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MARMARA UNIVERSITY

INSTITUTE FOR GRADUATE STUDIES IN

PURE AND APPLIED SCIENCES

MOLECULAR RECOGNITION USING FLUORESCENCE SPECTROSCOPY

Müge SENNAROĞLU

(Chemical Engineering)

THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

IN

CHEMICAL ENGINEERING PROGRAMME

SUPERVISOR

Prof.Dr. Mehmet S. Eroğlu

Prof.Dr. Sevil Ünal

İSTANBUL 2006

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06/2006 **Müge Sennaroğlu**

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ÖZET

MOLEKÜLER ALGILAMANIN FLORESANS SPEKTROSKOPİSİ İLE İZLENMESİ

Moleküler baskılanmış jel içerisindeki hedef molekülün absorpsiyonunun yüksek hassasiyetle izlenebilmesi amacıyla floresans tekniği kullanılmıştır.

Floresans prob dahil edilmemiş 3-amino benzoik asit baskılanmış, akrilik asit esaslı, sistemler hazırlanmış ve değişik çapraz bağlayıcı/monomer (68.37, 60, 50, 40) oranlarının hedef molekül absropsiyonu üzerindeki etkileri test edilmiştir. Bunun için, hazırlanan polimerik sistemler 3-amino benzoik asit ve 2-hidroksi-1-naftonoik asit içeren çözeltilere daldırılarak, azalan 3-amino benzoik asit miktarları HPLC ile takip edilmiştir. En yüksek absorpsiyon, çapraz bağ/monomer oranının 68.37 olduğu polimerik sistemlerde tespit edilmiş ve optimum oran olarak belirlenmiştir.

Özel ESIPT reaksiyonları gerçekleştiren ve bunun sonucu olarak belirgin çift band yayılımı oluşturan 3-HF türevleri, 2-hidroksi-asetofenon ve paraformaldehit reaksiyonundan yola çıkılarak dört aşamada sentezlenmiştir. Son aşamada, flavon molekülün, baskılanmış sisteme dahil edilebilmesi amacıyla yapıya HEMA takılmış ve vinil gruplu 3-HF türevi bu şekilde elde edilmiştir. Sentezlenen ürünlerin saflıkları ve reaksiyonlardaki değişimler TLC (İnce Tabaka Kromatografisi) ,^{1}H-NMR (Proton Nükleer Magnetik Rezonans) ve MASS (kütle spektroskopisi) kullanılarak kontrol edilmiştir.

Absorpsiyonlardaki değişimler ve 3-HF lerin farklı polaritelerdeki floresans özellikleri florimetri yöntemi ile izlenmiştir. Farklı polarite ortamları, çözeltiye su ilave

edilerek (%0-%17,9) oluşturulmuştur. Prob'un N* band pozisyonu ve şiddetinde artış gözlemlenirken, T* bandı sabit kalmıştır.

Vinil gruplu 3-hidroksi flavonların dahil edilmesiyle hazırlanan cAMP baskılanmış polimerik yapıdan hedef molekül, cAMP, sokslet ekstraksiyonu ile uzaklaştırılmıştır.Bunun sonucu olarak hidrojel içerisinde, moleküler hafızaya sahip, cAMP şeklini almış, boşluklar oluşmuştur. Jeller sulu ortamda cAMP, 3-amino benzoik asit ve 2-hidroksi-1-naftonoik asit içeren çözeltiye daldırılarak floresans ölçümleri alınmış ve en iyi hedef molekül absorpsiyonunun 15 gün bekletilmiş örnekte gerçekleştiği gözlemlenmiştir. Ayrıca, yüksek polarite nedeniyle N* ve T* bandları birleşerek 400-650 nm aralığında tek band yayılımı göstermişlerdir.

Anahtar Kelimeler : Moleküler baskılanma, cAMP, floresans prob, ESIPT, 3-hidroksi flavon.

6 /2006 **Müge Sennaroğlu**

ABSTRACT

MOLECULAR RECOGNITION USING FLUORESCENCE SPECTROSCOPY

A combination of molecular imprinting and flourescence techniques were employed for the recognition of template molecule inside the hydrogel in high sensivity.

Imprinted systems including no fluorescence probe were prepared using 3-aminobenzoic acid as a template molecule and different crosslinker/monomer ratios (68.37, 60, 50, 40) were tested to investigate the effect of crosslinker/monomer ratios on their molecular recognition capability. For this purpose, different compositions of polymers were prepared and plunged into a solution containing 3-amino benzoic acid and 2-hydroxy naphtonoic acid. The decrease in the 3-amino benzoic acid concentration was followed by HPLC and optimum crosslinker/monomer ratios was determined. The highest template molecule recognition capability was obtained at the crosslinker/monomer ratio of 68,37.

The 3-HF derivatives realising special ESIPT reactions, resulting in prospective two bands, were synthesized. This was possible through five step reaction, starting from 2-hydroxy acetophenone and paraformaldehyde reaction. The vinylated 3-HF derivative was formed by attaching 2-hydroxyethylmethacrylate (HEMA) to final flavone melecule in order to insert it into the polymeric imprinted structure. Purity of products and progress of the reactions were controlled by TLC (thin layer chromatography), ^{1}H-NMR (Proton Nuclear Magnetic Resonance) and MASS (mass spectroscopy). Fluorimetry was used for exploring the variations of absorption and fluorescence properties of 3-HF with the change in environment polarity. Different percentages of water (% 0 - % 17,9) was added in order to change the environment polarity. It was observed that there were

distinct increases at N* band position and intensity of probe molecule, with increasing water percentages while T* band remained constant.

The imprinted systems were prepared by incorporation of cAMP and the synthesized vinylated 3-HF derivative into the hydrogels. The cAMP was removed by extraction, which lead to the formation of cavities in the shape of cAMP molcules inside the hydrogel. The gels so obtained were immersed into a solution of cAMP, 3-amino benzoic acid, 2-hydroxy-1-naphtonoic acid, methanol and water.

Results indicated that interactions outside the gel were more intense than those inside the gel. The fluorescence measurements taken after 15 days presented the best template molecule recognition capability. It was also observed that N* and T* bands have combined to yield one band between 400 and 650 nm because of highly polar medium.

Keywords: Molecular imprinting, cAMP, fluorescence probe, ESIPT, 3-hydroxy flavon.

6 /2006 **Müge Sennaroğlu**

CLAIM FOR ORIGINALITY

Fluorescence is the most sensitive and easily available method to study intermolecular interactions. Molecular imprinting provides a way to synthesise new materials containing artificial receptors that can be employed in a variety of applications. Polymeric receptors can be constructed with a flourescent tag built into them so that binding and dissociation of the analyte would change the flourescence emission sufficiently for the binding event to be monitored conveniently. Many attempts have been hampered by the lack of appropriate flourescent tags that could respond to the binding event with a significant flourescence intensity change.

MIPs are expected to dominate the worldwide sensor, seperation and catalysis markets, accounting for a high percentage share of chemical, pharmaceutical, biotechnological, space and military industries in the next decade since they have the advantages of high affinity and selectivity, unique stability and the simplicity in preparation with the combination of the ease of adaptation to different practical applications . Considering their possible industrial applications, synthesis of MIPs combined with a special fluorescence probe , desgined by us, was investigated within the scope of this study for the first time.

The succesful results from cAMP imprinted polymeric gels combined with 3-HF probe obtained in this work, is significant both from economical and scientific viewpoints considering the importance of these materials in the future.

06/2006 **Prof.Dr.Mehmet S.EROĞLU** **Müge SENNAROĞLU**

LIST OF SYMBOLS AND ABBREVIATIONS

µmol	: micro mole
3 ABA	: 3-amino benzoic acid
AIBN	: Azobisisobutyronitrile
AU	: Arbitary Unit
cAMP	: Adenosine 3'-5'-cyclic monophosphate
CE	: Capillary Electrophoresis
cGMP	: Cyclic guanosine monophosphate
EGDM	: Ethylene glycol dimethacrylate
ESIPT	: Excited State Intramolecular Proton Transfer
HC	: Hydroxychromone
HF	: Hydroxyflavone
HEMA	: 2-Hydroxyethyl-methacrylate
HPLC	: High Performance Liquid Chromatography
MASS	: Mass Spectroscopy
min.	**:** minute
MIPs	**:** Molecularly Imprinted Polymers
mL	**:** mililiter
mmol	**:** mili mol
N	**:** Normal ground state
N*	**:** Normal Exited Form
nm	**:** Nanometers (wavelength)
NMR	**:** Nuclear Magnetic Resonance
^{o}C	**:** Centigrade
PTI	: Photon Technology International
RB	**:**Round Bottom
rpm	: Round per minute
SPE	**:** Solid Phase Extraction
T	: Tautomer ground state
T*	: Phototautomer Exited Form
TEA	: Triethyl amine
THF	: Tetrahydrofuran
TLC	**:** Thin Layer Chromatography
TRIM	**:** Trimethylolpropane trimethacrylate
UV	**:** Ultraviolet
v/v	: Volume per volume

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PART I

INTRODUCTION AND OBJECTIVES

Recently, molecular imprinting or template polymerization, has gained much attention as a convenient method for the construction of binding sites for different analytes(Gao *et al.* 2001). This technique has been used for the preperation of selective recognition sites for a wide variety of molecules (Alexander *et al*. 2003).

Molecular imprinting can be employed in a variety of applications, such as separation, sensors or catalysis. The latest developments in the techniques of molecular imprinting have made available polymers that can be used in the detection of drugs, toxins, pesticides, food components, and other molecules that would be difficult to isolate otherwise. Considering the versatility and high level of specificity-recognition capacity that can be achieved, the future of these materials is very promising (Perez-Moral and Mayes 2004).

This polymeric receptors could be constructed with a flourescent tag built into them so that binding and dissociation of the analyte would change the flourescence emission sufficiently for the binding event to be monitored conveniently. However, such efforts have been hampered by the lack of appropriate flourescent tags that could respond to the binding event with a significant flourescence intensity change(Gao *et al.* 2001; Dickert *et al.* 1998; Turkewitsch *et al.* 1998) .

3-Hydroxyflavones belong to an unique organic dye family that undergoes an excited state intramolecular proton transfer (ESIPT) reaction resulting in two highyl intensive, well seperated and solvent-dependent emission bands. originating from the normal excited form (N*) and the phototautomer (T*). The intensity ratio of N* and T*

bands and the distance between two bands changes characteristicaly depends of the environment polarity. Thus they can be used for recognition of molecules in a large spectrum from polar to non polar structure(Klymchenko *et al*. 2002; Estiù *et al.* 1999).

Adenosine 3',5'-cyclic monophosphate (cAMP) is an important second messenger in cells, mediating the actions of many hormones, drugs and neurotransmitters that act via cell surface receptors. Methods aimed at measuring the levels of cAMP, as well as other intracellular second messengers, are therefore highyl desirable. Other methods often require special handling and may suffer from poor stability and a complicated costly production procedure. An alternative approach is the use of molecularly imprinted polymers (MIPs) combined with flourescence sensors, since they combine the advantages of easy tailor design with physical and chemical stability and durability(Turkewitsch *et al.* 1998).

In the present study we experimented a novel approach toward the design of mimetic sensors in which a flourescent MIP serves as both the recognition element and the measuring element for the flourescent detection of cAMP in aquoeous media (Turkewisch *et al.* 1998). We describe the preperation, using a non-covalent imprinting approach of a selective MIP with a flavonol probe (Suárez-Rodrígez and Díaz-García 2000).Molecular imprints were prepared against cAMP that contain a flourescent dye, 6-(2-hydroxymethylmethacrylate)-4'-N,N-diethylamino-3-hydroxyflavone, as an integral part of the recognition cavity, thus serving as both the recognition element and the measuring element for the flourescent detection of cAMP in aqueous media (Turkewitsch *et al.*1998).

PART II

GENERAL BACKGROUND

II.1. MOLECULARLY IMPRINTED POLYMERS

Molecular imprinting can be basically described as a way of making artificial locks for molecular keys (Mosbach and Ramström 1996). In the first step, the selected key molecule is mixed with a variety of lock building blocks. We can imagine key molecules as template molecules and the building blocks as monomers and cross-linkers. In the second step, building blocks and the key is allowed to, either firmly or loosely, attach to each other. Then as a third step, the formed complexes between the key and the building blocks are subsequently "glued" together in order to fix the building block positions around the key as a puzzle. Finally, by removing the molecular key, the leaves a construction which, if everything works properly, is selective for the original key and will not recognise any other key (Ramström 1996).

The preperation of imprinted polymers usually involves (i): prearrangement of the print molecule (template) and the functional monomers at low temperature so that complementary intermolecular interactions among functional groups can develop; (ii): polymerizatiın of the monomers under conditions that cause minimal disturbance to the print molecule-monomer interactions; and (iii) extraction of the print molecules from the polymers, which leaves behind “receptor sites” that are complementary to the templates or print molecules in terms of size, shape, and functional group orientations (Gao *et al*. 2001; Steinke *et al.* 1995).

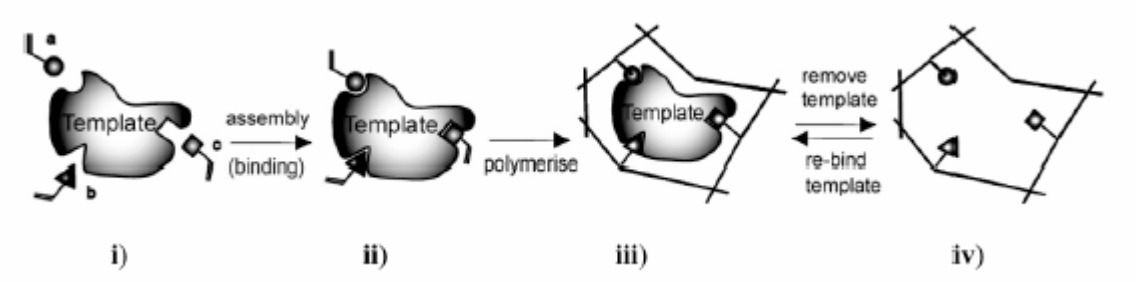


Figure II.1 General molecular imprinting process

Research activity in the area of molecular imprinting has accelerated markedly in the last decade. Molecular imprinting has come along way since its modest beginnings more than sixty years ago. Within the last five years in particular, interest in the area has surged, and it is now estimated that well over 100 academic and industrial research groups are active world-wide (Cormack and Mosbach 1999).

The concept of molecular imprinting has its roots in Linus Pauling's early theory of the formation of antibodies. Pauling suggested that antibodies were formed when serum proteins assembled around template antigen molecules. The assembled antibodies were thought to have specificity-endowing binding pockets complementary in shape to the antigens. Furthermore, strong antibody-antigen binding energy would result from multiple noncovalent binding interactions including hydrogen bonds, ionic bonds, and van der Waals forces. This theory led to the hypothesis by Pauling and Campbell that artificial antibodies could be assembled using these basic principles (Pauling and Campbell 1942).

Although Pauling's theory of antibody formation was later disproved, several groups subsequently tried to apply it to synthetic systems. Pauling's student, Frank Dickey, attempted to form specific absorbents using silica (Dickey 1955).

In the 1970s Wulff, formed covalent bonds between a monomer and the template molecule, followed by polymerization and template cleavage to yield a specific binding site. This method is limited by chemically cleaving the template molecule from the polymer (Wulff *et al.* 1973).

Pauling's original concept was finally applied to the synthesis of artificial antibodies with the development of molecular imprinting by Klaus Mosbach's group.

Mosbach's approach of preassembling a noncovalently associated monomer-template complex in solution prior to polymer formation was the breakthrough that enabled molecular imprinting to be used in a variety of applications (Andersson *et al*. 1984).

A molecularly imprinted membrane was recently developed that specifically bound theophylline over other purine bases. Such artificial affinity membranes should have applications for rapid analytical method development or for sample preparation methods. An additional benefit could be the combination of sample preparation and detection on the membrane (Kobayashi *et al.* 1995).

The advantages of the MIPs are their ease of preperation, stability, high selectivity and high affinity constants. To date, MIPs have been exploited in a number of applications including their use as seperation materials, as antibody mimics in binding assay systems, and as recognition elements in biosensors for assay of various analytes. Recently, beacuse of their compatibility with organic solvents MIPs have been utilized as solid-phase extraction (SPE) sorbents for environmental applications is rapidly increasing (Zhu *et al.* 2002**;** Yan and Kapua 2001).

Because MIPs have outstanding advantages such as predetermined selectivity, simple and convenient preparation, robustness in organic solvents and acidic or basic reagents, and durability to high temperature, molecular imprinting has drawn extensive attention in recent years (Zhang *et al*. 2001).

In order to obtain a MIP having high selectivity, which kind of structure a template should have is one of the hot topics (Hjerten *et al.* 1997). A summary of most important advantages and disadvantages of imprinted polymers are shown below and the characteristics of most important features are list in Table II.1.

Advantages

- Target defines own recognition site,
- Stability of synthetic materials,
- Specificity of natural systems,
- Adaptability/flexibility in use,
- Facile, “one-pot” synthesis,
- Use in non-aqueous media/aggressive environments.

Disadvantages

- Diversity of binding sites,
- Poor processibility.

Table II.1 The characteristics of MIPs features.

FEATURE	CHARACTERISTICS
Physical Stability	Resistant against mechanical stress, high pressures and elevated temperatures
Chemical Stability	Resistant against acids, bases, various organic solvents and metal ions.
Storage Endurance	Several years without loss of performance
Capacity	0.1-1 mg print molecule/g polymer in enantioselective chromatography
Recovery Yield	> 99 %
Binding Strength	M_n range

II.1.1. Approaches to Molecular Imprinting

Two basic approaches to molecular imprinting may be distinguished : (1) the pre-organised approach, mainly developed by Wulff and coworkers, where the aggregates in solution prior to polymerisation are maintained by covalent bonds, and (2) the self-assembly approach, mainly developed by Mosbach and coworkers, where the pre-arrangement between the print molecule and the functional monomers is formed by non-covalent or metal coordination interactions (Andersson *et al.* 1989; Ramström *et al.* 1994). Approaches to molecular imprinting technology has shown schematically in Figure II.2

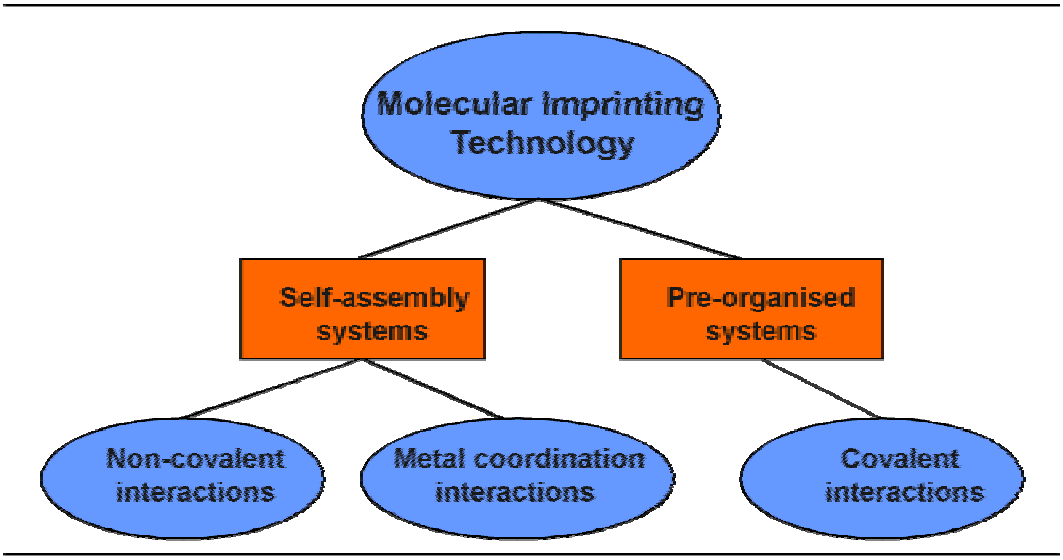


Figure II.2 Approaches to molecular imprinting technology

II.1.1.1. The Covalent Bonding

The covalent approach to molecular imprinting promises the most homogeneous binding site distribution. The binding constants for the template molecule are high, since the interaction between template and functional monomer is based on covalent interactions. However, despite the high affinities of the polymeric material, the range of functional groups which can be targeted is restricted and removal of the template molecules is very difficult and limits the application of covalently prepared MIPs (Molinelli 2004).

