Indian Journal of Chemical Technology
Vol. 26, September 2019, pp. 431-436

Synthesis and characterization of magnetic imprinted polymer for selective extraction of cephalexin from food matrices

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Received 19 July 2017; accepted 14 August 2019

Magnetic imprinted polymer having selectivity for cephalexin has been synthesized and evaluated for extraction of the antibiotic from selected food matrix. Fe_3O_4 magnetite has been prepared by co-precipitation of $FeCl_2.4H_2O$ and $FeCl_3.6H_2O$ in 1:2 molar ratios. Imprinted polymer has been synthesized by polymerization of methacrylic acid and ethylene glycol dimethacrylate in the presence of cephalexin over the surface of Fe_3O_4 magnetite. Selectivity of imprinted polymer over non-imprinted polymer is determined under different solvent conditions. Selectivity in both methanol and acetonitrile is found to be > 35. Selectivity is *p*H dependent in aqueous buffer solution and is in range of 2.33 to 21.3, maximum being at *p*H 5.0. Electrostatic interaction between amino group of cephalexin and carboxylic group in polymer appears to be main force contributing to selectivity of imprinted polymer. Cephalexin imprinted magnetic polymer extracted 80, 88, 63 and 78% cephalexin from water, milk, honey and egg white, respectively.

Keywords: Antibiotic extraction, Cephalexin, Imprinted polymer, Magnetic imprinted polymer, Selectivity

Cephalexin (CFX) is a beta-lactam antibiotic of cephalosporin family useful for the treatment of a number of bacterial infections and is active against gram-positive cocci and some gram-negative bacilli[1,2]. It is bactericidal and acts by inhibiting synthesis of peptidoglycan layer of the bacterial cell wall. As CFX closely resembles D-alanyl-D-alanine, an amino acid ending on the peptidoglycan layer of the cell wall, it is able to irreversibly bind to the active site of penicillin binding protein which is essential for the synthesis of the cell wall[3]. Bacterial cells expressing ß-lactamase are immune to CFX. It is used in the treatment of respiratory and urinary tract infection. CFX is also used for prevention and treatment of bovine mastitis and other infectious diseases[4]. Its use on lactating animal suffering from mastitis can contaminate milk. The presence of antibiotic in milk even at low concentration can cause problem in preparation of fermented products from such milk. Also, antibiotic residues in food can cause allergic reactions in sensitive individuals and alter gut micro biota of consumers. European Union (EU), Food and Drug Administration (FDA) and CODEX have defined permissible level of 100 ppb CFX in milk.

Imprinted polymers are fully synthetic polymers which have cavities wherein print molecule can fit and interact with functional groups or atoms of polymerized matrix. Such polymers are essentially synthesized from monomer and cross-linker, employing free radical reactions in presence of print (target) molecule.The polymerized product is solid mass and knocking of target creates cavity to which new incoming target molecule can bind. Imprinted polymer exhibits mechanical and thermal stability. Thus, the polymers can be stored at room temperature and can be used at higher operating pressure. The polymers are reusable. Monomer is allowed to interact with print molecule before initiation of polymerization. Binding between print molecule and polymer can be through hydrogen bond, hydrophobic, ionic bond andvander Waals interaction. These polymers are proving extremely useful for extraction of analytes prior to their quantitative analysis.

A range of monomer and crosslinker monomer[5] have been used for synthesis of imprinted polymer and choice is determined by possible non-covalent interaction between target and monomer/crosslinker. Imprinted polymers have been prepared against large number of small target molecules differing in chemical nature[5] which include antibiotics[6-11], chemical sensors[12,13], catalyst[14] and artificial antibodies[15] and to limited extent against larger-size

molecules such as proteins.Extraction of analytes using imprinted polymers require (i) incubation of sample with imprinted polymer under appropriate binding conditions, (ii) separation of imprinted polymer usually involving centrifugation step, (iii) releasing bound analytes by changing solvent conditions. Imprinted polymer can be reused after complete knocking off of analytes.

