrane was examined using NaCl solutions with concentration up to 100 mM. Fig. 6 shows that the total amount of drug diffused through the MIP-based membrane was larger than that through blank membrane at every salt concentration. Also, when the salt concentration in the feed solution is increased, TC permeation by MIP-based membrane increases strongly and then starts to level off at beyond 50 mM, while that of the blank membrane hardly changes. These results indicate that the concentration of salt in the feed solution can affect the release of TC from MIP-based membrane. Also, it was found that the selectivity of extraction was greater with increasing salt concentration up to 50 mM. Above 50 mM of salt concentration, no significant change in selectivity of extraction was observed. The affinity extraction shown for TC by the MIP membrane suggests the interaction of the MIP incorporated in the membrane with TC molecule. The increased TC release by the MIP membrane with increased salt concentration may be explained by the formation of transient cross-links of Na^+ ion with the PVC chain, changing the structure of the bulk polymer membrane. This effect often occurs with PVC-based membranes [39,40]. Also, the result demonstrated that the saturation of the salt within the membrane leads to the constant release of bound drug in the membrane.

4. Conclusions

The present study has demonstrated the possibility of creating a specific recognition MIP by using a mixture of tetracycline and its degradation products formed in situ as mixed templates. The molecular recognition produced with this imprinting mainly relates to the structure of template compounds in terms of size and shape. The binding selectivity observed in the imprinted polymer prepared with the use of TC free from degradation products as the template suggests the influence of the polymerisation procedure, which is responsible for the functionality fixed on the created cavity. The selectivity of the multiple tetracycline imprinted polymer was specific to the group of structurally related template compounds, namely tetracycline, oxytetracycline, doxycycline and chlortetracycline. The application of the receptor prepared as a selective adsorption phase in affinity membrane extraction has been shown to be useful for the removal of tetracyclines, which often contaminate the aqueous environment.

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