Furthermore, slow binding kinetics restrict the analytical application of covalently imprinted MIPs, if rebinding is based on reversible covalent bonds. Alternatively, in the semi-covalent approach rebinding occurs via non-covalent interactions taking advantage of faster rebinding kinetics (Molinelli 2004).

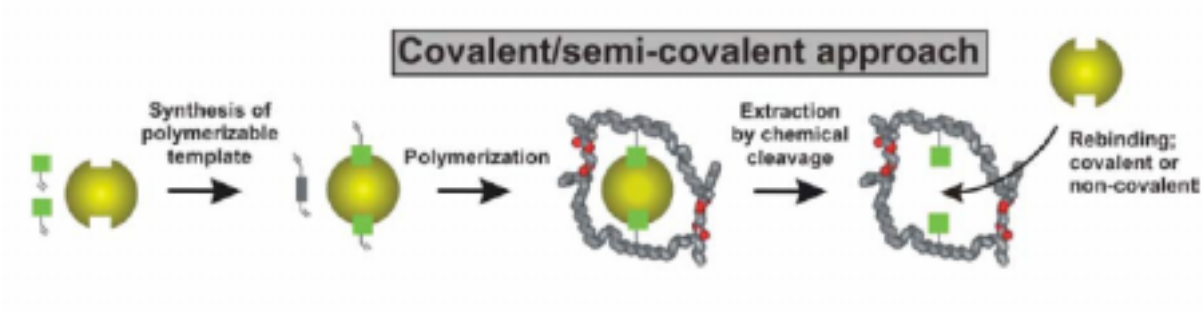


Figure II.3 The covalent and semi covalent approaches of MIPs

II.1.1.2. The Non-Covalent Bonding

In non-covalent molecular imprinting, complexes assembled by non-covalent interactions are formed in the pre-polymerization mixture. Complexation is achieved by mixing template, functional monomer, and cross-linker in a porogenic solvent matrix. (Muldoon and Stanker 1996 ; Molinelli 2002). In contrast to covalent imprinting, the self-assembly approach is characterized by a more heterogeneous binding site distribution. Resulting, sample overload may occur due to rapid saturation of the comparatively low amount of high-affinity binding sites, which results in a significantly decreased overall polymer performance. Nevertheless,the simple preparation procedures, the wide range of imprintable compounds, and reversible host-guest binding based on non-covalent interactions ('biomimetic binding') render the non-covalent imprinting approach the most widespread method for MIP preparation (Molinelli 2002**;** Siemann *et al.* 1996). A non-covalent approach has shown schematically in figure II.4.

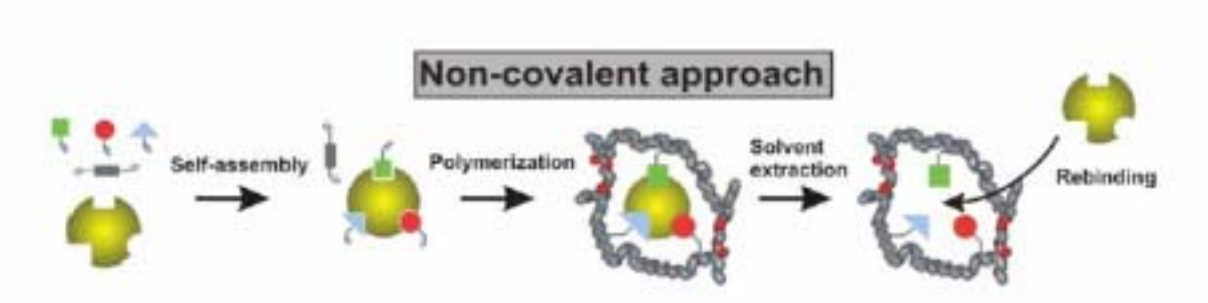


Figure II.4 Non-covalent approach to MIPs

The summary of most important advantages and disadvantages of covalent and noncovalent bonds for MIPs are listed in Table II.2 (Alexander and Davidson 2003). By the evaluation of this table the non-covalent route was followed in this study.

Table II.2 The advantages and disadvantages of covalent and non-covalent bonds.

Covalent Imprinting	Non-covalent Imprinting
• Ability to fix template in place during polymerization - lower dispersity in binding sites.	• Inability to fix template in place during polymerization - polydispersity in binding sites, poor definition.
• Can be carried out in any solvent flexibility.	• Generally requires low-polarity aprotic solvents - incompatible with aqueous polymerisations .
• Can be difficult to remove template from polymer - low recovery of valuable templates and low number of binding sites.	• Easy to remove template from polymer- good recovery of valuable templates and accessible binding sites.
• Limited number of chemistries for fixing template to polymer reversibly - reduction in number of templates that can be imprinted.	• Very large number of templates amenable to non-covalent imprinting
• Poor kinetics of re-binding	• Rapid kinetics of re-binding

II.1.2. Components of Molecular Imprinting

There are four main ingredients that must be included in a polymerization reaction and must understood clearly in order to accomplish a molecular imprinting process. These are template molecule, functional monomers, cross-linkers and porogens (solvents).

II.1.2.1. Template Molecule

The template is the molecule to be purified, catalyzed or recognized and the most important constituent since it directs the organization of functional groups pendent to functional monomers(Cormack and Elorza 2004; Markowitz *et al.* 2000). Template molecules can change from small molecular weight organic molecules to large biopolymers but larger molecules are more difficult to imprint (Henricksen and Martin 1996). Template molecules should be in pure form. Templates used for catalysis are generally transition state analogues, meaning that they behave similarly to high energy intermediates in the chemical reaction to be catalyzed (Markowitz 2000).

Because of many different reasons, all templates are not directly suitable for molecular imprinting. Templates should be chemically inert under the polymerization conditions in order to be compatible with free radical polymerization.

There are some essential questions to ask of a template ;

1) Does the template carry any polymerisable groups
2) Does the template carry functionality that could potentially inhibit or retard a free radical polymerisation.
3) Will the template be stable at moderately elevated temperatures or upon exposure to UV irradiation (Cormack and Elorza 2004).

It has become evident that the use of non-covalent interactions between the print molecule and the functional monomers is the more versatile.Furthermore, the use of non-covalent interactions in the imprinting step closely resembles the recognition pattern observed in nature(Ramström *et al*. 1996).

Molecular imprinting have been demonstrated against many classes of molecules. These include drugs (Vlatakis *et al.* 1993), hormones(Anderrson *et al.* 1995), pesticides (Muldoon *et al.* 1996; Siemann *et al.* 1996), proteins(Kempe *et al.* 1995), amino acids (Kriz *et al.* 1995), peptides (Ramström *et al.* 1994), carbohydrates (Mayes *et al.* 1994), coenzymes (Andersson 1989), nucleotides (Norrlöw *et al.* 1987), nucleotide bases (*Shea et al* 1993), steroids (Ramström *et al* 1996), dyes(Norrlöw *et al* 1984) and metal ions (Rosatzin *et al.* 1990). Some of these molecules and corresponding templates have been used in various imprinting protocols and displayed in Table II.3

Table II.3 Different substances used in various imprinting protocols

Compound Class	Example		Compound Class	Example
drugs	Timolol		amino acids[a]	Phenylalanine
	Theophylline			tryptophan
	Diazepam			tyrosine
	morphine			aspartic acid
	Ephedrine			
			carbohydrates[a]	Galactose
Hormones	Cortisol			glucose
	Enkephalin			Fucose
Pesticides	Atrazine		Co-enzymes	pyridoxal
proteins	Rnase A		Nucleotide bases	adenine
	Urease			

II.1.2.2. Functional Monomers

The functional monomers have a special role in molecular imprinting process and responsible for the binding interactions in the imprinting binding sites (Cormack and Elorza 2004). The functional monomers should each have 2 functional groups. On one end, they should interact with the template with weak interactions (non-covalent) such as hydrogen bonding and Van der Waals forces. On the other side of the monomers (the side that is not interacting with the template) should be a group that is able to bind covalently with the cross-linker (Markowitz *et al.* 2000).

Most used polymer systems for imprinting technology are either polyacrylate-based or polyacyrilamide-based systems and polystyrene-based systems (a lesser extend) (Ramström 1996).

In non-covalent systems functional monomers usually used in excess amounts relative to template molecule. In order to maximise complex formation and icrease the imprinting effect it is important to match the functionality of the functional monomers(Cormack and Elorza 2004).

Various functional monomers having chemically different structures and polarities are commercially available and also can be prepared by rational design. In Figure II.5 , the chemical structures of the more important functional monomers are shown used in non-covalent approach.

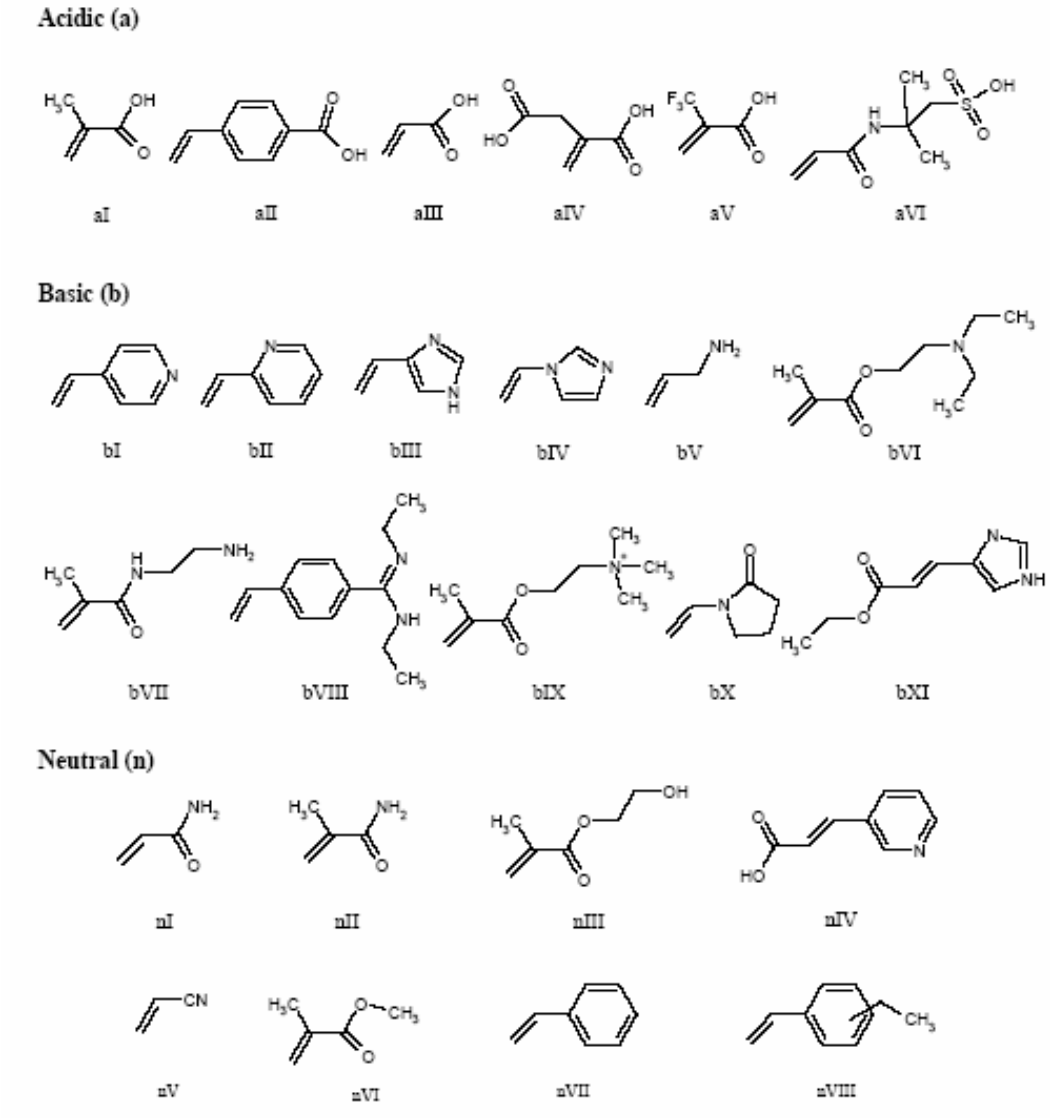


Figure II. 5 Selection of monomers used in the non-covalent imprinting

Acidic; aI: methacrylic acid (MAA); **aII:** *p*-vinylbenzoic acid; **aIII:** acrylic acid (AA); **aIV:** itaconic acid; **aV:** 2-(trifluoromethyl)-acrylic acid (TFMAA); **aVI:** acrylamido-(2-methyl)-propane sulfonic acid (AMPSA). **Basic; bI:**4-vinylpyridine (4-VP); **bII:** 2-vinylpyridine (2-VP); **bIII:** 4-(5)-vinylimidazole; **bIV:** 1-vinylimidazole; **bV:** allylamine; **bVI:** *N*,*N*_-diethyl aminoethyl methacrylamide (DEAEM), **bVII:** *N*-(2-aminethyl)-methacrylamide; **bV.III:** *N*,*N*_-diethyl-4-styrylamidine; **bIX:** *N*,*N*,*N*,-trimethyl aminoethylmethacrylate; **bX:** *N*-vinylpyrrolidone (NVP); **bXI:** urocanic ethyl ester. **Neutral; nI:** acrylamide; **nII:** methacrylamide; **nIII:** 2-hydroxyethyl methacrylate (2-HEMA); **nIV:** trans-3-(3-pyridyl)-acrylic acid; **nV:** acrylonitrile (AN); **nVI:** methyl methacrylate (MMA); **nVII:** styrene; **nVIII:** ethylstyrene.

II.1.2.3. Cross-linkers

The cross-linker is a molecule that can be polymerized around the template, binding covalently to the functional monomers and holding them in place after the template is removed (Haupt and Mosbach 1998).

A major problem that can be minimized by selecting the appropriate cross-linker is accessibility to the binding site. If the entire binding site is covered, then it may be difficult or impossible to remove the template, or for the binding site to be used at all. Thus, porous cross-linkers (with networks of small holes) or ones that can be broken into small pieces (pulverized) are typical (Markowitz *et al* 2000).

In polymer imprinting process the cross-linker plays three important roles. First of all, the cross-linker is important in controlling the morphology of the polymer matrix, whether it be gel-type, macroporous or a microgel powder. Secondly, it serves to stabilize the imprinted binding site. Finally, it imparts mechanical stability to the polymer matrix. The chemical structures of well known cross-linkers are shown in Figure II.6 . High cross-link ratios are generally preferred in order to access permanently porous (macroporous) materials and in order to be able to generate materials with adequate mechanical stability (Cormack and Elorza 2004). Acrylic or methacrylic acid based systems could be prepared with much higher specificity. Ethylene glycol dimethacrylate (EGDM) and trimethylolpropane trimethacrylate (TRIM) are presently commonly employed in several system (Ramström 1996).

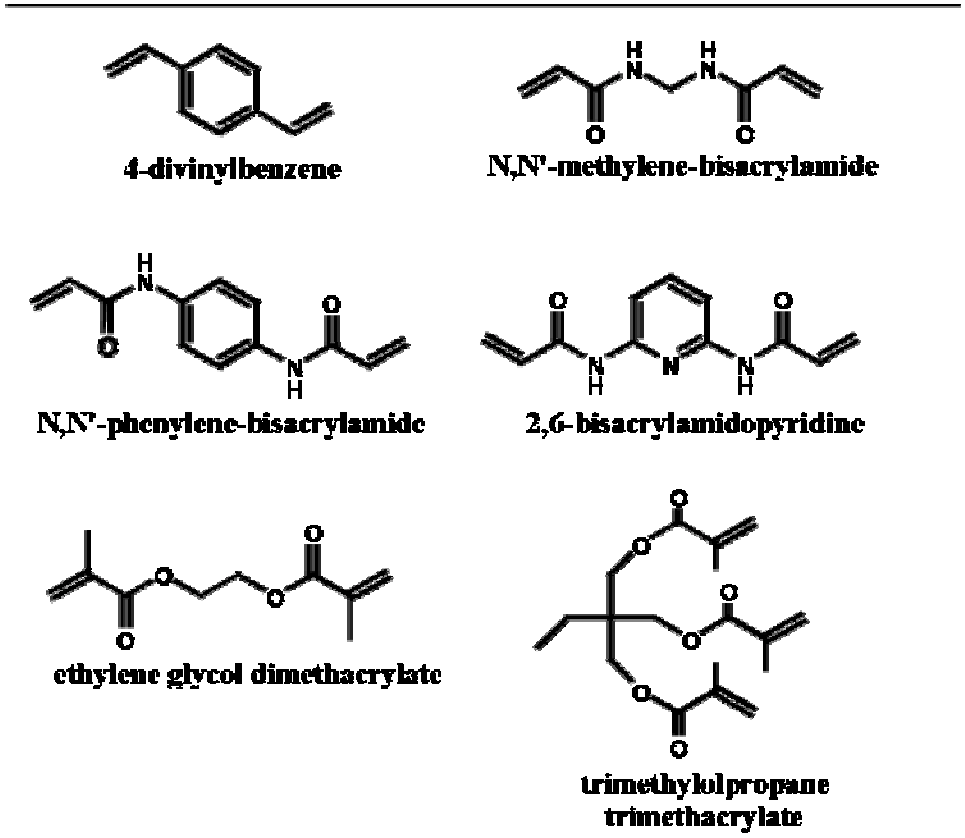


Figure II.6 Chemical structures of well known cross-linkers

II.1.2.4. Solvents (Porogens)

The solvent plays an important role in the outcome of a molecular imprinting process in self-assembly systems. As porogen in the polymerisation, the solvent governs the strength of non-covalent interactions in addition to its influence on the polymer morphology (Ramström 1996).Generally, the more polar the porogen, the weaker the resulting recognition effect becomes, as a consequence of the influence of the solvent polarity on non-covalent interactions. In the recognition step similar questions about the choice of solvent arise. Since all non-covalent forces are influenced by the properties of the solvent, non-polar solvents normally lead to the best recognition. When applying the polymers to gradually more polar solvents, the recognition is diminished. Also, the morphology is affected since the swelling of the polymers is dependent on the surrounding medium (Ramström 1996; Molinelli 2004).

According to literature, only few attempts of imprinting in the presence of polar solvents such as methanol and water were successful. Nevertheless, imprinting at aqueous conditions would allow maintaining the same conditions during the rebinding step and - most importantly – operating in aqueous environments would facilitate imprinting of ***biomolecules*** (Molinelli 2004; Cormack and Elorza 2004).

II.1.2.5. Initiators

In principle, any of the methods of initiation can be used to initiate free radical polymerisations in the presence of templates. However, there may well be drivers for selecting one over another arising from the system under study. For example, if the template were photochemically or thermally unstable then intiators that can be triggered photochemically or thermally. When complexation is driven by hydrogen bonding then lower polymerization temperatures are preferred, and under such circumstances photochemically active initiators may well be preferred as these can operate efficiently at low temperature (Cormack and Elorza 2004). The chemical structures of most known polymerization intiators are shown in Figure II.7

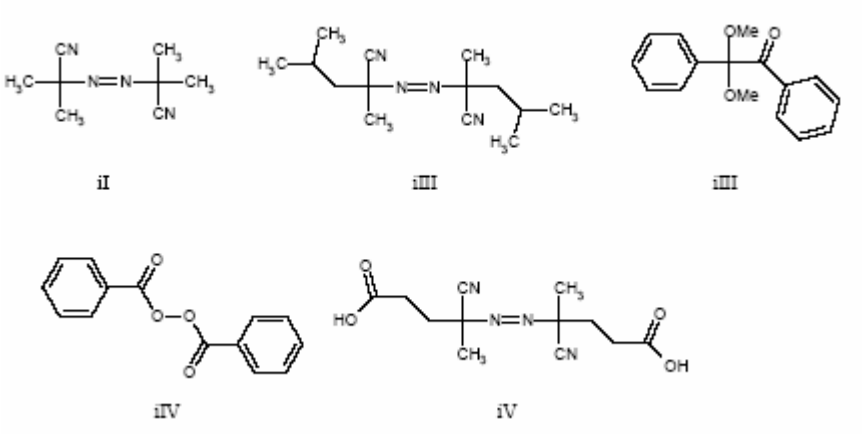


Figure II.7. Chemical structures of most known polymerization initiators:

iI: azobisisobutyronitrile (AIBN); **iII:** azobisdimethylvaleronitrile (ABDV); **iIII:** dimethylacetal of benzil; **iIV:** benzoylperoxide (BPO); **iV:** 4,4_-azo(4-cyanovaleric acid).