Magnetic imprinted polymers(MagIPs) are advantageous over non-magnetic imprinted polymers in terms of ease with which these can be separated out after binding to analytes. These require magnetic field instead of centrifugation for separation of polymer. These polymers have been synthesized against large number of molecules such as chloramphenicol[16], sulfonamides[17], tetracycline[17-19], oxytetracycline[20], cefquinome[6], fluoroquinolone[21], triazines[22], water-soluble acid dyes[23], bisphenol A[24], asprin[25] and β-sitosterol[26].

In the present study, synthesis and use of magnetic imprinted polymer for extraction of cephalexin from food have been described.

Experimental Section

Materials

Cephalexin, methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), oleic acid, iron (II) chloride ($FeCl_2.4H_2O$), iron (III) chloride ($FeCl_3.6H_2O$), polyvinylpyrrolidone (PVP), azobisisobutyronitrile (AIBN) were purchased from Sigma Aldrich USA. Methanol (HPLC grade), ethanol, acetic acid glacial, acetonitrile (HPLC grade) and water (HPLC grade) were procured from Hi-media India.

Food samples

Milk samples were obtained from cattle yard of National Dairy Research Institute, Karnal. Eggs were obtained from local market. Honey was obtained from Dabar India, Ltd.. These food samples were spiked with CFX stock solution (100 μg/mL) prepared in water.

Preparation of magnetic imprinted polymer

(i) Preparation of iron magnetite –EGDMA mix

Fe_3O_4 magnetite was prepared by co-precipitation method[16]and as discussed by Aggarwal *et al.*[20]. In brief, 10 mMoles of $FeCl_2.4H_2O$ and 20 mM of $FeCl_3.6H_2O$ were solubilized in 100 mL degassed water. Temperature of solution was raised to 80°C and simultaneously nitrogen gas was continuously bubbled through stirred solution. Then, 2M NaOH (40 mL) was added drop wise. The solution was further stirred for 1 h at 80°C under nitrogen gas environment and then cooled to room temperature. Iron magnetite (Fe_3O_4) was separated from supernatant with the help of external magnet and then washed 6 times with degassed water and dried at 60°C. One gram Fe_3O_4 powder was mixed with 1 mL oleic acid for 10 min. Then, 20 mMoles EGDMA was added and mixed.

(ii) Preassembly solution of monomer and cephalexin

CFX (1.0 mM) and 344 MAA (4.1 mM) were added to 10 mL methanol to allow interaction between MAA and CFX. The contents were mixed for 10 min.

(iii) Pre-polymerization mix.

Iron magnetite –EGDMA mix and preassembly solution were mixed and sonicated for 30 min.

(iv) Polymerization reaction

PVP (400 mg, average molecular weight 40000 Dalton) was solubilized in 80% ethanol (100 mL) at 60°C and the contents were purged with nitrogen gas. Then, pre-polymerization mix and 20.1 mM AIBN were added. Reaction was allowed to proceed at 60°C for 24 h. Polymer was washed several times with methanol: acetic acid (8:2 v/v) mixture, till washings were free from CFX. Polymer was further washed three times with deaerated water and dried at 60°C.

Preparation of Magnetic non-imprinted polymer

Magnetic non-imprinted polymerwas prepared similarly as MagIP, except CFX was omitted from pre-assembly solution.

Characterization of cephalexin polymers

Morphology of MagIPs was observed underscanning electron microscope (Carl Zeiss, Germany) and functional groups on MagIP surface were confirmed by Fourier transform infrared spectrometry (IR affinity, Shimadzu, Japan).

Determination of binding selectivity

Selectivity of prepared polymer was calculated by ratio of partition coefficient of CFX for imprinted and non-imprinted polymer in water, acetonitrile, methanol, 1 M NaCl, 20 mM acetate buffer (*p*H 4.0 and 5.0) and 20 mM phosphate buffer (*p*H 6.0 and 7.0)[27]. Polymers (20 mg) were incubated with 2 mL CFX (20 μg/mL) solubilized in respective solvent for 24 h at 25°C. Unbound CFX was assayed in

supernatant by measuring absorbance at 262 nm. Bound antibiotic was calculated by subtracting unbound antibiotic from total antibiotic added. Partition coefficient and selectivity were calculated as per the method described by Divya *et al.*[7].

