Towards the design of Molecular Imprinting Polymers selective to Flavonoids

A Minor Research Project submitted to UGC

By

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DECLARATION

I, SUBI JOSEPH, do hereby declare that this project entitled 'Towards the design of Molecular Imprinting Polymer selective to Flavonoids' has been undertaken by me as the Minor Research Project from UGC, I also declare that this is a genuine work and has not been submitted by me for the award of any degree, diploma, title or recognition before.

Vaikom

11/06/2015 **SUBI JOSEPH**

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Above all, I humbly solicits **Almighty** blessings for the strength and presence of mind showered upon me for completing the work successfully.

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CONTENTS

Towards the design of Molecular Imprinting Polymers selective to Flavonoids

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Abstract

Molecular imprinted polymers (MIPs) were prepared through thermal polymerisation by using chrysin as the template, 4-vinyl pyridine (4-VP) as functional monomer and ethylene glycol dimethacrylate (EDMA) as the crosslinker in the porogen of acetonitrile (ACN) and dimethyl sulphoxide (DMSO) in the ratio 3:2. The synthesised MIPs were identified by both Fourier transform infrared (FTIR) and scanning electron microscope (SEM). Systematic investigations of the influences of key synthetic conditions, including functional monomers, porogens and crosslinkers on the recognition properties of MIPs were conducted. .Besides chrysin ,a structurally similar compound morin was also employed for molecular regnition specificity teste of MIPs.

1. Introduction

The concept to develop a biomimic material capable of selectively recognizing desired molecules is being intensively discussed [1–3]. In the wake of the jump from a combinatorial method to the molecular self assembly, this field has significantly advanced recently by the so-called molecular imprinting technique [4, 5]. Described as a ‘‘from key- to-lock’’ process, molecular imprinting uses molecular self-assembly to position the groups of functional monomer around an inducible template [6, 7]. A photo- or thermal polymerization in the presence of a cross-linker is subsequently performed to fix this organized architecture. The template imprinted is then removed from the polymer, leaving behind binding sites complementary to this template in terms of the structure and function. The recognition of the binding framework constitutes an induced molecular memory, which makes the polymer capable of selectively recognizing the imprint molecule. Thus, comparable to interactions of some natural biomolecules toward their substrates, such as prepared antibody-antigen, receptor-ligand, or enzyme-substrate, the imprinted material is an antibody-like polymer capable of three dimensionally recognizing the imprint species. However, relative to these natural biomolecules, the molecularly imprinted polymer is a rigid cross-linked substance. Thus, the significant advantages of its properties include physical robustness, high strength,

resistance to elevated temperature and pressure, and inertia usually to acid, base, metal ion, and organic solvent as well. Owing to these traits, the imprinted polymer can be used not only partially as the substitute of natural biomolecules but also as substrate-selective or separation material under harsh conditions [8–11].

In the present paper we demonstrate the feasibility of preparing flavonoid imprinted and non-imprinted polymers with high specificity and selectivity towards the imprinted molecule through non-covalent approach. The primary goal of the present study is to investigate how a successful non-covalent imprint is obtained in a polar solvent (acetonitrile/DMSO) considering that most imprinting protocols require a non-polar solvent to maximise non-covalent interactions (Sellergren et al., 1997). Flavonoid was chosen as a representative target compound because they are currently the subject of extensive analysis in food samples [12–14] . Flavonoids are polyphenolic compounds . These compounds have long been recognized to possess antihepatotoxic, anti-inflammatory, antiatherogenic, antiallergic, antiosteoporotic and anticancer activities. Among the biophenols, the flavonoids are the most active compounds. The selected template is a flavonoid Chrysin. It was an attractive model for the purpose of this investigation on account of two molecular features, namely the presence of an aromatic π-electron system which renders the molecule hydrophobic and the presence of hydroxyl and carbonyl groups for intermolecular interactions with the functionalized monomers during non-covalent imprinting.