II.1.3. Application areas of MIPs

In recent times, four main areas of application for molecularly imprinted polymers have been pursued : 1)Affinity Seperation, 2) Antibody Binding Mimics, 3) Enzyme Mimics and 4) Bio-Mimetic Sensors. These four areas continue to be the focus of the most research efforts (Cormack and Mosbach 1999; Vidyasankar and Arnold 1995).
Other than these four main application areas nowadays the MIP society is highly sensitive towards contaminants in foodstuff. Such contaminants may be pesticides, herbicides, hormones and antibiotics (Brüggemann *et al.* 2000).

Three particular features have made MIPs the target of intense investigation:
(1) their high affinity and selectivity, which are similar to those of natural receptors;
(2) their unique stability which is superior to that demonstrated by natural biomolecules;
(3) the simplicity of their preparation and the ease of adaptation to different practical application (Piletsky *et al.* 2001).

A great variety of chemical compounds have been imprinted successfully (Table II.4). The resulting polymers are robust, inexpensive and, in many cases, possess an affinity and specificity that is suitable for industrial application.

Table II.4 Typical examples of MIPs design and application

TEMPLATE	**APPLICATION**
Amino acids and amino acid derivatives	Separation and binding Synthesis Assay and sensors
Aniline, phenol and their derivatives	Assay and sensors
Drugs	Separation and binding Assay and sensors
Gases and vapors	Sensors

Herbicides	Seperation and binding Assay and sensors
Heterocycles	Seperation and binding
Metal ions	Seperation and binding Assay and sensors
Micro-organisms	Seperation and binding
Nucleic acids and nucleic acid derivatives	Seperation and binding Assay and sensors
Polynuclear aromatic hydrocarbons	Seperation and binding Assays and sensors
Proteins	Seperation and binding
Steroids	Seperation and binding Detection
Sugars and sugar derivatives	Seperation and binding Assays and sensors
Alkaloids, toxins and narcotics	Seperation and binding Assays and sensors

II.1.3.1. Affinity Seperation

Affinity seperation is the biggest application for imprinted polymers at present. Their use as stationary phases in high performance liquid chromatography (HPLC) continues to be an important research area, in spite of its current limitations: low capacity and heterogeneous binding sites. In the research laboratory, HPLC using an

imprinted stationary phase remains one of the most convenient methods for assessing the efficiency of a new imprinting protocol. Besides liquid chromatography, imprinted polymers as selective solid-phase extraction (SPE) media are very much in vague. It may well be that this is the area in which we will see the first commercial application of these materials. Other key lines of research in the affinity seperation area include membranes (Rogers 1998) and capillary electrophoresis (CE) (Cormack and Mosbach 1998).

II.1.3.2. Antibody Binding Mimics

The demonstration that the strength and the selectivity of binding between an imprinted polymer and an analyte could be comparable, and in some cases even beter than that between an antibody and an antigen generated enormous interest.(Vlatakis *et al.* 1993).

In an application , these antibody binding mimics (‘plastic antibodies’) offer a rapid and inexpensive route into stable and robust molecular recognition matrices. They promise a real alternative in applications that use antibodies in their soluble form, e.g. immunoaffinity chromatography, immunosensors and immunoassays. Some recent immunoassay-related studies have focussed on developing new assay formats that do not rely on radioligands, e.g. fluorescence and elcetrochemical assays(Cormack and Mosbach 1998).

II.1.3.3. Enzyme Mimics

In the area of molecular imprinting a lot of work have been carried out for developing the catalytically active imprinted polymers (‘plastic enzymes’) that are capable of mimicking enzymes (Wulff *et al.* 1995). As a result of these works several organic reactions have been succesfully catalysed with molecularly imprinted polymers, including aldol condensations, ester hydrolysis, diels-alder reactions and β-eliminations.While molecularly imprinted polymers may not yet be able to compete with enzymes in terms of catalytic rate enchancements, they offer properties that are quite distinct from those of enzymes, for example their ready compatibility with organic solvents and their high temperature stability. Thus they are more useful as a complement

to enzymes rather than as a replacement to enzymes rather than as a replacement, at least for now(Cormack and Mosbach 1998).

II.1.3.4. Bio-Mimetic Sensors

In the last decade, a lot of attempts have been focused on to the use the MIP technique into the biosensor applications(Piletsky *et al* 1998). The idea is, to replace molecular recognition entities based on biomolecules with imprinted polymers. Although the biosensor field is a very competitive area, molecularly imprinted polymers would expected to challange very strongly in this area given their many and varied attractive properties. Several potential applications of bio-mimetic sensor application have been demonstrated on a laboratory scale, but nothing has found its way into the marketplace, since it is relatively new approach(Cormack and Mosbach 1998).

II.1.3.5. Robust food analysis

The MIPs are applicable for the determination of food additives, such as carbohydrates, peptides, or flavor additives. Galactose, fructose and mannose as well as glucose have been used as templates, or amino acids and proteins like ribonuclease A or transferrin (a globulin in blood plasma that carries iron) and also caffeine (Kempe *et al*. 1995). Furthermore vitamins and nucleotids can specifically be analyzed utilizing MIPs. A semi-covalent approach of molecular imprinting has been applied to analysis of cholesterol. Therefore MIPs have been applied for the determination of atrazine and 2,4-dichlotophenoxyacetic acid contamination. Furthermore, drugs, like local anaesthetics and in particular antibiotics have been employed as template molecules, such as macrodile antibiotics, chloramphenicol or clenbuterol. Even surface imprints of bacterial cells have been realized (Brüggemann *et al.* 2000).

II.2. MOLECULAR FLUORESCENCE

Photoluminescence is a type of optical spectroscopy in which a molecule is promoted to an electronically excited state by absorption of ultraviolet, visible, or near infrared radiation. The excited molecule then decays back to the ground state, or to a

lower-lying excited electronic state, by emission of light. The emitted light is detected (Wehry 1997).

Photoluminescence processes are subdivided into fluorescence and phosphorescence. These two processes differ mechanistically in their origin of emission (Singlet - Triplet). They also differ in the lifetime of their excited states; the fluorescence ceases immediately after irradiation is discontinued, while phosphorescence persists for a length of time (Soutar 1995). Fluorescence spectrometry is a very sensitive method in quantitative determination of traces amount of inorganic, organic and bio-molecules. Fluorometry may achieve limits of detection several orders of magnitude lower than those of most other techniques. This is known as the fluorescence advantage.



10^{-5} to 10^{-8} s fluorescence

10^{-4} to 10 s phosphorescence

Theory of Fluorescence and Phosphorescence:

-Excitation of *e*- by absorbance of hυ.

- Re-emission of hυ as *e*- goes to ground state.

- Use $h\upsilon_2$ for qualitative and quantitative analysis

Fluorescence – ground state to *single* state and back.

Phosphorescence -ground state to *triplet* state and back

Excitation of a molecule does not automatically produce fluorescence; many molecules exhibit very weak fluorescence. Most intensely fluorescent organic molecules contain large conjugated π-electron systems. To extend the applicability of fluorometry to the many compounds that do not exhibit intense native fluorescence, chemical reactions can be used to convert (derivatize) nonfluorescent molecules to fluorescent derivatives, or a nonfluorescent molecule may have chemically attached to it a fluorescent tag or label (Wehry 1997).

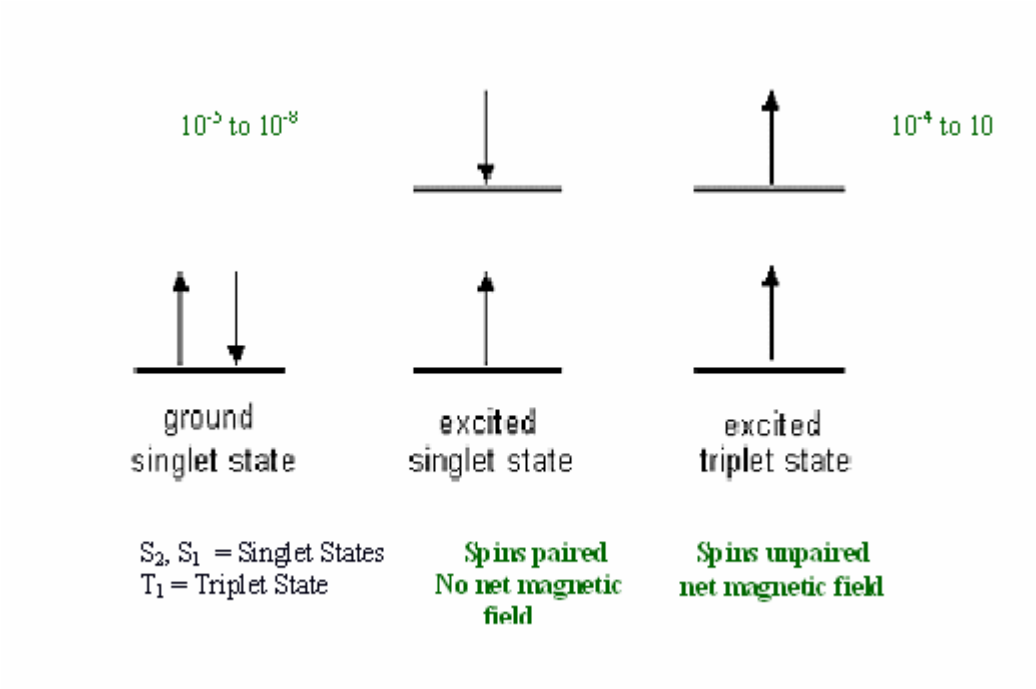


Figure II.8 Theory of fluorescence and phosphoresence

II.2.1. Chemical and Physical Principles of Fluorescence

The initial step in a fluorescence measurement is electronic excitation of an analyte molecule via absorption of a photon. Once formed, an excited molecule has available a variety of decay processes by which it can rid itself of the energy imparted to it by absorption. In addition to fluorescence (the desired decay route), there are nonradiative decay processes, leading to release of energy in the form of heat rather than light. Other sample constituents may interact with an excited analyte molecule in such a way as to prevent it from fluorescing; such processes are called quenching. Also, an electronically excited molecule may undergo chemical reaction (photodecomposition). The fraction of electronically excited molecules that decay to the ground state by fluorescence is called the fluorescence quantum yield (Wehry 1997).

DEACTIVATION PROCESSES

a) Vibrational relaxation: The loss of vibrational excitation energy by a molecular entity through energy transfer to the environment caused by collisions. The molecular entity relaxes into vibrational equilibrium with its environment (Wehry 1997).

solvent collisions

- l emission > l excitation (Stokes shift)

- vibrational relaxation is efficient and goes to lowest vibrational level of electronic state within 10-12s or less.

- significantly shorter life-time then electronically excited state

- fluorescence occurs from lowest vibrational level of electronic excited state, but can go to higher vibrational state of ground level.

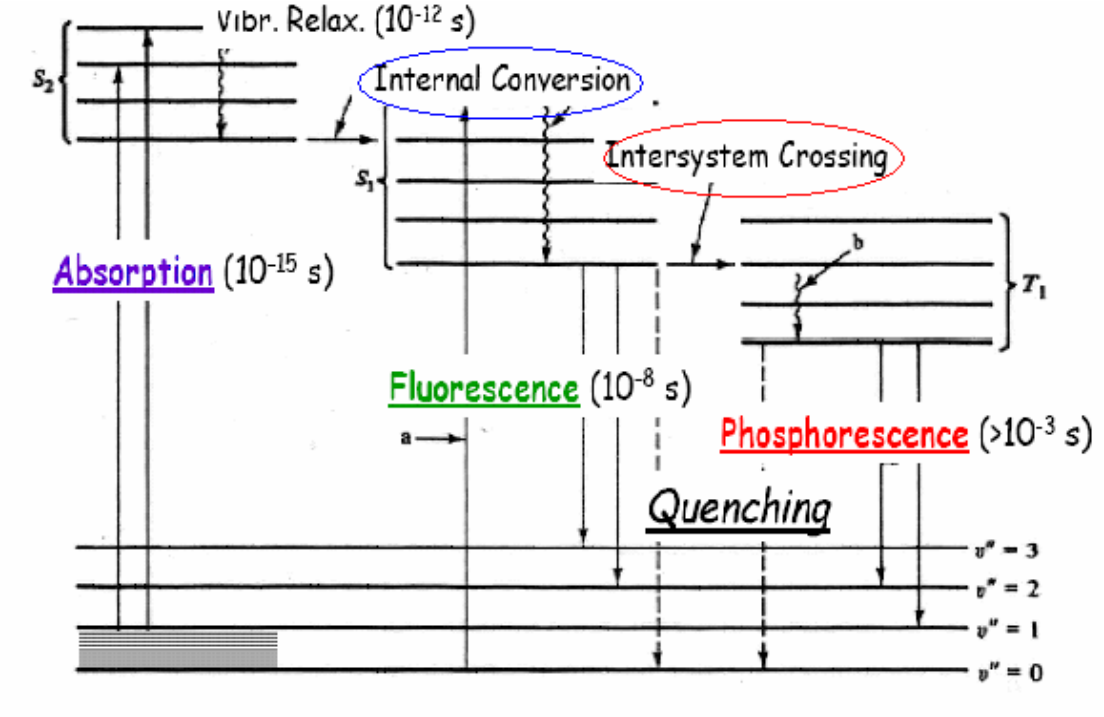


Figure II.9 Deavtivation process

***b) Internal conversion*:** encounters radiationless processes where molecules in an excited singlet state may return to the ground state without the emission of a photon, converting all the excitation energy into heat. Generally internal conversion is an inefficient process and is probably only a small fraction of the total excitation energy in most molecules

- not well understood

- crossing of *e*- to lower electronic state.

- efficient since many compounds don't fluorescence

- especially probable if vibrational levels of two electronic states overlap, can lead to predissociation or dissociation.

c) External conversion: This is the deactivation via collision with solvent and excess energy given to the solvent.

-deactivation via collision with solvent (collisional quenching)

-decrease collision → increase fluorescence or phosphorescence

-decrease temperature and/or increase viscosity

-decrease concentration of quenching (Q) agent

d) Intersystem crossing. The mechanism for intersystem crossing involve vibrational coupling between the excited singlet state and a triplet state. Thus this is a spin-dependent internal conversion process

- spin of electron is reversed

- change in multiplicity in molecule occurs (singlet to triplet)

- enhanced if vibrational levels overlap

- more common if molecule contains heavy atoms (I, Br)

- more common in presence of paramagnetic species (O2)

e) Phosphorescence: Deactivation from an 'triplet" electronic state to the ground state producing a photon.

II.2.2. Factors Effecting Fluorescence

The fluorescence property of a molecule and the fluorescence light intensity depends upon the molecule structure and it's chemical environment .

A variety of environmental factors affect fluorescence emission, including interactions between the fluorophore and surrounding solvent molecules (dictated by solvent polarity), other dissolved inorganic and organic compounds, temperature, pH, and the localized concentration of the fluorescent species. The effects of these parameters vary widely from one fluorophore to another, but the absorption and emission spectra, as well as quantum yields, can be heavily influenced by environmental variables(Rhys-Williams 2000; Gaigalas *et al.* 2001).

Temperature: Increasing the temperature will decrease fluorescence because the rate of dynamic quenching is increased.

Solvent: The viscosity, polarity, and hydrogen bonding characteristics of the solvent can significantly affect fluorescence. Fluorescence usually increases with an increase in solvent viscosity due to reduced rate of bimolecular collisions and rate of dynamic quenching. Solvent polarity and hydrogen bonding are critical because they affect the nature of the excited state. There is a rapid (10^{-11} to 10^{-12} s) reorientation of solvent molecules around the excited state species. Causes a shift in the excitation wavelength.

pH: pH of solutions in protic solvents can be critical for aromatic molecules with acidic or basic functional groups (phenols, amines). In some cases only the protonated or unprotonated form of the acid or base may be fluorescent. For example, many phenols are fluorescent only in the nonionized form(Rhys-Williams 2000).

II.2.3.The Spectrometry And Fluorimetry

All fluorescence instruments contain three basic items: a source of light, a sample holder and a detector. In addition, to be of analytical use, the wavelength of incident radiation needs to be selectable and the detector signal capable of precise manipulation and presentation. In simple filter fluorimeters, the wavelengths of excited and emitted light are selected by filters which allow measurements to be made at any pair of fixed wavelengths.

Simple fluorescence spectrometers have a means of analysing the spectral distribution of the light emitted from the sample, the fluorescence emission spectrum, which may be by means of either a continuously variable interference filter or a monochromator. In more sophisticated instruments, monochromators are provided for both the selection of exciting light and the analysis of sample emission. Such instruments are also capable of measuring the variation of emission intensity with exciting wavelength, the fluorescence excitation spectrum(Rhys-Williams 2000).

A general fluorescence spectromety has been shown in figure.

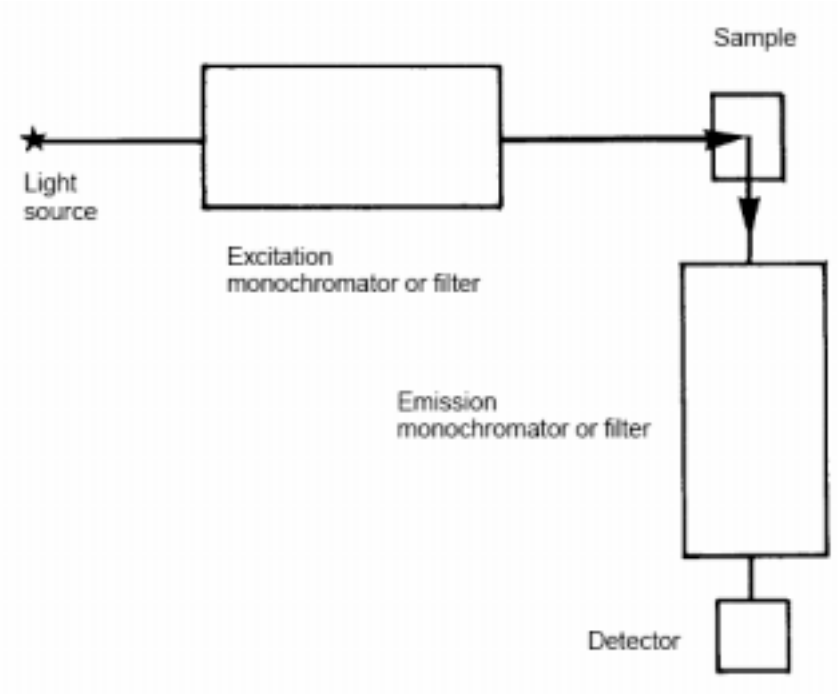


Figure II.10 Essential components of a fluorescence spectrometer

II.2.4. Fluorescence Probe

Fluorescence probing is one of the major research tools in molecular and cellular biology, biotechnology and related areas. The probes are usually organic fluorophores that correlate optimal spectroscopic properties with optimal functional performance. Their spectroscopic properties involve the ability to generate strong and easily measurable signal reporting on different intermolecular interactions in the probe environment and on the dynamic processes in the studied system. Response can be obtained in different parameters characterizing fluoresence emission : position of spectral bonds, intensity, anisotropy and lifetime (Lakowicz 1999; Valeur 2002; Turkmen *et al.* 2005).