Cross recognition of cephalexin imprinted polymer

The cross recognition of CFX imprinted polymer was determined by incubating the CFX imprinted polymer with ampicillin, cefquinome, penicillin, CFX, gentamicin and tetracycline. Forty micrograms each of antibiotic was incubated with 20 mg polymer in 2 mL, 20 mM acetate buffer (*p*H 5.0) at 30°C for 24 h. Bound antibiotic was calculated by subtracting unbound antibiotic from total antibiotic. Concentrations of ampicillin, cefquinome, penicillin, CFX, gentamicin and tetracycline were measured by recording absorbance at 325, 221, 322, 262, 420, and 320nm, respectively.

Extraction of cephalexin from food matrix

Two grams of honey or 2 mL of egg-white or 2 mL of water was diluted to 20 mL with waterand then spiked with 200 µg CFX. Diluted sample or water was mixed with 40 mg imprinted or non-imprinted polymer. Then, polymer was ten times washed with 3 mL methanol and supernatant from each washing was collected. Bound CFX was eluted six times with 3 mL 0.5M NaCl. Prior to use, imprinted and non-imprinted polymers were conditioned by treating them with methanol and water in sequence. CFX concentration in each washing or elution was monitored.

Results and Discussion

Results

Particle characterization

Yield of polymer from 344 mg MAA and 3.77 mL EGDMA was 4.2 g. MagIPs were spherical in shape and appeared like a conglomeration of porous beads (Fig. 1). The particles were of 227nm ± 52.8 (mean ± SD). Polymer exhibited peak at 534 cm^{-1}(characteristic of Fe-O bond stretching), 1120 cm^{-1} (C-O stretch), 1260 cm^{-1} (C-H stretch), 1370 cm^{-1} (C-H rock), 1450 cm^{-1}(C-H bend) and 1724 cm^{-1} (C=O stretch) in FTIR (Fig. 2). C=O stretch peak and C-H (stretch, rock, bend) peaks imply that carboxyl and methyl groups from MAA are present on the surface of polymerized matrix. Also, methyl groups from EGDMA can contribute to surface structure. Absorption peak at 1120 cm-1 suggests that EGDMA is also present on the surface[28]. Thus itcan be concluded that methyl and carboxyl groups are present on the surface of polymer including cavity.

Effect of solvent and pH

Effect of solvents viz. water, methanol, acetonitrile and 1M NaCl and *p*H (4.0, 5.0, 6.0 and 7.0) on binding of CFX to imprinted and non-imprinted polymers are shown in Table 1. The selectivity values which measures binding characteristics of imprinted polymer over non-imprinted polymer in different solvents and *p*H are also shown in Table 1. Nature of solvent markedly influenced binding of CFX to imprinted polymer and its selectivity. Binding of CFX to MagIP in methanol and acetonitrile was ≥ 65% whereas binding to non-imprinted polymer was just 5%. This resulted in high selectivity (≥35) in methanol and acetonitrile, both of being apolar solvents. In contrast, there was low binding of both imprinted and non-imprinted polymer in water as well as in 1M NaCl. This resulted in little or no selectivity in water or aqueous NaClsolution.