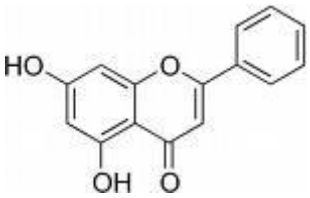


Fig. 1 5, 7-dihydroxy-2-phenyl-4H-chromen-4-one (Chrysin)

Due to the occurrence of flavonoids in natural samples in low concentrations, and because of the complexity of the sample matrices, selective sample preparation methods are necessary prior to chromatographic analysis. Preconcentration and/or isolation of compounds are frequently performed using SPE [17, 18] .In that aspect, new selective materials employing molecular recognition mechanisms may enable selective analyte isolation from complex real world matrices. This mechanism can improve significantly the method specificity and sensitivity. As such, attempts to develop suitable molecularly imprinted polymers (MIPs) for flavonoids have recently been reported. [19-26]

As can be seen in Fig. 2, the Chrysin molecules encompass functional groups that may form hydrogen bonds with the functional monomer, during the pre-organisation of monomer and template in the pre-polymerisation solution. A major limitation faced in the preparation of the MIPs was the low solubility of the Chrysin in non-polar organic solvents. The Chrysin is a polar molecule readily soluble in ACN : DMSO (3:2) mixture but, insoluble in ACN alone. The presence of polar protic solvents in the polymerisation mixture is reported to be disadvantageous for imprinting by non-covalent interactions. Such solvents hinder the formation of hydrogen bonds between the template and the functional monomers, desired for the arrangement of selective binding sites[27] DMSO was found to be the only aprotic solvent to provide adequate solubility for the flavonoid Chrysin as a template **.**A significant proportion of template may remain trapped in the highly dense polymeric network and is not used to form cavities useful for shape-selective adsorption. Finally, during the post polymerization processing (extraction of template and grinding), population of the affinity sites in the polymer matrix may be damaged. As a result, only a fraction of the high-affinity imprinted sites that combine proper size, shape, and orientation of the functional groups for the interaction with the target molecule remain available to bind the target analytes[28].

2. Experimental

2.1. Materials and Methods

5,7-dihydroxyflavone (chrysin), azobisisobutyronitrile (AIBN), 4-vinylpyridine (4-VP), ethylene glycol dimethacrylate (EGDMA), were obtained from Sigma–Aldrich (Milwaukee, WI, USA). 4-Vinylpyridine was distilled under vacuum prior to use; all other chemicals were used as supplied. Solvents used were of HPLC grade and obtained from Merck (Germany). Water was double de-ionised and filtered though a 20 _m filter (Schleicher & Schuel, Dassel Germany). Fourier Transform Infrared Spectrophotometer 8400 S Shimadzu, Japan was used to record FT-IR spectra of imprinted and nonimprinted polymers.

2.2. Preparation of MIP

For polymerization, 4-vinylpyridine was chosen as functional monomer. The template target molecule (chrysin) is not readily soluble in common porogen solvents typically used in the polymerization process. Based on preliminary solubility experiments, the following porogen solvent mixtures of DMSO/ACN 2:3 v/v were used. Excess ethyleneglycoldimethylacrylate (EDMA) was used as the crosslinker to enhance the

imprinting effect. Azo-bis-isobutyl nitrile (AIBN) was used as the initiator of the polymerization reaction (50 mg AIBN in 12.5 mL mixture DMSO/ACN 2:3). Several adsorbents were prepared from different polymerization mixtures (Table 1) at the following polymerization conditions. The prepolymer solutions were degassed with nitrogen for 5 min in glass tubes, which were then carefully sealed and heated in a water bath at60˚C for 24 h. At the end of the polymerization, the glass tubes with the polymers were crushed and the organic polymers were ground in a laboratory mortar and pestle under wet conditions (with the addition of deionized water). The solid particles were sieved through 71 and 20 lm sieves with the assistance of deionized water. The particles that passed through the 71 lm sieve were collected; the retained particles were reground. The collected polymer particle fraction was washed to remove the nonreacted monomer compounds and template as follows: The polymers were dispersed in the polymerization solvent and were sonicated twice for 30 min. Then the polymers were extracted in a Soxhlet apparatus with a mixture of methanol/acetic acid 9:1 v/v for 18 h in 30– 35 solvent cycles (each Soxhlet cycle in approximately 30 min). To estimate the effectiveness of the Soxhlet extraction, the concentrations of rutin or quercetin in the extracts were determined by UV spectroscopy. Fine particles were removed from the polymer by repeated sedimentation in methanol/water 1:1 v/v. Finally, the particles were dried under vacuum and stored at ambient temperature. To better evaluate the molecular recognition properties of the produced polymers, control nonimprinted polymers (NIPs) were fabricated using the same polymerization and protocol and postpolymerization treatment, but without addition of the template molecule in the prepolymer mixture.