It is common perception that fluorescence probes exhibiting two bands in emission together with two band ratiometric sensivity should have a strong advantage over wavelength-shift probes in fluorescence sensing techniques(Klymchenko *et al.* 2002b).

II.2.5. Excited State Intramolecular Proton Transfer (ESIPT)

ESIPT reactions are of great scientific and technological interest. ESIPT reactions are used in organic synthesis and in many biological processes(Gerssen 2003). Different from various classes of fluorescent dyes investigated so far, the one based on the ESIPT has an intrinstic advantage for stimulated emission because it is harnessed with a favorable four-level photophysical cycle.

The ESIPT reaction, a fast Normal (N) – to tautomer (T) protropy occuring in the excited states of intramolecularly H-bonded molecules, is thus gaining ever increasing interest due to the fundamental importance. Because the ESIPT molecules are more stable in N forms in the ground states but in T* forms in the excited states, photoexcitation of them is immediately followed by the four-level cyclic proton-transfer reactions (NfN*fT*fTfN) mediated by the intramolecular H-bonds. It is characteristically observed, therefore, that the absorption from N and emission from T* results in an abnormally large Stokes shift with no self-absorption, providing an ideal scheme for the proton-transfer lasers and UV photostabilizers (Park *et al.* 2005).

The acidity or basicity of certain structural features of molecules are enhanced in the excited state compared to the ground state. If two of these features (one acidic and one basic) are in close proximity to each other in a molecule it is possible, on excitation, for a proton to “hop” from the acidic site to the basic one -ESIPT.In most examples of molecules which undergo ESIPT the acidic proton donor is a hydroxyl group and the basic acceptor is a nitrogen atom or a carbonyl function (Ameer-Beg *et al.* 1998; 2001).

II.2.6. Esipt Reactions Of 3-Hydroxy Flavones

The 3-hydroxyflavon (3-HF) derivatives are very attractive fluorescence sensors due to their ability to respond to small changes in their microenvironment via a dramatic alteration of the relative intensities of their two well-seperated emission bands (Klymchenko *et al* 2002a).

ESIPT reactions, known to produce emmisive tautomer forms with dramatic Stokes shifts. Among all fluoropores exhibiting ESIPT, only 3-Hydroxychromones (3HCs) and their derivatives 3-hydroxyflavones (3HFs) are the compounds, in which the

observation of two seperate bands in emission is not coupled to the peresence of two or more ground-state or excited-state conformers or to a slow ESIPT reaction. In 3HCs the 3-hydroxyl group (proton-donor) and the 4-carbonyl group (proton acceptor) are attached to a rigid skeleton and coupled by an intramolecular hydrogen bond. In 3HCs with electron-donor substituents the ESIPT reaction is faster than the emission, and the observation of two emission bands belonging to initially excited normal (N^*) form and reaction product tautomer (T^*) form is due to the rapid establishment of a dynamic equilibrium between these forms. Increase of the dipole moment of the N^* form by proper substitutions allows to make not only the position of the N^* band but, most importantly, the ratio of band intensities sensitive to interactions with the environment.

In order to be sensitive to the two-band ratiometric probe, the dye is required to exhibit an excited-state reaction : isomerization, electronic charge transfer, or proton transfer. In addition, both initial and product forms of the reaction should be present in emission and provide high-intensity and well seperated fluorescence bands. (Klymchenko *et al.* 2002a)

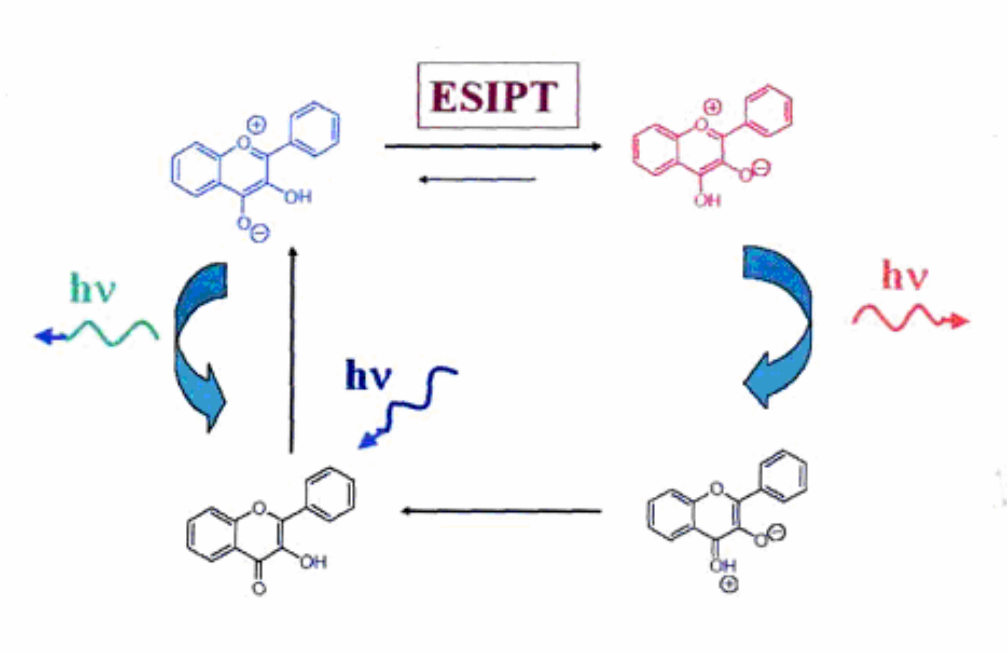


Figure II.11 ESIPT reactions of 3-hydroxy flavones

The ESIPT reaction site is strictly localized between 3-hydroxl and 4-carbonyl groups, which form a hydrogen bond that closes a low-stable five-membered ring. Therefore, this reaction shows extreme sensitivity to intermolecular hydrogen bond

perturbations that should have a certain directionality in space. Moreover, because of the asymmetric nature of the chromophore and uniderectional nature of ESIPT reaction, the fluorescence spectra are expected to be sensitive to anisotropic properties of the environment and in particular to electrostatic fields. This is extremely attractive in the studies of different structurally anisotropic systems such as micelles, monolayers, biological membranes and polymeric structures (Klymcenko *et al.* 2002b).

II.3. MOLECULAR IMPRINTING USING FLUORESCENCE PROBE

The developement of fluorescent sensors for organic molecules is of great practical importance in chemical, biological, and pharmaceutical sciences. Recently, many very sensitive fluorescent sensors have been developed for peptides, metal ions, saccharides, and others. One approach that does not require the prior knowledge of the 3-dimensional structure of the analyte and the complete *de novo design* of the complementary binding site is the construction of polymeric receptors through template polymerization or molecular imprinting. (Liao *et al.* 1999)

Obviously, fluorescent sensors have the advantages of ready detectability and high sensitivity. Efforts have been made to prepare fluorescent sensors using molecular imprinting methods with limited success. Most of these systems rely on the use of fluorescent ligands or fluorotag-ligand conjugates for the fluorescent detections. Therefore, such approaches can only be used for the detection of fluorescent molecules, but cannot be used as a way to build fluorescent sensors for nonfluorescent molecules.

Developing a way to prepare polymeric receptors with a fluorescent probe built into the receptor could provide a general approach to the preperation of fluorescent sensors using molecular imprinting methods. One difficulty in this approach is that the fluorescent probe built into the polymer may or may not be sensitive enough to the binding event (Liao *et al.* 1999).

Shouhai Gao, Wei Wang, and Binghe Wang have designed and synthesized a fluorescent monomer that allows for the preparation of fluorescent sensors of *cis* diols using molecular imprinting methods. This monomer has been used for the preparation of imprinted polymers as sensitive fluorescent sensors for D-fructose. The imprinted

polymers prepared showed significant fluorescence intensity enhancement upon binding with the template carbohydrate. Recently, they have reported a method of making fluorescent sensors using molecular imprinting methods through the use of an external fluorescence quencher to manifest the fluorescence intensity change upon binding of the target compound .Furthermore they have been interested in the synthesis of fluorescent sensors for carbohydrates because of their importance as biomarkers in different biological systems (Gao *et al.* 2001).

Gin and coworkers have constructed a polymeric receptor for cholesterol with a fluorescence probe built into it. However, the fluorescence of the polymeric receptor was not sensitive to the binding event (Gao *et al* 2001).

Yuan Liao and coworkers made efforts in searching for ways to make fluorescent sensors through template polymerization of fluorescent monomers. They used an external fluorescence quencher in studying the binding of the imprinted fluorescent polymer. In conclusion, using template polymerization or molecular imprinting techniques, they have demonstrated that a polymeric fluorescent sensor can be prepared for the template molecule (Liao *et al.* 1999).

A novel design for template-selective recognition sites in polymers prepared by molecular impring was reported by Petra Turkewitsch and coworkers. Molecular imprintings were prepared against cAMP that contain fluorescence dye, trans-4-[p-(N,N-dimethylamino)styryl]-N-vinylbenzylpyridinium chloride, as an integral part of the recognition cavity, for the fluorescence detection of cAMP in aqueous media. This fluorescent molecularly imprinted polymer displays a quenching of fluorescence in the presence of aqueous cAMP, whereas almost no effect is observed in the presence of the structurally similiar molecule, cGMP (Turkewitsch *et al.* 1999).

Fluorescence signals from the chromophores covalently bound to a solid matrix can reveal information about their local environment through changes in their photophysical properties studied by steady state emission and/or time dependent fluorescence measurements (Wandelt *et al.* 2002; Turkewitsch *et al.* 1999).

In our work, we will describe the preperation, using a non-covalent imprinting approach, of a selective MIP using a flavonal probe, 6-(2-hydroxyethylmethacrylate)-4'-N,N-diethylamino-3-hydroxyflavone. The flavonol probing is 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 fluorescent and the presence of hydroxyl and carbonyl groups for intramolecular interactions with the functionalized monomers during non-covalent imprinting.Several other MIP systems have used fluorescence detection methods based either on the use of fluorescent-labeled analytes or on the formation of fluorescent complexes between added reagents and residual functional groups on the polymer. However we have created template-selective sites in a HEMA based polymer for cAMP which contain a fluorescent reporter group as an integral part of the recognition cavity. Obtaining binding and selectivity in aquoeus media is a major requirement for MIP-based sensors designed for biological systems, because the binding event occurs under conditions compatible with biological molecules.

PART III

EXPERIMENTAL WORK

III.1. EXPERIMENTAL PLAN

In the experiments fluorescence probe, molecularly imprinted polymers without fluorescence probe and imprinted polymers with the incorporation of fluorescence probe was synthesized respectively. Also testing of probe and measurements of recognition capabilities performed. Experimental plan is shown in the following list.

1. **Synthesis of Fluorescence Probe**
    - 5-chloromethyl-2-hydroxyacetophenone synthesis
    - 6-etoxymethyl-4'-N,N-diethylamino-3-hydroxyflavone synthesis
    - 6-chloromethyl-4'-N,N-diethylamino-3-hydroxyflavone synthesis
    - 6-(2-hydroxyethyl-methacrylate)-4'-N,N-diethylamino-3-hydroxyflavone synthesis
2. **Preperation of molecularly imprinted systems**
    - Molecular imprinting without fluorescence probe (3-ABA template)
    - Molecular imprinting with fluorescence probe (cAMP template)
3. **Testing & Measurements**
    - Fluorescence probe testing
    - Measurements of recognition capability
        - Imprinted polymers without probe
        - Imprinted polymer with probe

III.2. MATERIALS & EQUIPMENTS

III.2.1. Chemicals

2-Hydroxyethylmethacrylate (HEMA)

HEMA was obtained from MERCK and used with further purification. HEMA was purified by vacuum distillation. Since HEMA is in equilibrium with ethylene glycole dimethacrylate, (EGDM), freshly distilled HEMA was used in preparation of molecularly imprinted polymer (with probe) as functional monomer. Chemical formula and some properties of HEMA is shown below.

Formula: $H_2C=C(CH_3)COOCH_2CH_2OH$

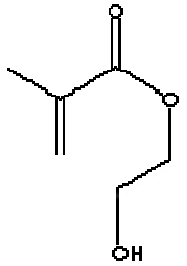


Figure III.1 The chemical structure of 2-hydroxyethylmethacrylate

Molecular Weight :	130.14 g/mol
Physical State :	clear liquid
Melting Point :	-12 ^oC
Boiling Point :	205 ^oC
Specific Gravity :	1.072 – 1.076 g/cm^3
Solubility in Water :	Miscible
Autoignition :	435 ^oC
Flash Point :	107 ^oC

Acrylic Acid

Acrylic acid was obtained from ACROS and used with further purification. Acrylic acid was used at preperation of molecularly imprinted systems (without probe) as functional monomer. Chemical formula and some properties of acrylic acid is shown below.

Formula : CH_2=CHCOOH

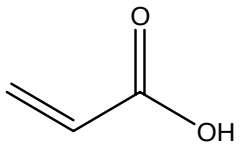


Figure III.2 The chemical structure of acrylic acid

Molecular Weight : 72.06 g/mol

Physical State : clear liquid

Melting Point : 12 ^oC

Boiling Point : 141 – 142 ^oC

Specific Gravity : 1.047 – 1.051 g/cm^3

Solubility in Water : soluble

Autoignition : 435 ^oC

Flash Point : 48 ^oC

Stability : Polymerization will occur under light and heat

Ethylene Glycol Dimethacrylate (EGDM)

EGDM was obtained from Aldrich and used with further purification. EGDM was used at preperation of molecularly imprinted systems (without probe and with probe) as crosslinking agent. Chemical formula and some properties of EGDM is shown below.

Formula : $C_{10}H_{14}O_4$

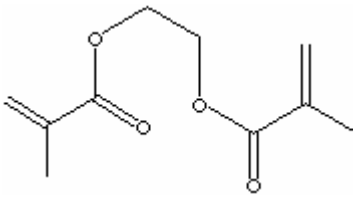


Figure III.3 The chemical structure of ethylene glycol dimethacrylate

Molecular Weight : 198.22 g/mol

Physical State : clear liquid

Boiling Point : 85 ^oC at 2 hPa

Specific Gravity : 1.05 g/cm^3

Flash Point: > 95 ^oC

Stability : The stability depends upon dissolved oxygen and MEHQ inhibitor. The presence of oxygen is necessary for inhibitor to prevent polymerization.

Benzoylperoxide

Benzoylperoxide was obtained from ACROS and used with further purification. Benzoylperoxide was used in preperation of molecularly imprinted system (with probe) as polymerization initiator. Chemical formula and some properties of benzoylperoxide is shown below.

Formula: $(C_6H_5CO)_2O_2$

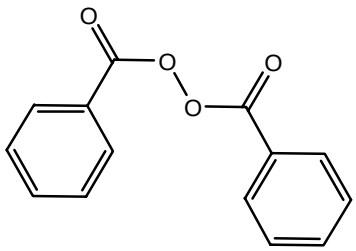


Figure III.4 The chemical structure of benzoyl peroxide

Molecular Weight : 242.23 g/mol

Physical State : white powder

Melting Point : 103-105 ^oC

Boiling Point : Explodes

Specific Gravity : 1.33 – 1.35 g/cm^3

Solubility in Water : Slightly Soluble

Flash Point : 107 ^oC

Azobisisobutyronitrile (AIBN)

AIBN was obtained from ALDRICH and used with further purification. AIBN was used in preperation of molecularly imprinted system (with probe), as initiator. Chemical formula and some properties of AIBN is shown below.

Formula : $(CH_3)_2C(CN)N=NC(CH_3)_2CN$

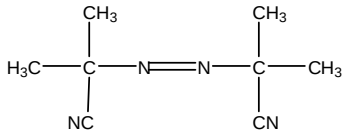


Figure III.5 The chemical structure of azobisisobutyronitrile

Molecular Weight :	164,21 g/mol
Physical State :	white to off-white powder
Melting Point :	Decomposes
Specific Gravity :	1.1 g/cm^3
Solubility in Water :	Insoluble
Autoignition :	64 ^oC

Adenosine 3'-5'-cyclic monophosphate (cAMP)

cAMP was obtained from ACROS and used without further purification. cAMP was used in preperation of molecularly imprinted system (with probe), as template molecule. Chemical formula and some properties of cAMP is shown below.

Formula : $C_{10}H_{12}N_5O_6P$

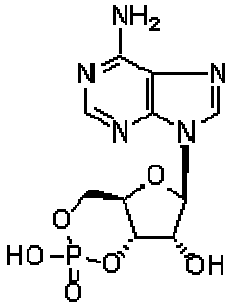


Figure III.6 The chemical structure of adenosine 3'-5'-cyclic monophosphate

Molecular weight : 327.20 g/mol

Physical State : white powder

Melting Point : 260 ^{o}C

Solubility in Water : Soluble

3-amino benzoic acid (3-ABA)

3-ABA was obtained from Aldrich and used without further purification. 3-ABA was used at preperation of molecular imprinted systems (without probe), as template molecule. Chemical formula and some properties of 3 ABA is shown below.

Formula : $H_2NC_6H_4COOH$

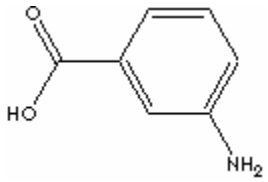


Figure III.7 The chemical structure of 3-amino benzoic acid

Molecular weight : 137.14 g/mol

Physical State : white to yellowish crystal

Melting Point : 178 – 180 ^{o}C

Solubility in Water : Soluble

Stability : Stable under ordinary conditions. Oxidizes in light

Trietylamine (TEA)

TEA was obtained from Aldrich and used without further purification. TEA was used during the synthesis of 6-(2-hydroxymethylmethacrylate)-4'-N,N-diethylamino-3-hydroxyflavone as a hydrochloric acid holder. Chemical formula and some properties of TEA is shown below.

Formula : $N(C_2H_5)_3$

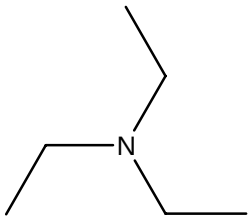


Figure III.8 The chemical structure of triethylamine

Molecular weight : 101.19 g/mol

Physical State : Clear, colorless liquid, strong ammonia-like odor.

Melting Point : -115 ^{o}C

Boiling Point: 89 ^{o}C

Solubility in Water : 5.5 g./100 g

Vapor Density : 3.5 g/cm^3

Stability : Stable under ordinary conditions

Tetrahydrofuran (THF)

THF was obtained from Aldrich and dried by refluxing with metalic sodium and benzophenone under argon until the intense blue colour of benzophenone ketyl appears.

THF was used during the preperation of molecularly imprinted system (with probe) as a porogen and as solvent in other tests. Chemical formula and some properties of THF is shown below.

Formula: C_4H_80

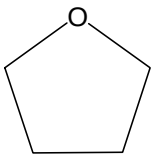


Figure III.9 The chemical structure of tetrahydrofuran

Molecular Weight : 72 g/mol

Physical State : colorless liquid

Boiling Point : 65.965 ^{o}C

Specific Gravity : 0.8892 g/cm^3

Freeze Point : -108.39

Vapor Pressure : 162 mmHg (25 ^{o}C)

Other Solvents

Solvents used for extraction and column chromatography were all distilled before use and for spectral analysis spectroscopic solvents were used.

Benzophenone (Aldrich),Concantrated HCl (Fluka), 2-hydroxy asetephenone (Aldrich), p-formaldehyde (Aldrich), sodium hydroxide (Sigma), H_2O_2 ($_{\%30}$) (Fluka), sodium carbonate (Sigma), sodium sulphate (Sigma), sodiumhydrid (aldrich), methanol (Merck), dicloromethane (Merck), acetic acid (Merck), acetone (Merck), chloroform (Merck), ethyl acetate (Merck).

All solvents used for adsrobtion and flourescence measurements were all obtained from Aldrich and Fluka and have suitable purity for spectroscopy. Also used acetonitril and ethyl acetate solvents were dried by phosphor pentoxide and sodium sulphate.