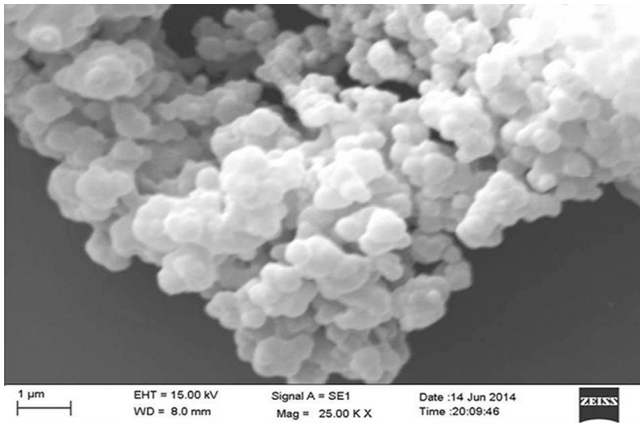

Fig 1 — SEM image of cephalexin imprinted magnetic polymer

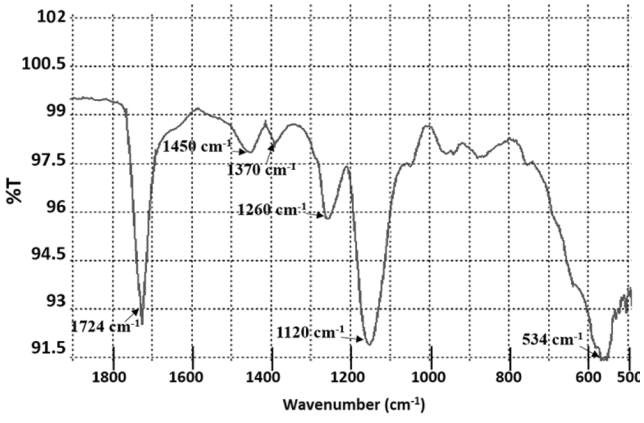

Fig. 2 — FTIR spectrum of cephalexin imprinted magnetic polymer

Table 1 — Effect of solvent on binding and partition coefficient of CFX imprinted and non-imprinted polymersandon selectivity

Solvent	Polymer	% Bound	Partition coefficient	Selectivity
Water	Magnetic Imprinted	5	5.26	1.26
	Non-imprinted	4	4.16	
Methanol	Magnetic Imprinted	66	194.11	36.19
	Non-imprinted	5	5.26	
Acetonitrile	Magnetic Imprinted	65	185.71	35.30
	Non-imprinted	5	5.26	
1M NaCl	Magnetic Imprinted	13	14.94	1.51
	Non-imprinted	9	9.89	
20mM Acetate buffer *p*H 4.0	Magnetic Imprinted	60	150	13.51
	Non-imprinted	10	11.11	
20mM Acetate buffer *p*H 5.0	Magnetic Imprinted	79	376.19	21.32
	Non-imprinted	15	17.64	
20mM phosphate buffer *p*H 6.0	Magnetic Imprinted	65	185.71	7.42
	Non-imprinted	20	25	
20mM phosphate buffer *p*H 7.0	Magnetic Imprinted	50	100	2.33
	Non-imprinted	30	42.85	

Binding of CFX to imprinted polymer or non-imprinted polymer was dependent on *p*H. Binding of CFX to non-imprinted polymer increased with increase in *p*H from 4.0 to 7.0 and was in the range from 10.0 to 30.0%. In contrast, binding of CFX to imprinted polymer was in the range of 50 to 79%., maximum being at *p*H 5.0. This has resulted in maximum selectivity at *p*H 5.0 and value was 21.32. It may be noted that selectivity in methanol and acetonitrile was similar but higher than at *p*H 5.0. Selectivity was not noted in 1M NaCl.

Cross recognition of CFX imprinted polymer

Cross reaction of imprinted polymer was checked with common antibiotics viz. ampicillin, cefquinome, penicillin, gentamycin and tetracycline in use. Binding of the antibiotics including CFX was evaluated at *p*H 5.0 in 20 mM acetate buffer. In comparison to 79% CFX binding, the binding of ampicillin, cefquinome, penicillin, gentamycin and tetracycline was between 10-20%. It may be noted that binding of CFX to non-imprinted polymer at *p*H 5.0 was also 15% and at other conditions between 4 to 30% (Table 1). Low level binding of these antibiotics perhaps arises from non-specific interaction with surface structure (other than in cavity) on polymer. This indicates CFX imprinted polymer was largely specific or exhibited low cross reactivity with other antibiotics.