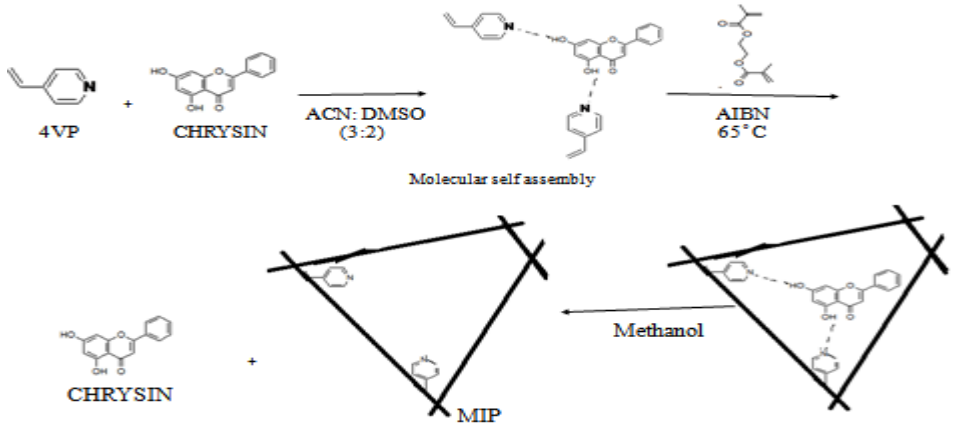


Fig 2. Technical outline for the preparation and recognition of imprinted polymers

3. Results and discussion

3.1 IR Spectra and SEM Images

The flavonoid imprinted and non-imprinted polymers were characterized by FT-IR and SEM. The surface morphology of the polymers was followed by SEM (fig.3),. it was observed that the MIP becomes more porous with cavity of smaller size. Correlating to the preparation process these can be logically a consequence of imprinting. This structure enabled chrysin to diffuse easily during the binding experiments The SEM micrograph of non-imprinted polymer (NIP) was found to be more compact with rough morphology and no cavity was observed. The compact structure indicates no specific binding sites had been created by template.