All molecular imprinting experiments were carried out in the Polymer Laboratories of Chemical Engineering Department at Marmara University, where fluorescence probe synthesis were carried out in the Organic Laboratories of Chemistry Department at Istanbul Technical University.

III.2.3.Electronic Equipment

Ultraviolet Spectrophotometry	Shimadzu UV-1601
Nuclear Magnetic Resonance	250 MHz AC Aspect 3000 model NMR
Flourescence Spectrophotometry	Quanta Master (PTI) and SLM 48000
Refrigerator	Vestel No Frost S 6450 HG
Vacuum pump	KNF Lab, LABOPORT
Balances	OHAUS,model EP214 C, Max 210g
Oven	MMM-Group, Ecocell
Vacuum Oven	MMM-Group, Vacucell
Magnetig Stirrer (Heater)	Mr3001 k, Heidolph, Germany

III.3. EXPERIMENTAL TECHNIQUES

In the experiments synthesis of fluorescence probe, preperation of molecularly imprinted polymers, testing of probe molecule and measurements of recognition capability of imprinted molecules was performed. Experimental route was listed in experimental plan (section III.1)

III.3.1. Flourescence Probe Synthesis

The fluorescence probe, 6-(2-hydroxyethylmethacrylate)-4'-N,N-diethylamino-3-hydroxyflavone was synthesized starting from 2-hydroxyacetophenone in four steps. The synthesis route is in figure III.10

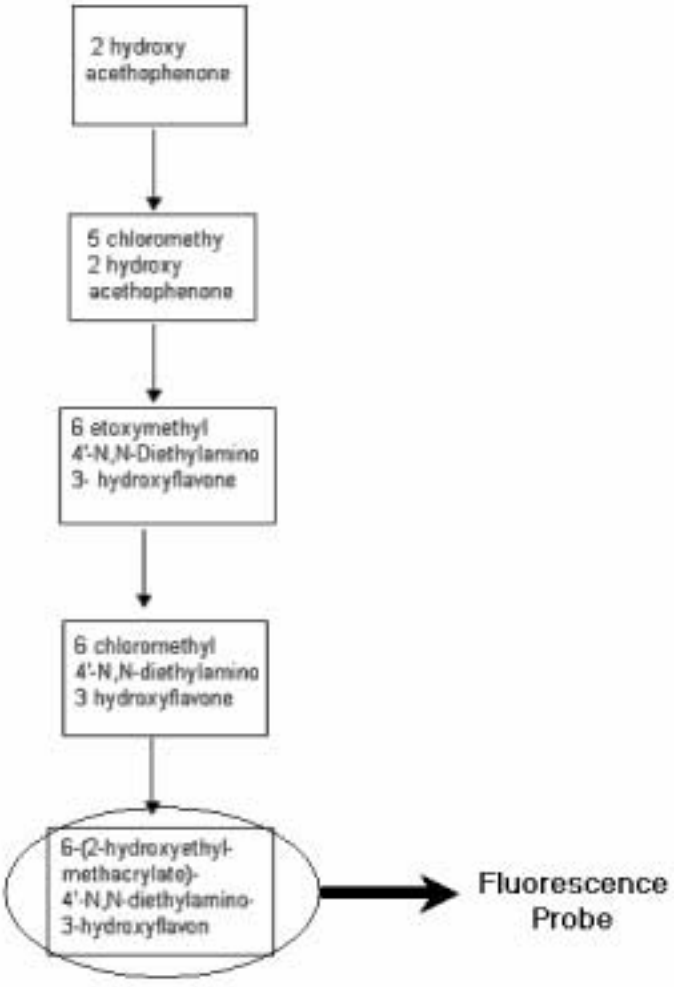


Figure III.10 The experimental route of fluorescence probe synthesis

III.3.1.1. 5-chloromethyl-2-hydroxyacethophenone synthesis

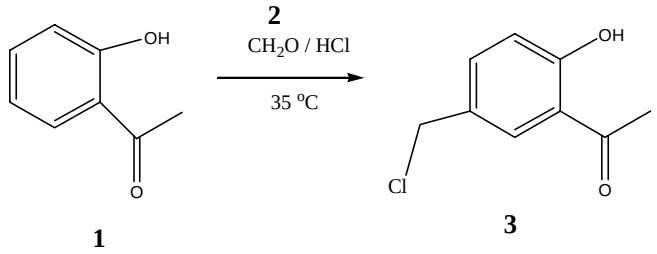


Figure III.11 The 5-chloromethyl-2-hydroxyacethophenone synthesis

A suspension of 5 mL 2-hydroxyacethophenone **(1)** and 1.35 g paraformaldehyde **(2)** in 25 mL of concentrated hydrochloric acid was stirred at 35 °C (water bath at ~55 °C) for 5 hours until the formation of yellow precipitate,which was filtered and washed throughyl with water until the yellowish color disappeared. In the course of the reaction, paraformaldehyde **(2)** must be dissolved well and stirred quickly. The precipitate was dried and washed with minimum amount of methanol. At the end of the reaction 5-chloromethyl-2-hydroxyacethophenone **(3)**was obtained (Klymchenko et al. 2002b).

III.3.1.2. 6-etoxymethyl-4'-N,N-diethylamino-3-hydroxyflavone synthesis

i) Calcone Synthesis

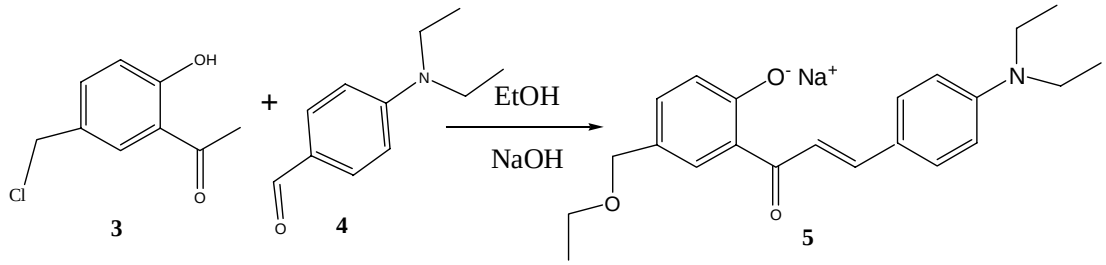
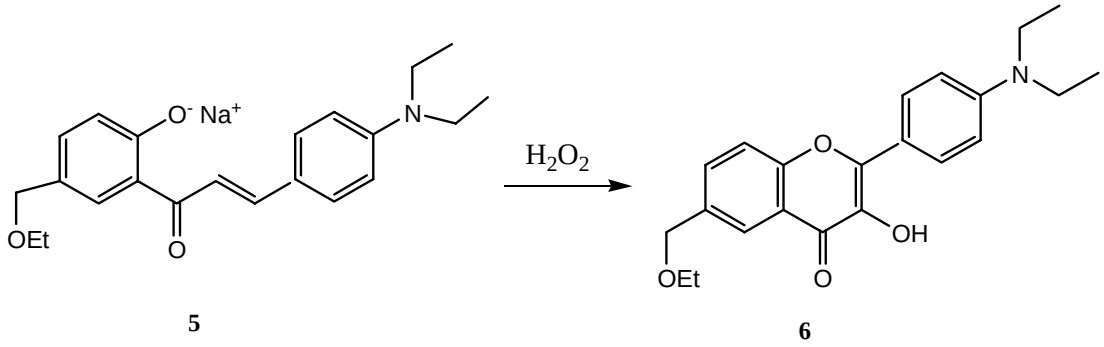


Figure III.12 The calcone synthesis from 5- chloromethy-2-hydroxyacethophenone

1 g of 5-chloromethyl-2-hydroxyacetophenone **(3)** was dissolved in 20 mL of ethanol. The solution was treated with 1.08 g of sodium hydroxide in 10 mL water, and the mixture was refluxed for 30 min until it becomes homogeneous. To the resultant

solution, 0.97 g of 4'-N,N-diethylaminobenzaldehyde **(4)** was added.The mixture was stirred for 8 hours and then left for 3 days at room temperature(Klymchenko 2002b).

ii)Ring Closing

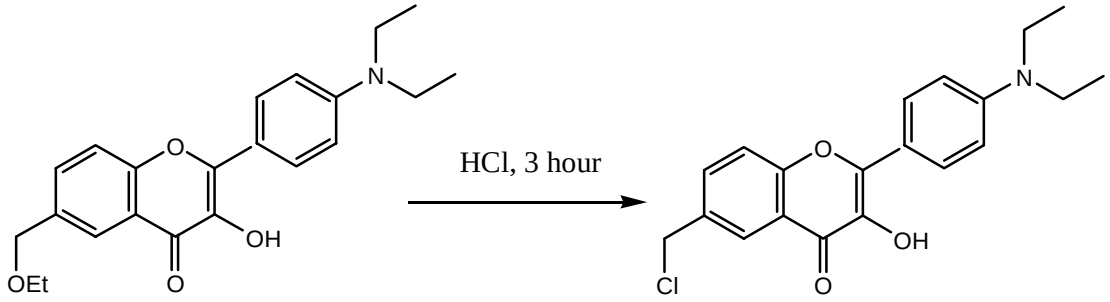


5 **6**

Figure III.13 The 6-etoxymethyl-4'-N,N-diethylamino-3-hydroxyflavone synthesis by ring closing

The red solution of sodium salt of 6-etoxyethylamino-4'-diethylamino-2-hydroxychalcone **(5)** was cooled on an ice bath and treated with 14,328g (0,1475 mol) of 30% hydrogen peroxide while stirring vigorously. The progress of the reaction was controlled by TLC with chloroform : methanol = 9:1 as mobile phase. The solution of the product was neutralized by acetic acid, and the precipitate was filtered and purified by column chromatography on silica gel (ethyl acetate : hexane= 1:1).

III.3.1.3. 6-chloromethyl-4'-N,N-diethylamino-3-hydroxyflavone synthesis



6 **7**

Figure III.14 The 6-chloromethyl-4'-N,N-diethylamino-3 hydroxyflavone synthesis

6-etoxymethyl-4'-N,N-diethylamino-3-hydroxyflavone **(6)** in hydrochloric acid (10 mL) was heated on an oil bath at 100 ^{o} C for 3 hours. The cooled solution was neutralized with %20 sodium carbonate, and the resultant precipitate was filtered and washed with water. The reaction was controlled by TLC with methanole : ethylacetate = 9:1 as mobile phase and purified by column chromatography on silica gel (methanole : ethyl acetate=1:1).

III.3.1.4. 6-(2-hydroxyethylmethacrylate)-4'-N,N-diethylamino-3-hydroxyflavone synthesis

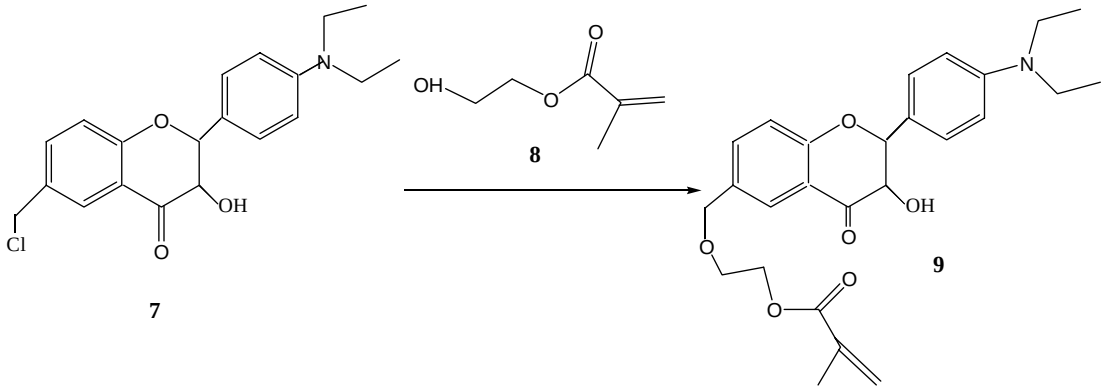


Figure III.15 The 6-(2-hydroxyethylmethacrylate)-4'-N,N-diethylamino-3-hydroxyflavone synthesis

The reaction was carried out in a 25 mL round bottom flask (RB) equipped with a teflon coated magnetic stirring rod and a rubber septa. 0.1 g of chlorinated flavone (**7**) was placed into the RB flask. After being sealed, the RB flask was purged with dry argon for 0.5 hour and 5 mL of dry THF was transferred under argon atmosphere, which resulted in a clear and yellowish solution. After successively addition of 3 mL of triethylamine (TEA) and 1.5 mL of HEMA (**8**), the reaction was left stirring for 1 week at room temperature. The solution was filtered and the solvent was evaporated under reduced pressure until approximately 2 mL of mixture remained. The mixture was dissolved in dichloromethane and the excess HEMA and TEA were extracted with cold water. Column chromatography was used for purification. The solvent system was dichloromethane /hexane (7/2 and 7/1, v/v) and dichloromethane.

III.3.2. Preperation Of Molecularly Imprinted(Mip) Systems

Two types of MIP systems with 3-ABA only and cAMP + fluorescence probe was prepared. Molecular recognition capability of 3-ABA imprinted systems was tested in 3-amino benzoic acid + naphtonoic acid by HPLC. The recognition capability of the cAMP imprinted system was followed by fluorescence measurement.

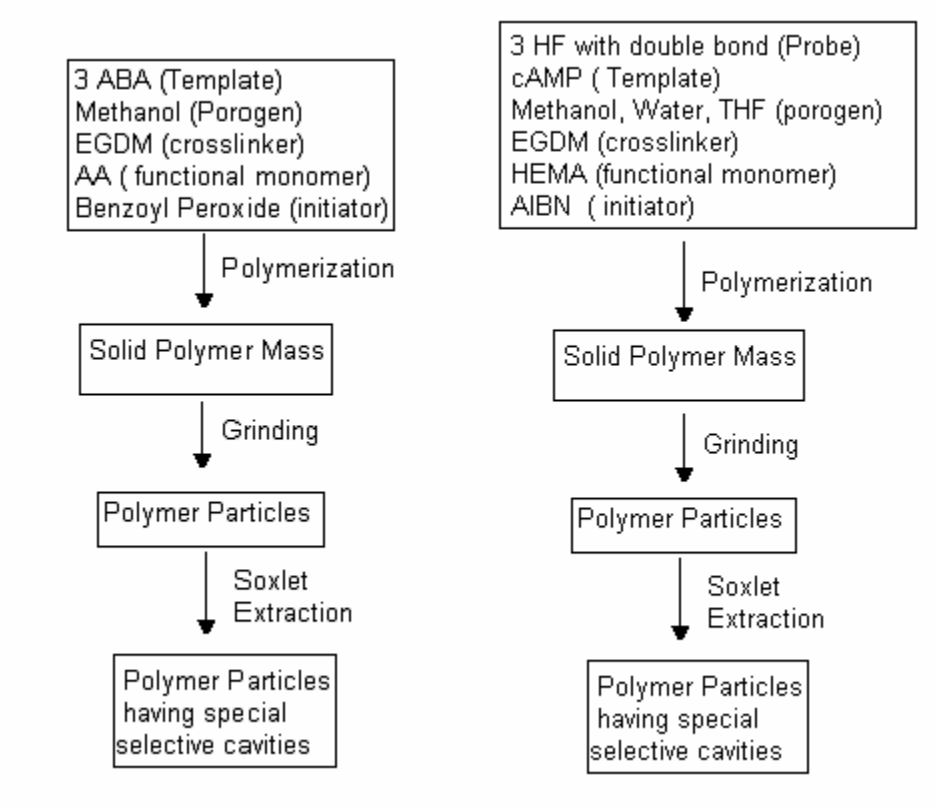


Figure III.16 Impriting process of polymers without a probe and with a probe respectively

III.3.2.1.Molecular Imprinting without fluorescence probe

The 3-aminobenzoic acid imprinted system was prepared by adding 30 ml methanol to the template molecule, 3-aminobenzoic acid (16.303 mg, 119 μmol) in a glass specimen bottle. The mixture was stirred until all the 3-aminobenzioc acid was dissolved. The cross-linker, EGDM (25,542 g, 129 mmol), the functional monomer , Acrylic Acid (0.3736 g, 5.19 mmol) and the initiator , benzoil peroxide (0.981 g, 4.05 mmol) , were then added. The mixture was purged with nitrogen for 10 min and was subsequently placed in an oil bath at 60 ^{o}C for 3-4 hours. The white solid polymer was

obtained by breaking the reaction tube and the polymer finely grinded using mortar and pestle. The unreacted monomers and cAMP were removed by continuous soxhlet extraction with water/methanol (1:1, v/v) for 1 week. The polymer particles were dried under vacuum (20 mm Hg) at 40 ^{o}C overnight and stored in airtight vials. Different compositions of imprinted polymers were prepared by the same way and all are shown in table III.1.

Table III.1 Compositions of 3-amino benzoic acid imprinted polymers

	Template	Crosslinker	Monomer	Initiator	Ratio	
	3-amino benzoic acid	EGDM	Acrylic acid	Benzoyl peroxide	Template/ Monomer	Crosslinker/ Monomer
1.	0,0163 g	25,542g	0,3736 g	0,981 g	0,0436295	68.37
2.	0,0163 g	25,542	0,4257 g	0,981 g	0,0382898	60
3.	0,0163 g	25,542g	0,51084 g	0,981 g	0,031908229	50
4.	0,0163 g	25,542g	0,63855 g	0,981 g	0,025526583	40

III.3.2.2. Molecular Imprinting with fluorescence probe

0,9 mg of probe dissolved , 1 mL THF was added into 4 mL of methanol/water (3:1, v/v) mixture. Then cAMP (5,2125 mg, 14,875 µmol) was added in this solution in a glass specimen bottle and the mixture was stirred and sonicated until all the cAMP was dissolved. The crosslinker monomer, EGDM (2,55 g, 12,9 mmol), the functional monomer, HEMA (0,0675g, 0,519 mmol) and the initiator, AIBN (0,0665g, 0,405 mmol) were successively then added into the reaction tube. It was purged with argon for 10 minute and subsequently placed in an oil bath at 60 ^{o}C for 3-4 hours. A yellowish polymer was obtained. The yellowish solid polymer was obtained by breaking the reaction tube and the polymer finely grinded using mortar and pestle. The unreacted monomers and cAMP were removed by continuous soxhlet extraction with methanol/water (1:1, v/v) for one week. The polymer particles were dried under vacuum (20 mmHg) at 40 ^{o}C overnight and stored in airtight vials.

III.3.3. Testing & Measurements

Fluorescence probe was tested with fluorescence spectroscopy and the measurements of template recognition capabilities of imprinted systems, which has been prepared without and with probe, was performed. The 3 amino benzoic acid imprinted systems were followed by HPLC while fluorescence spectroscopy was used for the determination of recognition capability of cAMP imprinted system.

III.3.3.1. Fluorescence Probe Testing

For testing the variations of fluorescence probe, 0,45 mg of probe molecule was dissolved in 1 mL of dry THF and placed into a 1 cm quartz cuvette. The water drops each weighted of 32.36 mg was added respectively and followed by a Photon Technology International (PTI) Quanta Master spectrofluorometer by front face illumination. For each sample the water percent was % 0, %3.5, %6.8,% 9.8, %12.7,%15.4 and %1,9 of the total mass, respectively.

III.3.3.2. Measurements of recognition capability

The recognition capabilities of imprinted systems without and with probe , was measured by different methods and explained below separately.