Extraction of CFX from water, milk, honey and egg white

Comparative performance of imprinted and non-imprinted polymers for extracting CFX from CFX from water, milk, honey and egg white is given in Figs 3 and 4. In typical experiment, food samples were spiked with CFX and incubated with imprinted or non-imprinted polymers. Un-bound CFX was washed with methanol (eluent 1 to 10) and bound CFX was eluted with 0.5 M NaCl (eluent 11 to 16). The pattern of CFX in eluent fractions from imprinted and non-imprinted polymer was distinctly different. CFX from water, milk, honey and egg-white did bind to imprinted polymer, not to non-imprinted polymer and bound CFX could be eluted with salt solution.

Discussion

Methacrylic acid has carboxylic group and therefore can interact with target through ionic interaction if print molecule possesses positive charge (Fig. 4). Methyl group in MAA and EGDMA is capable of forming hydrophobic bond. CFX has one amino (*p*Ka - 6.8) group and one carboxylic (*p*Ka – 3.1) group, besides hydrophobic moiety. *p*Ka value of carboxylic group in MAA is 5.5[29]. Absence of selectivity in water rules out possibility of hydrophobic interaction between imprinted polymer and CFX . Presence of methyl group in cavity can

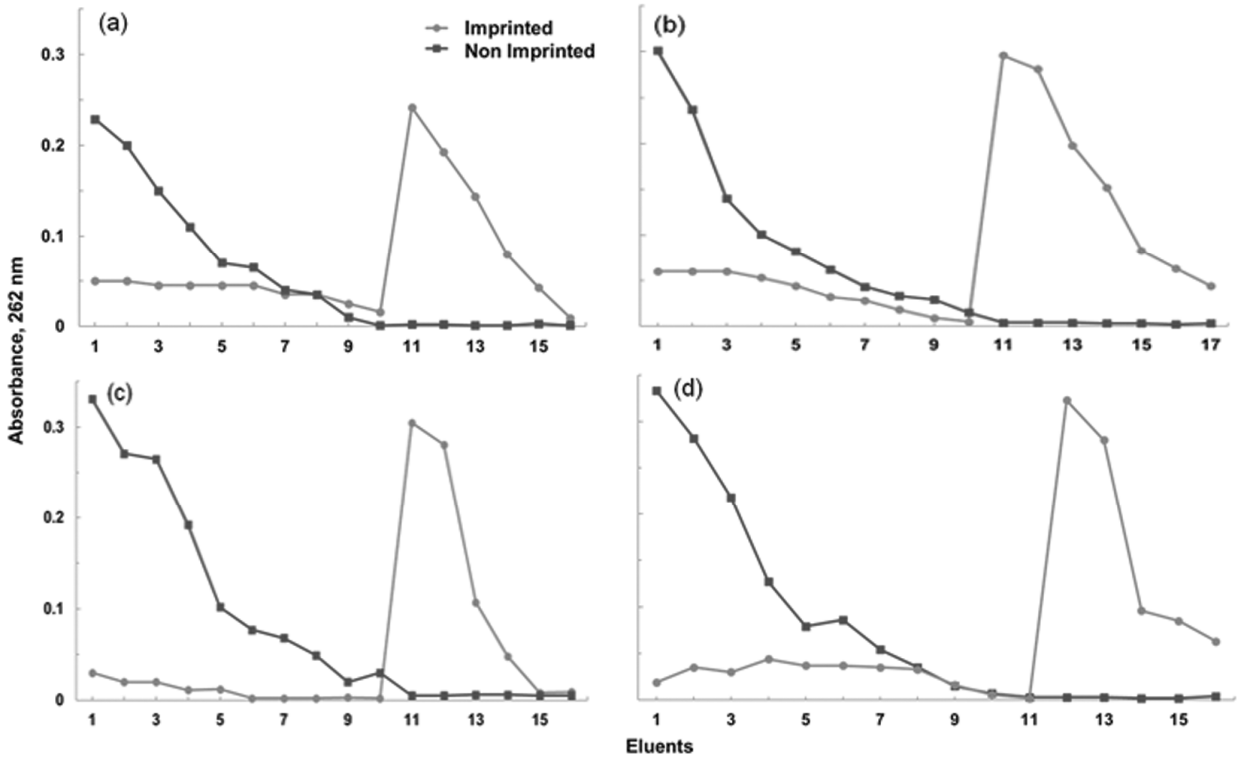

Fig. 3 — Binding and elution pattern of cephalexin from food matrix using imprinted and non-imprinted magnetic polymer. (a) Water, (b) Milk, (c) Honey and (d) Egg white