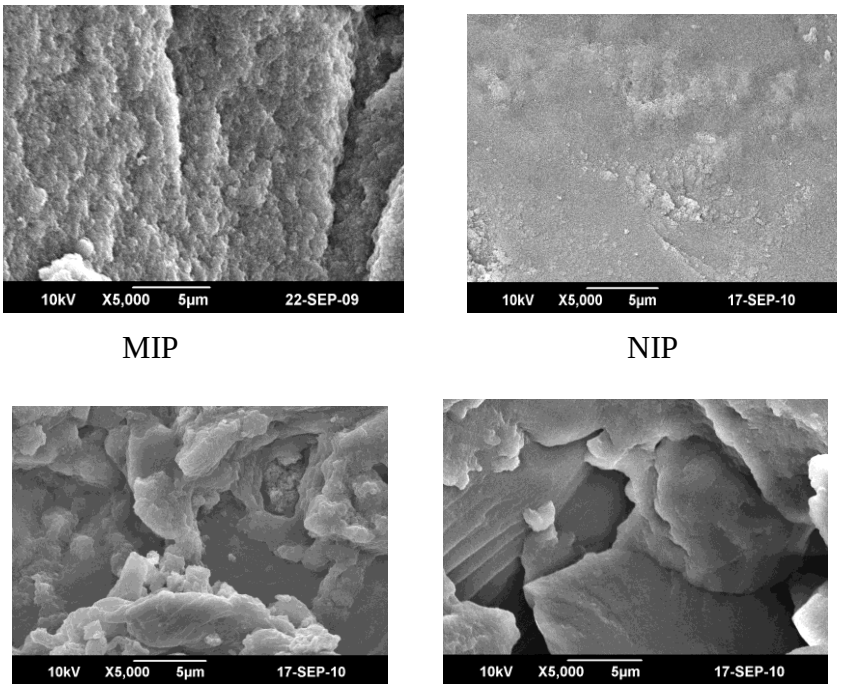


Fig. 3. SEM images of prepared materials

IR was also used to clarify the chemical structure and composition of the MIP. Vibrational spectroscopy has been used extensively to study intra- and intermolecular hydrogen-bonding interactions and was used in this study to characterize the host-guest complex The vibrational frequencies shift to lower frequencies upon hydrogen bonding and the intensity of the shifted stretching vibrations is a direct measurement of complex concentration. These changes in the spectrum due to hydrogen bonding make infrared spectroscopy a powerful tool for examining intermolecular interactions.. The absorption

peaks at 1598, 1562, and 1419 cm-1 can be assigned to the ring vibration of VP. Shift from 1562cm^{-1} to 1547cm^{-1} in bound MIP shows the hydrogen bonding of pyridyl Nitrogen with Chrysin. The strong peak at1725cm^{-1} is the stretching vibration peak of the C=O bond and a doublet at 1243cm^{-1}and 1222cm^{-1} shows the C-O stretching of the crosslinker EGDMA. After washing, the spectrum of the MIP becomes comparable to the NIP. In bound MIP peaks at 1658cm^{-1} and 3453cm^{-1} represents C=O stretching of Chrysin and hydrogen bonded –OH stretching of Chrysin respectively. As also noted, after washing, the spectrum of the MIP displays no visible difference from that of the blank polymer. This thus reveals that almost all templates are removed from the precursor, which therefore presents an advantage for the study of imprint effect.

Concentration effects

The extent of template binding increases regularly with concentration. (Fig.4) The specific binding results from the formation of recognition sites complementary to the chrysin in the shape and in the positioning of the functional groups as the effect of the imprinting process. The 40% EGDMA crosslinked MIP is having highest binding, specificity and selectivity compared to 50% and 75% MIPs. In all these cases the imprinted polymers are having highest binding compared to nonimprinted polymers.

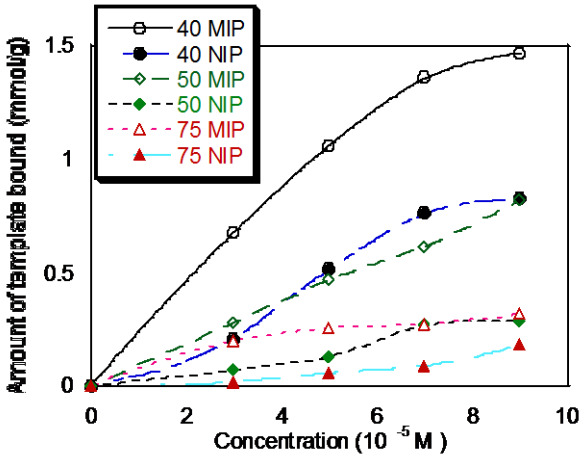


Fig.4. Variation of Chrysin binding with concentration by imprinted and non-imprinted polymers.

Effect of time

The imprinted polymers took more time for saturation of binding sites compared to the non-imprinted polymers.(Fig.5). This is because the template molecule has to be penetrated through highly crosslinked network to access the imprinted sites for binding whereas in the non-imprinted system there is no specific arrangement of the binding sites and nonspecific interactions occur at the available sites of the polymer which leads to a fast random binding of the template.