Imprinted polymers without probe

A solution of 50 mL methanol and 50 mL water containing 0,25 gr 3-aminobenzoic acid and 0.25 gr 2 Hydroxy-1-naphthoic acid was prepared. The imprinted systems having different ratios of crosslinker/monomer (68.37, 60, 50, 40) were plunged to the solutions seperately and kept at constant 40 ^{o}C for 2 weeks. The recognition capabilities of each imprinted system was followed by HPLC

Imprinted polymer with probe

A solution of 50 mg cAMP, 50 mg 3-amino benzoic acid,2-hydroxy-1-naphtoic acid, 50 mL methanol and 50 mL water was prepared. The imprinted system with fluorescence probe was plunged , suspended in the mixture and saved in a well sealed bottle by continious stirring with a magnetic stirrer. Suspended sample was centrifuged at 4500 rpm for 15 minute and the fluorescence of the polymer was evaluated on the same 1mL of centrifuged samle. The measurements of recognition capability of

imprinted system, were carried out with a Photon Technology International (PTI) Quanta Master spectrofluorometer by front face illumination in 4 different days (5^{th},8^{th}, 12^{th} and 15^{th}).

PART IV

RESULTS AND DISCUSSION

IV.1. FLUORESCENCE PROBE

The fluorescence probe, 6-(2-hydroxyethylmethacrylate)-4'-N,N-diethylamino-3-hydroxyflavone, was synthesized starting from 2-hydroxyacetophenone in four steps as described in section III.3.1. Purities of products at each step was controlled with ^{1}H-NMR and mass spectroscopy,thus their chemical properties were determined. A Photon Technology International (PTI) Quanta Master spectrofluorometer was used for testing the fluorescence properties of final product, vinylated 3 HF fluorescence probe,with the change in environment polarity . The interaction of fluorescence probe and cAMP molecule was also tested by using spectrofluorometer. Results obtained are presented seperately for each step below.

IV.1.1. The 5-chloromethyl-2-hydroxyacetophenone

The 5-chloromethyl-2-hydroxyacetophenone was synthesized as described in section III.3.1.1. Product purity was followed by ^{1}H-NMR and it was pure enough to be used in the next step. ^{1}H-NMR spectrum and some chemical properties has been shown below.

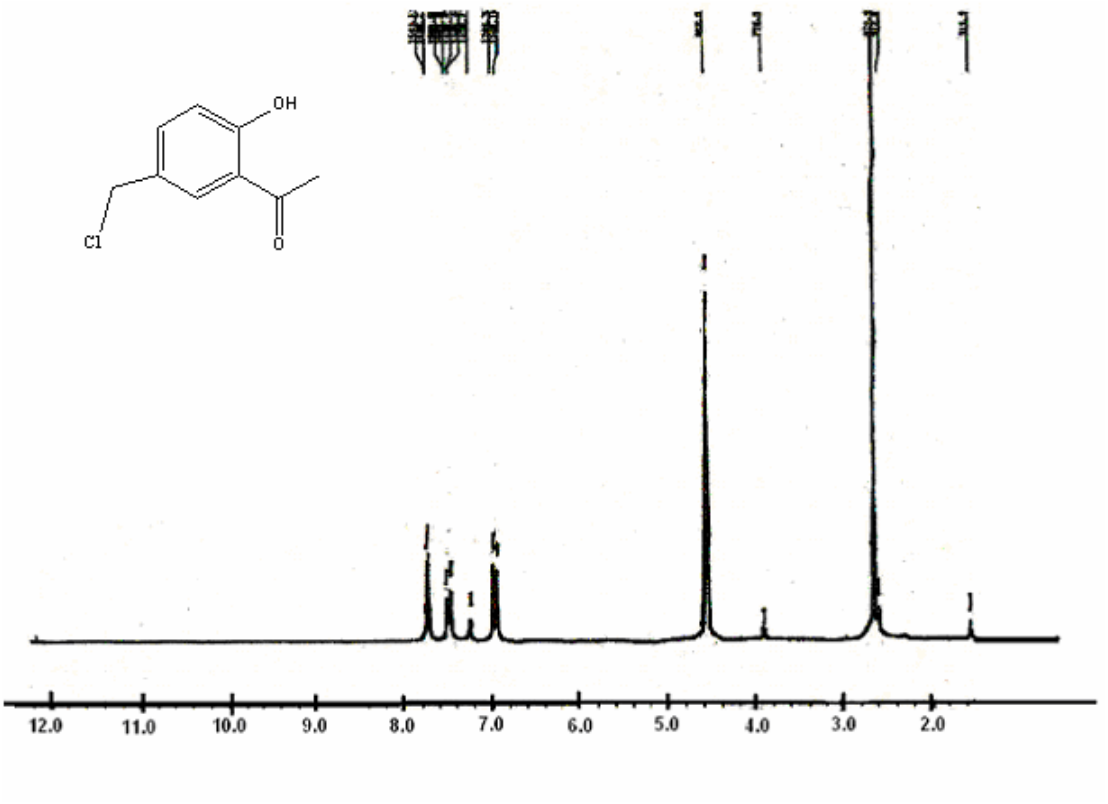


Figure IV.1 ^{1}H-NMR spectrum of 5-chloromethyl-2-hydroxyacetophenone

Product %60 , mp: 78-81 ^{o}C, ^{1}H-NMR (250 MHz, $CDCl_3$) δ 2,65(s,3H), 4.65 (2H, s), 6.97 (1H, dd, *J* 8.6 Hz, 2.1 Hz),7,49 (1H,dd,J 8.6 Hz,2.1Hz), 7.73 (1H, d, *J* 2.1 Hz), 12.31 (1H, s) ; Anal Calcd. $C_9H_9ClO_2$C 58.55 %, H 4.91;
Found C 58.74 %, H 4.99 %.

IV.1.2. The 6-etoxymethyl-4'-N,N-diethylamino-3 hydroxyflavone

The 6-etoxymethyl-4'-N,N-Diethylamino-3-hydroxyflavon was synthesized as described in section III.3.1.2. Product purity was followed by MASS and ^{1}H-NMR and it was pure enough to be used in the next step. Spectra and some chemical properties have been shown below.

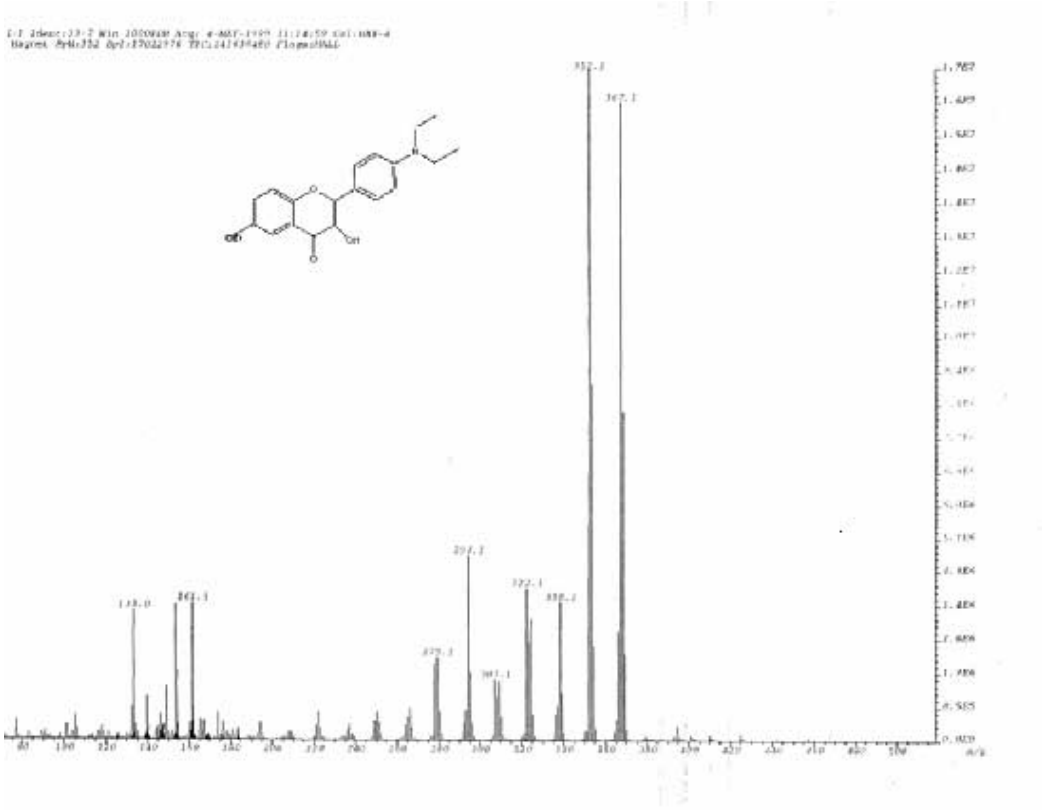


Figure IV.2 MASS Spectrum of 6-etoxymethyl-4'-N,N-diethylamino-3-hydroxyflavone

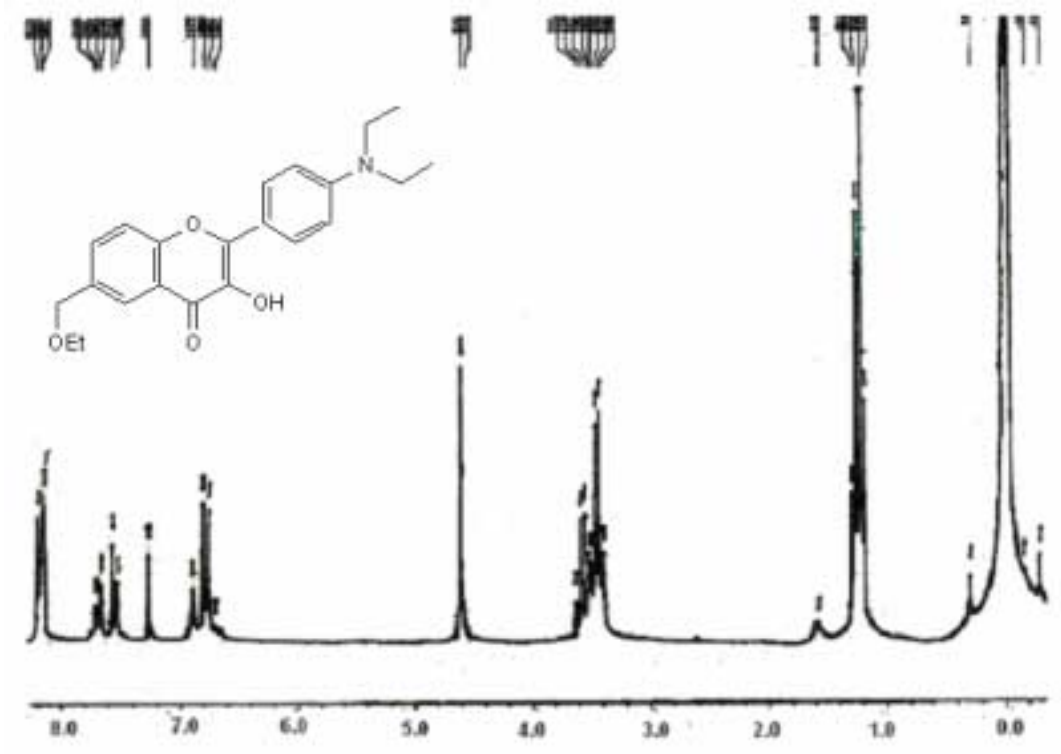


Figure IV.3 ^{1}H-NMR Spectrum of 6-etoxymethyl-4'-N,N-diethylamino-3-hydroxyflavone

Product : 34 % ; m.p.132 ^{o}C ; ^{1}H-NMR (250 MHz, $CDCl_3$) δ 1.19-1.3 (9H, m), 3.45 (4H, q, *J*=7.14 Hz), 3.57 (2H, *J*=7.16 Hz), 4.61 (2H, s), 6.77 (2H, d, *J*=9.04 Hz), 6.89 (1H, s), 7.54 (1H, d, *J*=8.68 Hz), 7.68 (1H, dd, *J*=8.68 Hz, 1.78 Hz), 8.15(1H,d,J=1.78), 8.18(2H, J=9.04 Hz) ; MS (EI) *m/z* 367 (M+) ; Anal Calcd. $C_{22}H_{25}NO_4$C 71.90 %, H 6.85 %, N 3.80; found C 71.54 %, H 6.79 %, N 3.47.

IV.1.3. The 6-chloromethyl-4'-N,N-diethylamino-3 hydroxyflavone

The 6-chloromethyl-4'-N,N-diethylamino-3-hydroxyflavone was synthesized as described in section III.3.1.3. Product purity was followed by MASS and ^{1}H-NMR and it was pure enough to be used in the next step. Spectra and some chemical properties have been shown below.

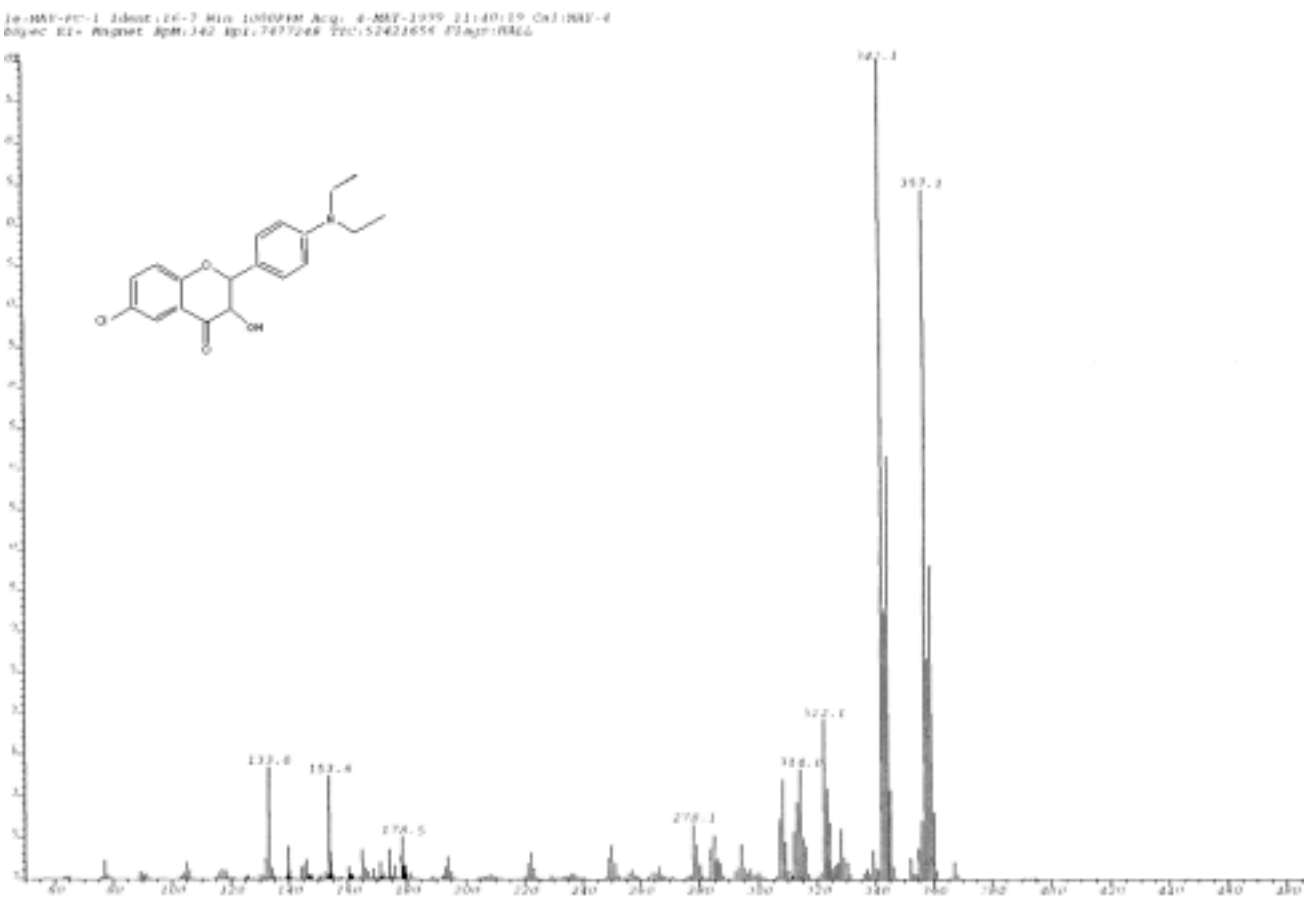


Figure IV.4 MASS Spectrum of 6-chloromethyl-4'-N,N-diethylamino-3 hydroxyflavone

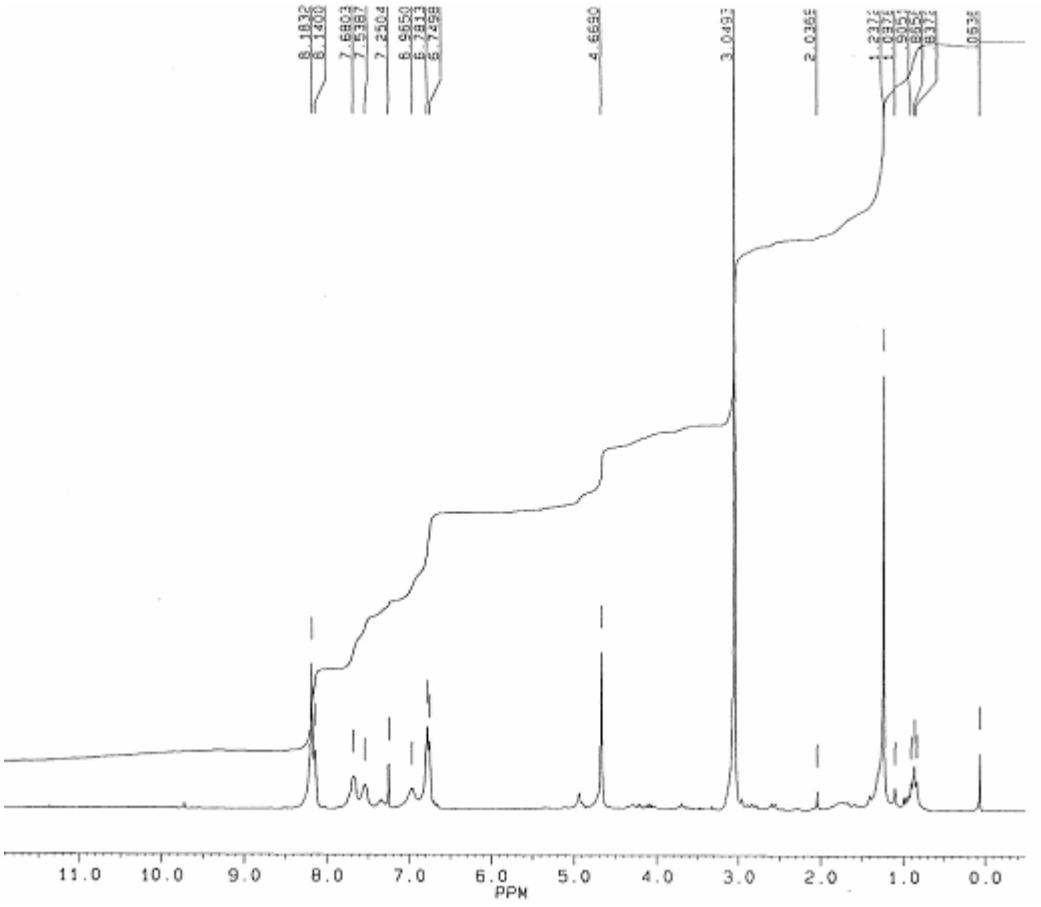


Figure IV.5 ^{1}H-NMR Spectrum of 6-chloromethyl-4'-N,N-diethylamino-3-hydroxyflavone

Product : 74 %, m.p. 138-140; ^{1}H-NMR (250 MHz, $CDCl_3$)δ 1.23 (6H, J=7.0, t), 3.46 (4H, q, J=7.0 Hz), 4.62 (2H, s), 6.67 (2H, d, J=8.50 Hz), 6.87 (1H, s), 7.55 (1H, d, J= 8.73 Hz), 7.68 (1H, dd, J=8.73 Hz, 1.93 Hz), 8.16 (2H, d, J=8.5 Hz), 8.21 (1H, J=1.93 Hz, d) ; MS (EI) m/z 357 (M+); Anal Calcd. $C_{20}H_{20}ClNO_3C$ 67.13 %, H 5.63 %, H 5.63 %, N 3.91 ; found C 67.55 %, H 5.81, N 3.49 %.