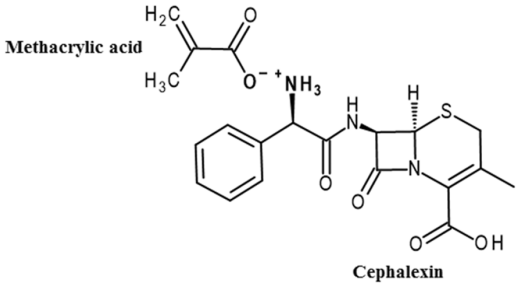

Fig. 4 — Schematic representation of interaction between methacrylic acid and cephalexin.

strengthen other non-covalent interaction. Loss of selectivity in NaCl solution points towards breakdown of ionic interaction between polymer and CFX. Hydrophobic interaction of methyl group in polymer with hydrophobic site in CFX can bring $-NH_3^+$ from CFX closure to – COO^- group in polymer. Dependence of selectivity on pH also points towards ionic interaction being major force for recognition of CFX by imprinted polymer. Maximum selectivity was obtained at *p*H 5.0. At *p*H 4.0 and *p*H 6.0, selectivity was comparatively low and was further decreased at *p*H 7.0. Change-in-*p*H results change-in-dissociation of amino as well as carboxylic group and dissociated or undissociated status of these groups will decide charge on these groups (Fig. 5). It is hypothesized that at *p*H 5.0, maximum number of CFX will interact with dissociated carboxylic group (-COO^-) from polymer through its undissoicated amino group ($-NH_3^+$). As one moves away from *p*H 5.0, the number of CFX molecules involve in such interaction will decline. Thus, it appears that negative charge on cavity surface of polymer and positive charge on CFX is perhaps crucial for recognition of CFX by imprinted polymer.

Carboxylic groups in non-imprinted polymer are also to be present on surface and theoretically these can participate in interaction with CFX. Higher CFX binding to imprinted polymer does suggest that microenvironment must be non-polar or less polar which can strengthen ionic interaction. Methyl groups from MAA and EGDMA can contribute to this environment.

The recovery of CFX from water, milk, honey and egg-white was 80.0, 88.0, 65.9 and 82.7% respectively. Characteristic peak of Fe-O bond is visible in FTIR (Fig. 2). The possibility of interacting iron (Lewis acid) with the amino and carboxyl group (both are Lewis base) in CFX cannot be ruled out because such interaction can reduce recovery.

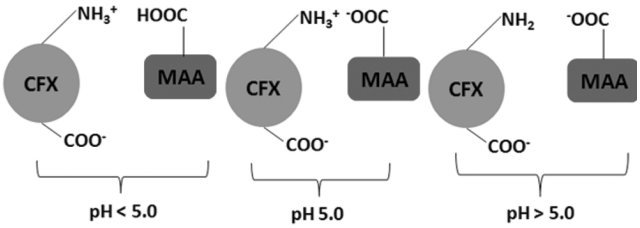

Fig. 5 — Schematic representation of dependence of interaction between cephalexin and methacrylic acid on pH.

Magnetic imprinted polymers have added advantage over non-magneticimprinted polymers as these remain in suspension while binding to target molecule and can be easily made to settle just by applying external magnetic field. Selectivity values in different solvent eventually help in defining extraction protocol.

Conclusion

MagIP retained recognition sites for CFX as well as paramagnetic properties. It is essential to evaluate imprinted polymer for binding to analyte in different solvents for optimizing extraction conditions. MagIP has inherent advantage of easy separation of bound analytes just by application of external magnetic field. These materials can also concentrate target analyte from complex biological samples for subsequent analysis.

Acknowledgements

The authors acknowledgethe financial support provided by National Agricultural Science Fund (NASF) (Grant No.30019), Government of India.

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