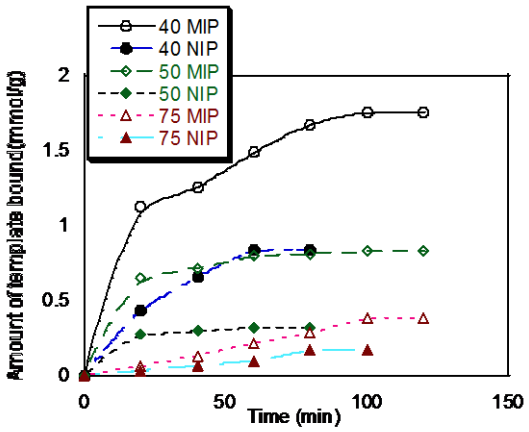


Fig.5. Effect of time on Chrysin binding by imprinted and non-imprinted polymers.

Effect of mass of polymer

A continuous increase in specific binding was observed with increasing amounts of polymer for all the imprinted polymers. (Fig.6) As the mass of the polymer increases the number of binding sites is expected to increase with the result template binding also increased. This may be attributed to the increased adsorbent surface area and availability of more adsorption sites resulting from the increased amount of the adsorbent.

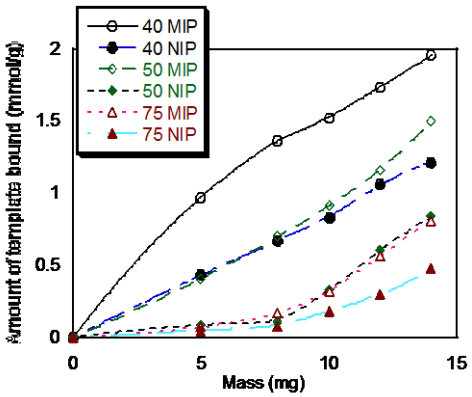


Fig.6. Effect of mass of polymer in Chrysin binding by imprinted and non-imprinted polymers,

Extent of crosslinking

The imprinted and non-imprinted polymers of Chrysin were synthesized with varying extend of EGDMA crosslinking (40%, 50% and75%) and binding studies were carried out. At very high crosslinking the polymer matrix is too rigid to allow access to the template into the site. At very low crosslinking the polymer cannot retain the binding site. We therefore wanted to know whether the degree of cross-linking can be reduced without abolishing the recognition properties of the polymer. This would make the polymer more flexible which could be an advantage for certain applications requiring thin imprinted films or membranes. At the same time it might be possible to incorporate more functional monomer into the polymer and to use more template, thus resulting in a higher binding capacity [28] . The 40% EGDMA- crosslinked system is found to be more specific. (Fig.7). The specificity of the sites was comparable for 40% and 50% crosslinked polymers, but less for 75% crosslinked polymers. With the corresponding control polymers (NIPs), low amount of non-specific binding was observed independent of the degree of cross-linking.

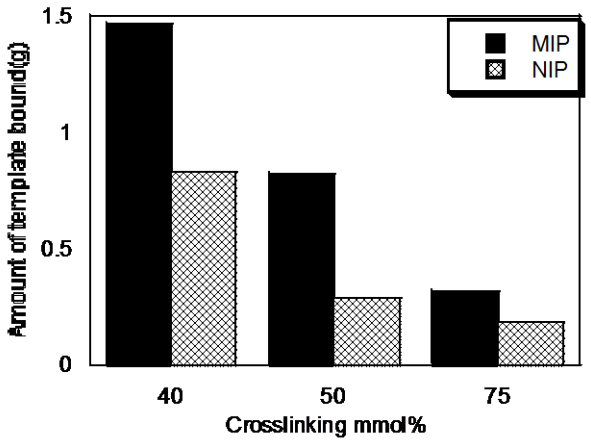


Fig7. Effect of extent of EGDMA crosslinking on Chrysin binding by imprinted and non-imprinted polymers

Solvent effects

Solvent plays an important role in the formation of the porous structure of MIPs,.The morphological properties of porosity and surface area are determined by the type of solvent , referred to as “porogen” , used in the polymerization. Porosity arises from the phase separation of the porogen and the growing polymer during polymerization.