IV.1.4. The 6-(2-hydroxymethylmethacrylate)-4'-N,N-diethylamino-3-hydroxyflavone

The probe molecule, 6-(2-hydroxymethylmethacrylate)-4'-N,N-diethylamino-3 hydroxyflavone, was synthesized as described in section III.3.1.4. Product purity was

followed by ^{1}H-NMR and it was pure enough to be used in the next step. Spectra and some chemical properties have been shown below.

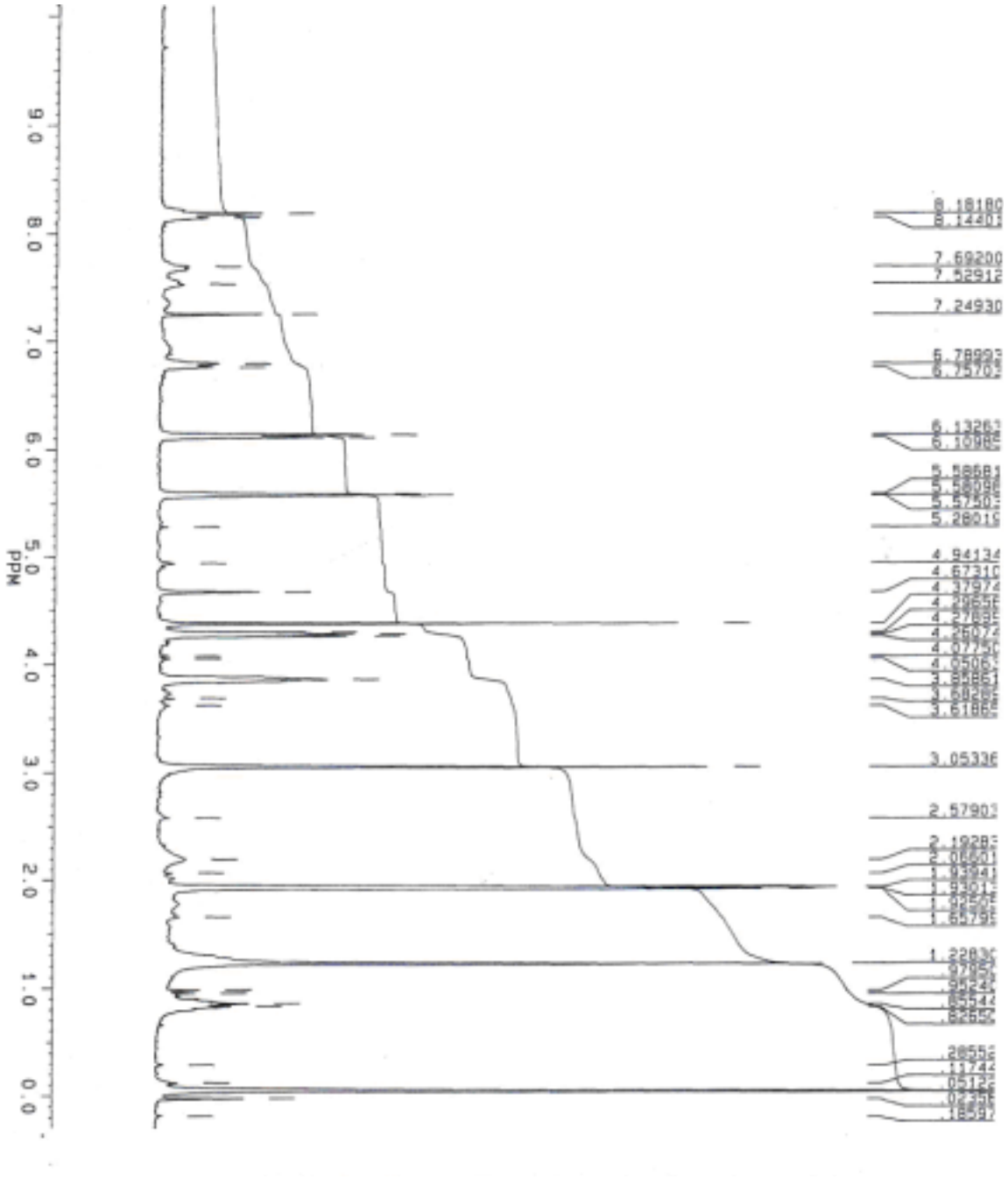


Figure IV.6 ^{1}H-NMR Spectrum of 6-(2-hydroxymethylmethacrylate)-4'-N,N-diethylamino-3-hydroxyflavone

^{1}H NMR (250 MHz, $CDCl_3$) δ 1.22 (6H, s, NCH_2CH_3), 1.93 (3H, s, CH_3), 3.05 (4H, q, *J*=6.5 Hz, NCH_2CH_3), 3.85 (2H, *J*=4.50 Hz, t, OCH_2CH_2O), 4.27 (2H, *J*=4.50 Hz, t, OCH_2CH_2O), 4.37 (2H, s, OCH_2Ph), 5.58 (1H, *J*=5.7 Hz, d, $=CH_2$), 6.12 (1H, *J*=5.7 Hz, d, $=CH_2$), 6.77 (2H, d, *J*=8.23 Hz, Ar), 6.80 (1H,bs), 7.53(1H,d,J=8,65 Hz), 7.70 (1H,d,J=8,65 Hz), 8.17 (2H, d, *J*=8.23 Hz, Ar), 8.19 (1H, s, Ar) ; MS (EI) *m/z* 352 (M+1).

Testing of probe with fluorescence spectroscopy

The variations in fluorescence intensity with increasing polarity, was followed with a Photon Technology International (PTI) Quanta Master spectrofluorometer as described in section III.3.3.1. The spectra is below.

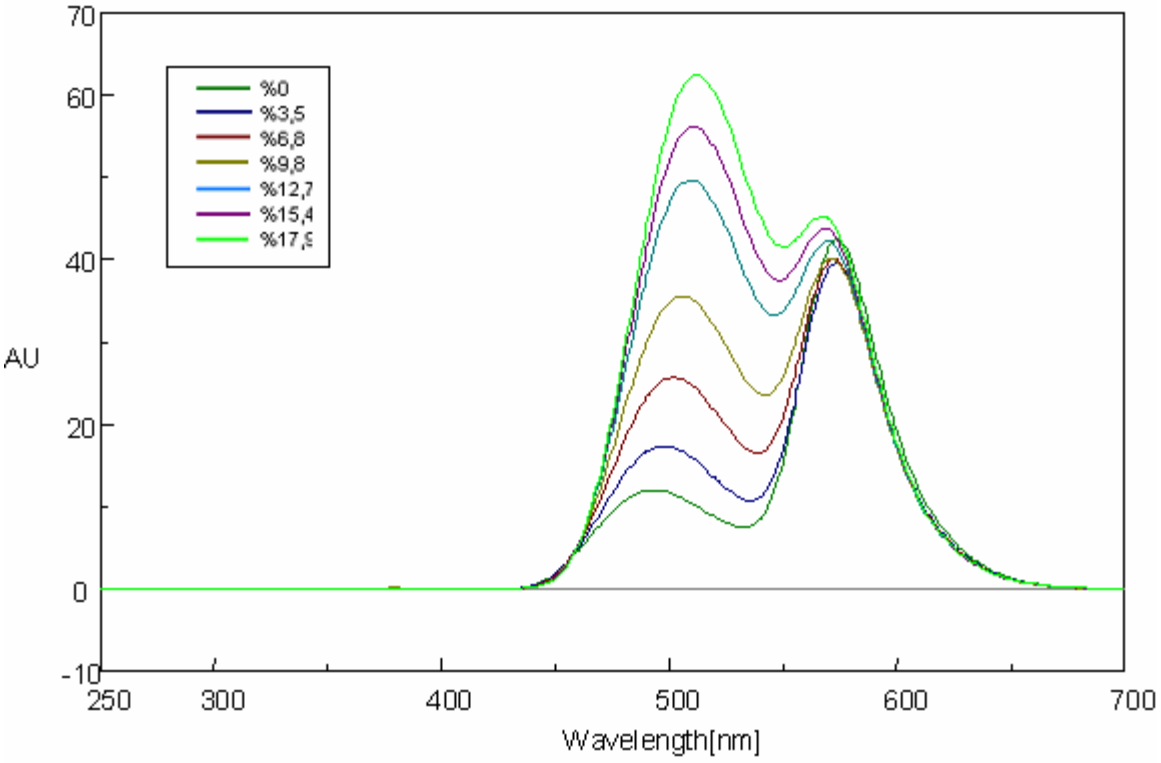


Figure IV.7 Changes in N* and T* bands with increasing percentage of water

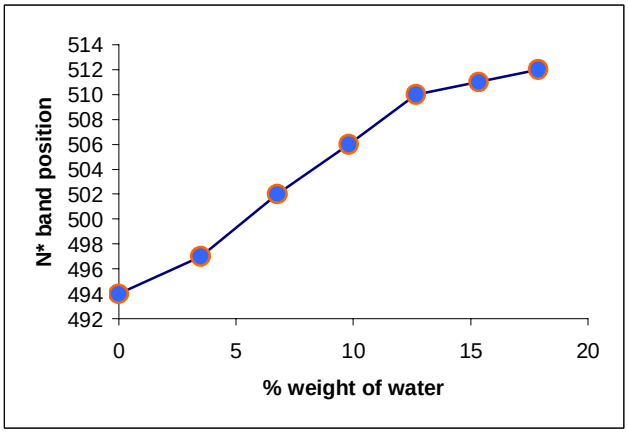


Figure IV.8: Changes in band positions of N* with increasing polarity

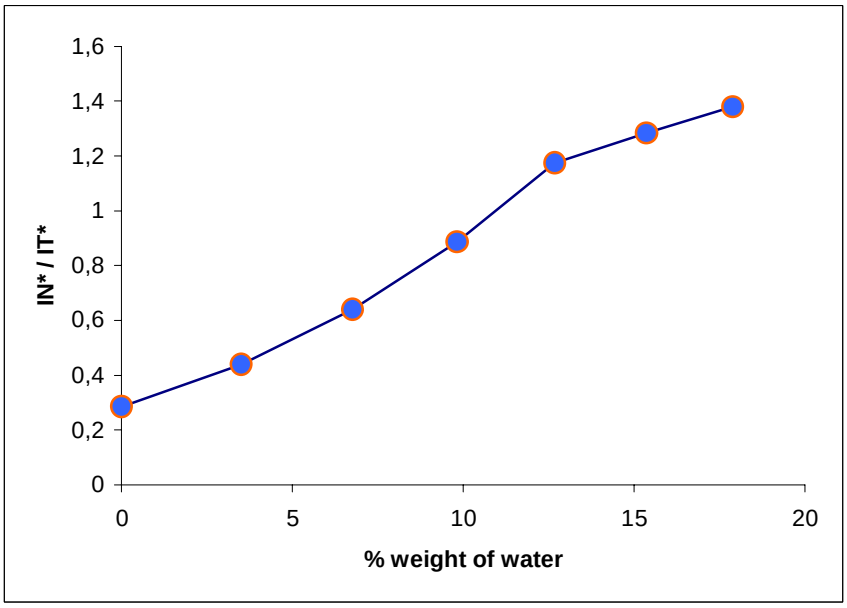


Figure IV.9 Changes in I_{N^*} / I_{T^*} with increasing polarity

From the fluorescence spectra of the 6-(2-hydroxymethylmethacrylate)-4'-N,N-diethylamino-3 hydroxyflavone, very high intensities of N* and T* bands were observed. These two bands formed as a result of ESIPT reaction and known as characteristic properties of all 3HF and 3HC derivatives like Demchenko, Klymenchenko and others agreed. The variations in fluorescence properties, N* band position and ratio of N* and T* band intensities, shown in Figure IV.8 and IV.9. It was observed that N* band of the fluorescence probe exhibited a dramatic increase both in

position and intensity while T* band remained constant as a function of increased water percent.Thus the intensity ratios of N* and T* bands were also increased. Water is a polar-protic solvent and an increasement in water percent also cause an increase in polarity. Thus probe variations was considered to be due to the increased environment polarity. Klymchenko,Pivovarenko, Demchenko, Bader, Ariese and Gooijer were also experimented other 3-HF derivatives and all concluded with the same result.

IV.2. MOLECULARLY IMPRINTED SYSTEMS

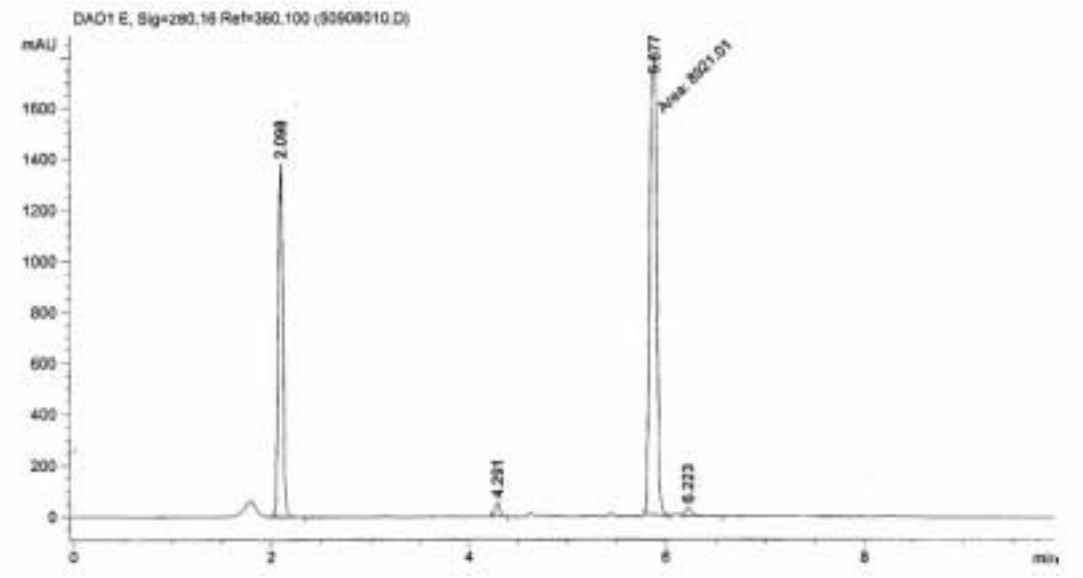
Two types of MIP systems with 3-amino benzoic acid only and cAMP + fluorescence probe was prepared as explained in section III.3.2 and each prepared to measurements seperately as it explained in III.3.3 The results obtained are presented separately for both imprinting systems below.

IV.2.1. Molecular Imprinting without fluorescence probe

The effect of crosslinker amounts on recognition capability of imprinted systems were investigated by crosslinler/monomer variation in polymer synthesis. Crosslinker/monomer ratios were 40, 50, 60 and 6.37. The recognition capability of imprinted systems was defined as the decreased amounts of 3 ABA in solution and followed by HPLC. The results obtained are presented separately for each ratio below.

Crosslinker / Monomer Ratio: 40

In Figure IV.10 crosslinker EGDM ratio to monomer Acrylic acid is 40.This was the smallest ratio and least rigid structure. The starting concentrations of 3-amino benzoic acid and 2-hydroxy-1-naphthoic acid were the 0.5 mg/mL.The 3-amino benzoic acid concentration decreased to 4.80737 mg/mL and 2 hydroxy-1-naphthoic acid was decreased to 4.92348 mg/mL. Decreased amount of 2-hydroxy-1-naphtoic acid was very low compare with 3-aminobenzoic acid. This means that 0.19263 mg/mL of 3-aminobenzoic acid was trapped within the imprinted polymer and the functionalised cavities can recognise template molecule. The 2-hydroxy-1-naphtoic acid can not be recognised since the cavities were only complementary with 3-amino benzoic acid.

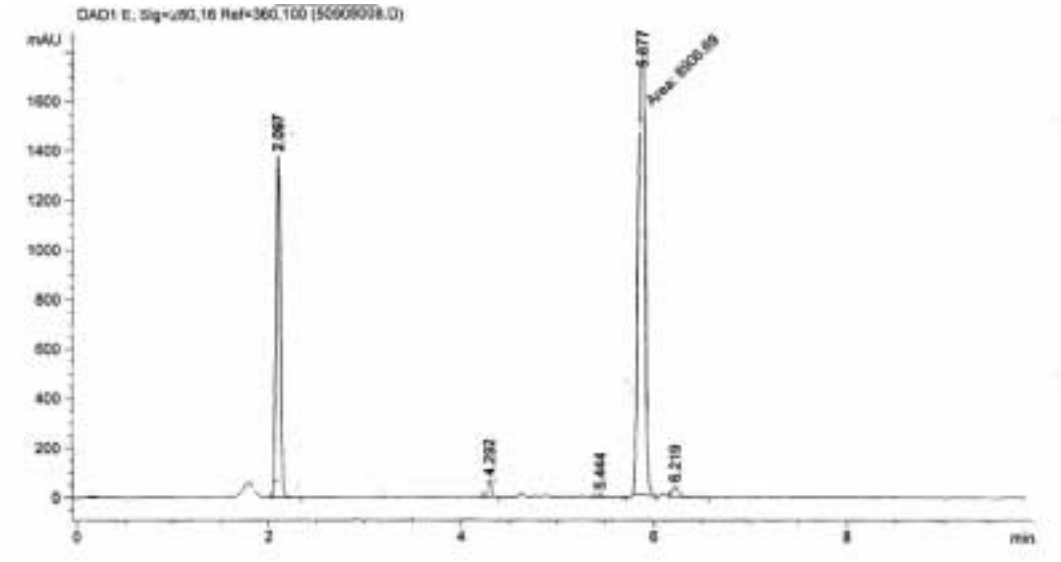


RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [mg/ml]	Grp	Name
2.098	VB	4433.52148	1.08432e-3	4.80737	3	amino benz ac
5.877	MM	8921.01172	5.51897e-4	4.92348	2	hydr napht ac
Totals :				9.73085		

Figure IV.10 HPLC results for crosslinker / monomer :40 ratio

Crosslinker / Monomer Ratio: 50

In Figure IV.11 the crosslinker EGDM ratio to monomer acrylic acid was 50. The starting concentrations of 3-amino benzoic acid and 2-hydroxy-1-naphthoic acid was 0.5 mg/mL. The 3-amino benzoic acid concentration decreased to 4.77211 mg/mL and 2 hydroxy-1-naphthoic acid was decreased to 4.91558 mg/mL. Decreased amounts of 2-hydroxy-1-naphtoic acid was very low compare with 3-amino benzoic acid. This means that 0.22789 mg/mL of 3-amino benzoic acid was trapped within the gel and the functional cavities can recognise template molecule (3-amino benzoic acid). The 2-hydroxy-1-naphtoic acid can not be recognised since the cavities were only complementary with 3-amino benzoic acid.