Another important role for solvent in the formation of MIPs is the effect it has on the complexation of functional monomers with the template before , during and after

polymerization .Before (and during) polymerization , the extent of the non-covalent pre-polymer complex is affected by the polarity of the porogen solvent. Less polar solvents will increase complex formation by Le Chatelier's principle, facilitating polar non-covalent interactionssuch as hydrogen bonding. On the other hand , more polar solvents tend to dissociate the non-covalent interactions in the pre-polymer complex. The MIP recognition abilities in ACN : DMSO (3:2) were superior to all other solvents, as it resembles best the conditions used during the imprinted polymer generation **.** Methanol a polar, protic solvent usually decreases binding dramatically by forming hydrogen bonds to both polymer and template, thus disturbing interactions taking place in the binding cavities (Kim and Guiochon,2005a,b).

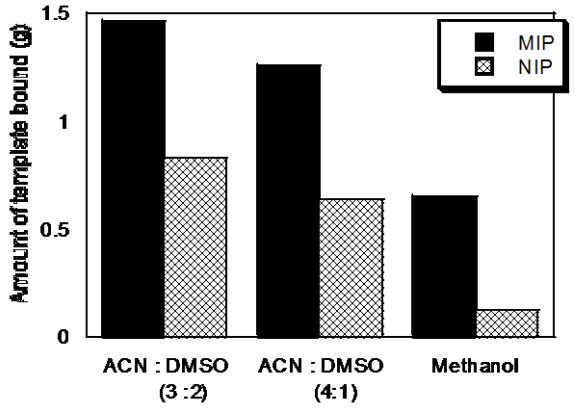
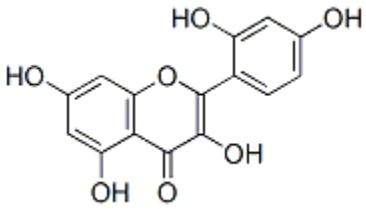


Fig 8. Solvent studies showing the high binding in ACN:DMSO (3:2)

Binding specificity of the polymers

Structurally related compounds have been used for verification of the selectivity, characterizing the recognition abilities of this novel separation material. The selectivity of imprinted polymers is based on the configuration of the binding sites and the interaction between the template and the functional groups in the sites. The selectivity of Chrysin is assessed with its structural analogue Morin.



Morin

Table:1

Monomer : Template	Selectivity Factor $\alpha_{chrysin/morin}$	
	MIP	NIP
1:8	1.32	1.09
1:4	1.46	1.33

Selectivity of MIP arises from the differences in the free energy of adsorption of one substrate verses another. The free energy of binding the substrate by the MIP is determined by :

$$\Delta G = -RT \ln Kp$$

where Kp is the partition coefficient of substrate

$$Kp = B / F$$

where B is the amount of template bound and F is the amount of template that is left free.

The selectivity of one substrate verses another is quantified by the ratio of the two partion coefficients $K_{pchrysin}$ and K_{pmorin}, which is referred to as separation factor α

$$\alpha = K_{pchrysin} / K_{pmorin}$$

The corresponding difference in free energy of binding is written as :

$$\Delta G_2 - \Delta G_1 = \Delta\Delta G = -RT \ln \alpha$$

Table :2

Template	$\Delta\Delta G$ (J / mol)
Chrysin	- 937.8
Morin	-706.7

Thus the binding of chrysin to the polymer is more thermodynamically favourable compared to the binding of morin . The value of $\alpha_{chrysin/morin}$ in this experiment showed the chrysin-imprinted polymers had special recognition ability to chrysin and implied that the imprinted polymers could be used as solid phase for chrysin enrichment Morin resembles

Chrysin in structure, but it did not exhibit high ‘‘cross-reactivity’’ on the MIP possibly resulting from intramolecular hydrogen bonding formed in morin at the test conditions[29]. More over it is not able to enter in smaller sites due to the presence of many hydroxyl group in its structure.

Monomer concentration

Monomer: template ratio also affects selectivity of the polymer.1:8 40% EGDMA crosslinked polymers of chrysin were prepared and the selectivity is compared with 1:4 40% EGDMA crosslinked chrysin polymer.The decrease in monomer : template ratio increases the selectivity of the polymer(Fig 9) .