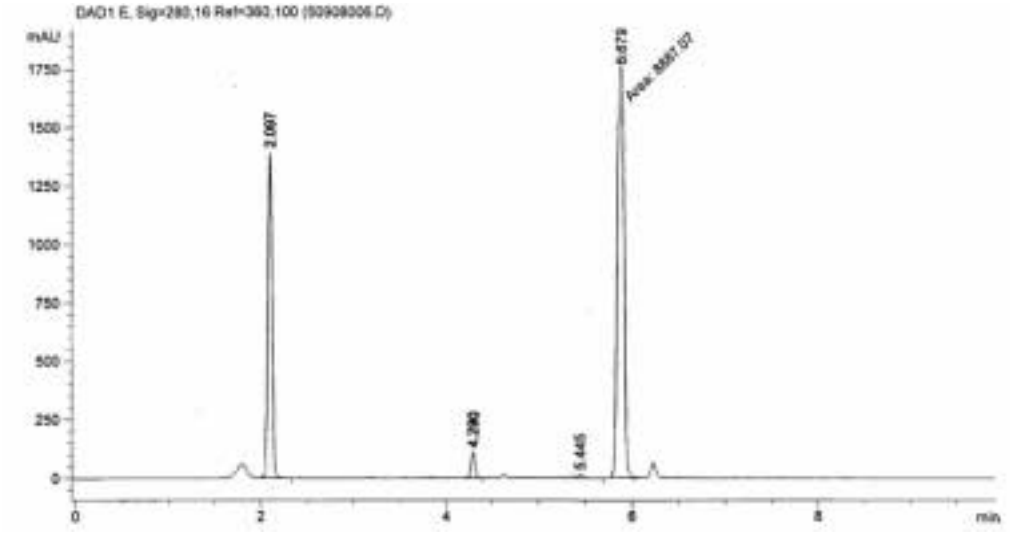


RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [mg/ml]	Grp	Name
2.097	VB	4401.00146	1.08432e-3	4.77211	3	amino benz ac
5.877	MM	8906.69336	5.51897e-4	4.91558	2	hydr napht ac
Totals :				9.68769		

Figure IV.11 HPLC results for crosslinker / monomer :50 ratio

Crosslinler /monomer Ratio: 60

In figure IV.12 the crosslinker EGDM ratio to monomer acrylic acid was 60.This was the second highest ratio and the second most rigid structure. The starting concentrations of 3-amino benzoic acid and 2-hydroxy-1-naphthoic acid was 0.5 mg/mL .The 3-amino benzoic acid concentration decreased to 4.83692 mg/mL and 2 hydroxy-1-naphthoic acid was decreased to 4.90474 mg/mL. Decreased amount of 2-hydroxy-1-naphtoic acid was very low compare with 3-aminobenzoic acid. This means that 0.16308 mg/mL of 3-aminobenzoic acid was trapped within the gel and functional cavites can recognise template molecule (3-amino benzoic acid). The 2-hydroxy-1-naphtoic acid can not be recognised since the cavities were only complementary with 3-amino benzoic acid.

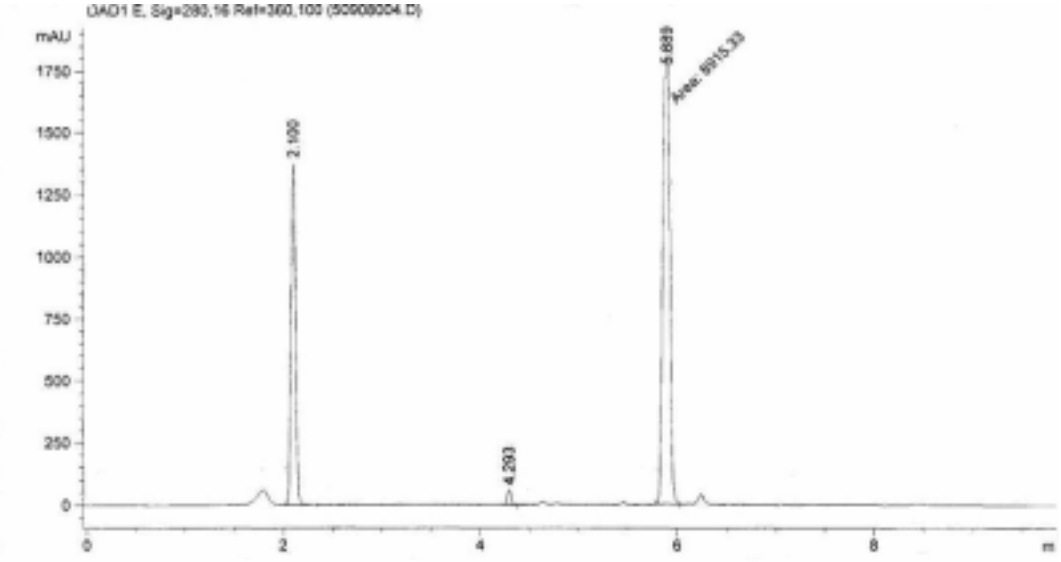


RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [mg/ml]	Grp	Name
2.097	VB	4460.77734	1.08432e-3	4.83692	3	amino benz ac
5.879	MM	8887.06836	5.51897e-4	4.90475	2	hydr napht ac
Totals :				9.74167		

Figure IV.12 HPLC results for crosslinker / monomer :60 ratio

Crosslinker/Monomer Ratio: 68.37

In figure IV.13 crosslinker EGDM ratio to monomer acrylic acid was 68.67. This ratio was the highest and reflects the presence of most croslinked and rigid polymer. The starting concentrations of 3-amino benzoic acid and 2-hydroxy-1-naphthoic acid were 0.5 mg/mL .The 3-amino benzoic acid concentration decreased to 4.73201 mg/mL and 2 hydroxy-1-naphthoic acid was decreased to 4.92035 mg/mL. Decreased amount of 2-hydroxy-1-naphtoic acid was very low compare with 3-aminobenzoic acid. This means that 0.26799 mg/mL of 3-aminobenzoic acid was trapped within the gel and functional cavities can recognise template molecule. The 2-hydroxy-1-naphtoic acid can not be recognised since the cavities were only complementary with 3-amino benzoic acid.



RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [mg/ml]	Grp	Name
2.100	VB	4364.01709	1.08432e-3	4.73201	3	amino benz ac
5.889	MM	8915.33496	5.51897e-4	4.92035	2	hydr napht ac
Totals :				9.65235		

Figure IV.13 HPLC results for crosslinker / monomer :68.37 ratio

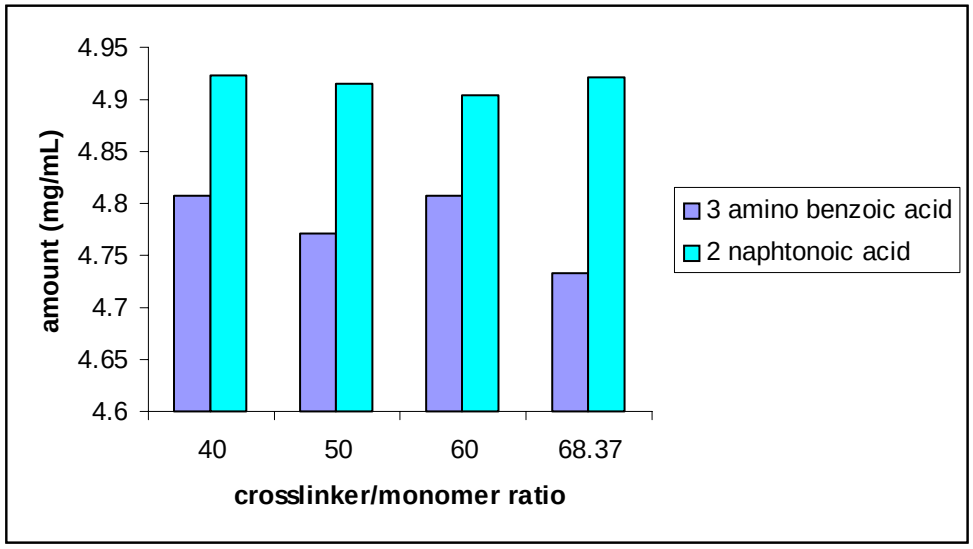


Figure IV.14 Changes in template concentration with increasing crosslinker /monomer ratio.

In Figure IV.14 the 3-amino benzoic acid concentration decreased with increasing crosslinker/monomer ratio. However, it increased when crosslinker/monomer ratio was

60.This was considered to be due to the experimental mistake. The 68,67 crosslinker/monomer ratio represents the maximum recognition capability for template molecule. For 2-hydroxy-1-naphtonoic acid, there was a slightly decrease when ratio was 60 and almost constant for the rest of the ratios. It can be concluded that the prepared imprinted polymers all have selectivity only for the template molecule 3-amino benzoic acid, since the cavities formed inside the polymer were in the shape of 3-amino benzoic acid. Also high crosslinker/monomer ratios are necessary since the lower ratios were resulted diminished rigidity and template affinity . Morphology and appearence were changing with deceasing crosslinker/ monomer ratios as a result of diminished rigidity. Highly crosslinked polymers were in white rigid chalk appearence while very low crosslinked ones were in transparent jelly appearence. Alexandra Molinelli (2004) studied the effect of varying crosslinker/monomer ratios on polymer recognition properties, and on polymer morphology and found similar behaviour.Varying crosslinker ratios enabled tuning of polymer recognition properties and affected polymer morphology.

IV.2.2. Molecular Imprinting with fluorescence probe

The effect of long interaction times on recognition capability of cAMP + fluorescence probe imprinted polymers were tested by fluorimetry in different days. Measurements was carried out for 5^{th} to 15^{th} days. The fluorescence spectra obtained from the measurements of recognition capability is presented for each days separately below.

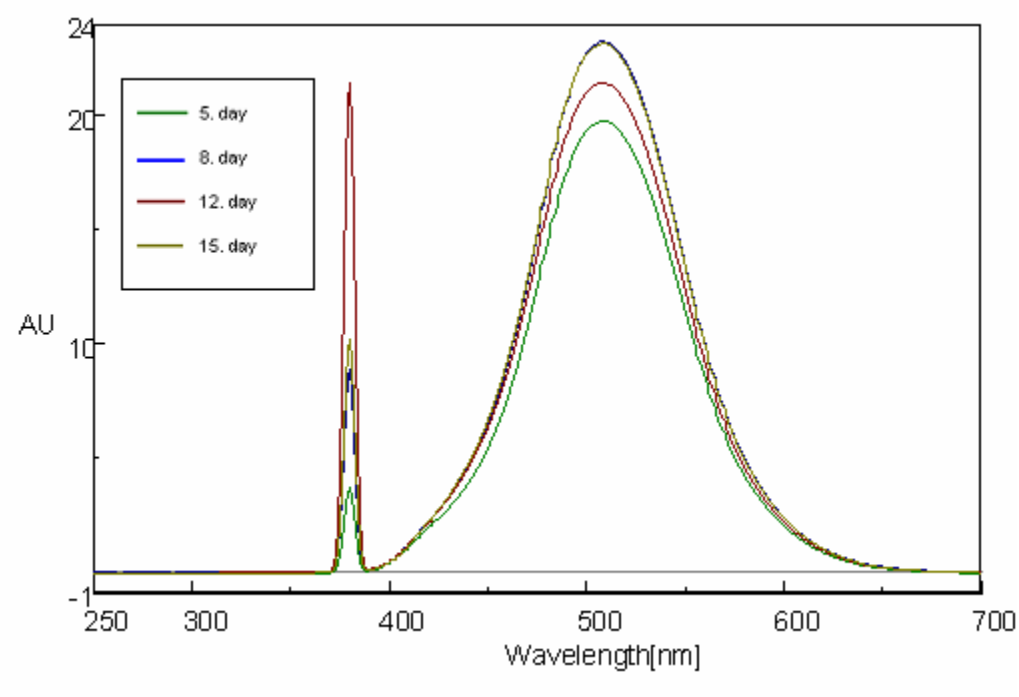
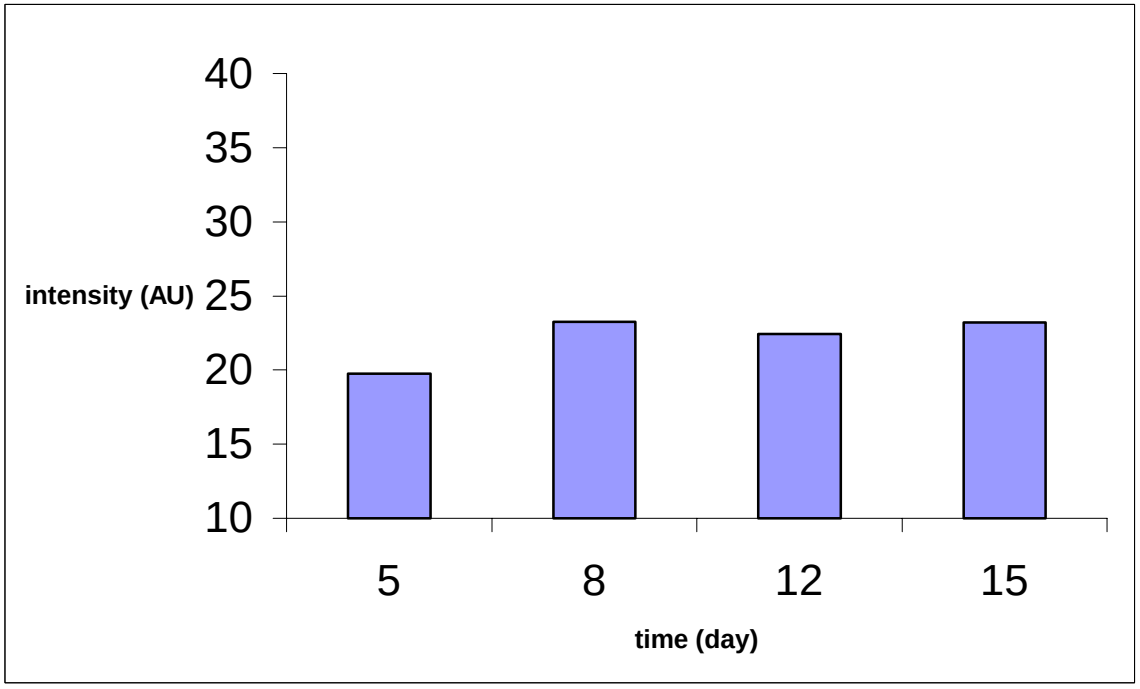


Figure IV.15 The fluorescence spectra of imprinted systems indicating template recognition

In figure IV.15 four sharp peaks at 380 nm and four normal peaks through 400-650 nm were seen. The sharp peaks at 380 nm are scattering peaks and are not characteristics peaks. The fluorescence emission take place 400-650 nm has taken into consideration as fluorescence emission .

The flourescence emission from the imprinted systems all result in only a single band although the 3HF derivatives is known to have two characteristic bands N* and T*. In this study this considered to be due to the high amount of water and methanol within the system, used which both in the preperation of imprinted systems and solutions in which imprinted polymers are suspended . Methanol and water are highly polar and aprotic solvents and cause N* band overlap to T* band and combined to yield only one band from 450 to 650 nm. Numeruous experiments have demonstrated that, in solutions, the occurance of these emmisive forms depends on both non-specific interactions between the 3-hydroxy group and the 4-carbonyl, which is the site of the ESIPT reaction. In the presence of a hydrogen-bonding solvent, mainly emission originating from N* is observed(Arjen N. Bader, Alexander P. Demchenko 2003). On the other hand Ricard Casadesu´ s (2005) suggested that the emission of the N* form cannot be

observed in aprotic solvents. For long, this behavior has been attributed to the very fast ESIPT process in 3HF that would impede the normal fluorescence.



IV.16 The variations of fluorescence emissions indicating recognition capabilities of imprinted polymers

The fluorescence emission increased between 5^{th} to 8^{th} days but later remained almost constant. This was considered to be due to the attainment of maximum absorption capacity of template molecule, cAMP. It can be concluded that the polymeric systems prepared in this study, being a biomolecule, need at least 8 interaction days with template molecule for the best recognition capability.

IV.3. CONCLUDING REMARKS

The objective of this study was to research the possibility of designing imprinted polymeric receptors constructed with a flourescent tag built into them. Thus the binding and dissociation of the template molecule would change the flourescence emission sufficiently for the binding event to be monitored conveniently.Obviously, these polymeric fluorescent sensors have the advantages of ready detectability and high sensitivity.

High cross-link ratios should preferred in order to access permanently macroporous materials, best template recognition capabilities, and in order to be able to generate materials with adequate mechanical stability.

The capability of operating in aqueous environments and organic solvents is a distinct advantage of MIPs in contrast to natural receptors.

According to literature, only few attempts of imprinting in the presence of polar solvents such as methanol and water were successful. Nevertheless, imprinting at aqueous conditions would allow maintaining the same conditions during the rebinding step and - most importantly – operating in aqueous environments would facilitate imprinting of ***biomolecules*** (Molinelli 2004), which was performed in this study.

Methods aimed at measuring the levels of cAMP, as well as other intracellular second messengers,are highyl desirable. The molecularly imprinted systems combined with flourescence sensors would be an alternative since their preparation is simple and cost effective combined with physical and chemical stability and durability (Shea *et al*. 1993; Turkewisch *et al.*2002).

Fluorescence sensors possess characteristic features that offer significant advantages in practical use. They are molecular sensors; their sensing and response takes place at the molecular level. They do not require any intermediate signal transduction factors or steps in recording the sensing event. In addition, their operation does not require the supply and consumption of additional reagents. (Demchenko 2005)

A very attractive additional feature of fluorescence sensors is the possibility of using nearly all possible biopolymer and mimetic systems in which molecular imprinting can be realized or simulated. (Swartzman *et al.* 1999; Mendoza *et al.*1999))

Within the scope of this study the following experiments were carried out and feasible results were obtained.

- 3 amino benzoic acid templated acrylic acid based imprinted systems were prepared with different cross link ratios and their template recognition capability was followed by HPLC. It was observed from experimental results that the imprinted gels has more selectivity to template molecule, 3-amino benzoic acid, than 2 hydroxy-1-naphtonoic acid. Thus their

selectivity for desired molecule and success of imprinting process was proved.

- 3-hydroxy flavone based probe molecule was synthesized and incorparated into the cAMP, template molecule, imprinted system
- The flavone based probe molecule was evaluated by variation in polarity. It was observed that N* band were sensitive to environment polarity while T* band was not. Thus changes in N* band position and intensity ratios of N*/T* bands leads to a multiparameter analysis. The synthesized probe molecule could find many application areas other than imprinted polymeric receptors.
- The absorption behaviour of template molecule, cAMP, was monitored by fluorescence spectroscopy. It was observed that N* and T* bands of 3-hydroxy flavones were combined to yield one band due to the increased environment polarity. Also long interaction times for a beter recognition capability was needed since the templated molecule was a biomolecule.

In this study, synthesis and design of a fluorescence probe belongs to 3-hydroxyflavone family, which recently attracted the attention of researchers due to their interesting photochemical behaviour and as prospective two-band flourescence sensors, and incorporation into a cAMP imprinted HEMA based polymeric system was performed successfuly.

It was established that the fluorescent sensor, which was incorporated into the polymer, responds effectively in interactions with the template molecule by changes in the polymer fluorescence. This fluorosensor was introduced as a functional monomer forming complexes with the template molecules in the polymerising mixture, whose fluoresence was sensitive to interactions with nucleotides caused the polymer itself to moderate the transducer signal by its own functionality.

With this method, single digit µM concentration detection could be achieved. Further fine-tuning of this method could conceivably lead to the developement of practical fluorescent sensors for a wide variety of compounds because the construction of such a fluorescent sensor does not require the print molecule to have certain specific

structural features or to be fluorescent itself. In conclusion, using template polymerization or molecular imprinting techniques, we have demonstrated that a polymeric fluorescent sensor can be prepared for the template molecule.

The design and optimization of fluorescence reporters remains a key determinant of the future success of direct sensing technologies. Suprisingly, there have been few reports on the design of sensor fluorophores and appropriate optical detection methods that could provide guidelines for future developements.The aim of this study is to fill this gap and to critically discuss fluorescence technologies that are currently used and may be useful for future developement of moleculer biosensors.

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APPENDIX A

DATA TABLES

Table A.1Changes in band positions of N* with increasing polarity

% water	N* band position
0	494
3.5	497
6.8	502
9.8	506
12.7	510
15.4	511
17.9	512

Table A.2 Changes in I_{N^*} / I_{T^*} with increasing polarity

% water	I_{N^*} / I_{T^*}
0	0.2844
3.5	0.4395
6.8	0.6398
9.8	0.8859
12.7	1.1747
15.4	1.2832
17.9	1.3788

Table A.3 Changes in template concentration with increasing crosslinker/monomer ratio

Crosslinker /monomer ratio	**3 amino benzoic acid**	2 hydroxy naphtonoic acid
40	4.80737	4.92348
50	4.77211	4.91558
60	4.83692	4.90475
68.37	4.73201	4.92035

Table A.4 The variations of fluorescence emissions indicating recognition capabilities of imprinted polymers

Time (day)	Intensity (AU)
5	19.7573
8	23.2395
12	21.4215
15	23.189

AUTOBIOGRAPHY

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Presented Announcements

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