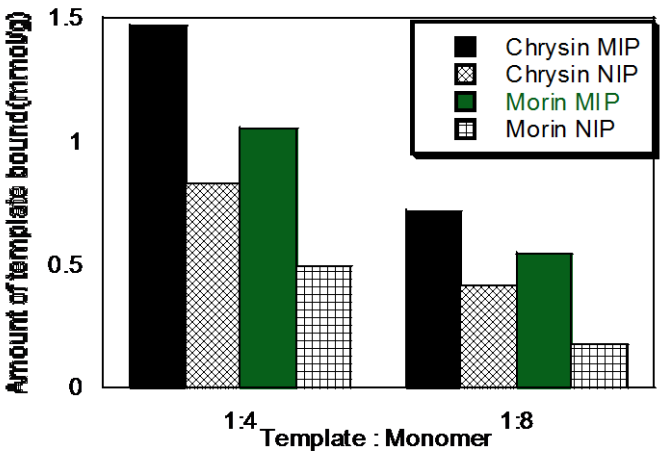


Fig.9. Effect of template: monomer ratio in the selectivity of MIP and NIP

As detailed by Wulff [30], a simple one point interaction is insufficient to induce selectivity regardless of the strength of this interaction. Resulting, selectivity of non-covalent MIP materials requires conditions mimicking biological receptor entities where molecular recognition relies on the interplay of electrostatic interactions in concert with secondary interactions such as hydrophobicity [31].

Characterisation of MIP swelling

Another type of elastic deformation very widely used to charecterise polymers is swelling. This is a three dimensional dilation in which the polymer absorbs solvent, reaching an equilibrium degree of swelling at which the free energy decrease due to the mixing of solvent with the polymer is balanced bythe free energy increase accompanying the stretching of the chains. Swelling characterisation of 1:4 40% EGDMA crosslinked polymers are

carried out in solvents of varying polarity. The MIPs and NIPs were incubated in solvents ACN: DMSO (3:2), (4:1), and Methanol for at least 24 h. The swelling ratios of the disk MIPs were estimated gravimetrically after wiping off the excess solvent on the surface. The swelling ratio was calculated from the following formula:

$$\text{Swelling ratio} = \{Ws-Wd\} / Wd$$

Table : 3

Solvent	Percentage crosslinking	Swelling ratio(%)	
		MIP	NIP
ACN:DMSO (3:2)	40%	4.251	3.856
	50%	3.103	2.832
	75%	4.385	3.942
ACN:DMSO (4:1)	40%	3.556	3.118
	50%	3.253	3.241
	75%	2.446	2.067
Methanol	40%	3.154	3.047
	50%	2.654	2.497
	75%	2.541	2.477

where Ws and Wd are the weight of swollen and dry MIP, respectively. The crosslinked ratio controls the behaviour of a polymer on contact with a solvent and is inversely proportional to the degree of swelling. The 40% EGDMA-crosslinked imprinted polymers gave maximum swelling in all the solvents that proved the relation between the swelling and binding capacity of the imprinted polymers. The high swelling of the system in ACN:DMSO (3:2), which is due to the optimum swelling in the like solvent, also supports the high chrysin binding in this solvent.

4. Conclusion

Molecular imprinted polymers can be effectively prepared and successfully applied for the specific and selective recognition of templates. The Chrysin imprinted polymer showed specificity to Chrysin. The extent of binding depends on time, concentration of template solution, amount of polymer , monomer-template ratio and degree of crosslinking in the polymer support. Since the binding studies require very dilute template solutions and small amount of imprinted polymer, any valuable substance can be imprinted. The nature of template and crosslinking agent influences the efficiency of imprinting. The preparation of the polymer is simple and inexpensive and it was able to distinguish flavonoids that differ slightly in the placement of hydroxyl groups. We have demonstrated that the FI can model the binding behavior of MIPs. The ability to measure the extent of heterogeneity, enables a greater understanding of the structure of MIPs and the imprinting mechanism. This understanding should lead to the rational optimization of the imprinting process and MIPs with improved binding characteristics.

ACKNOWLEDGMENT

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