

**RADIATION INDUCED MOLECULAR IMPRINTING
OF
D-GLUCOSE
ONTO POLY(2-HYDROXYETHYL METHACRYLATE)
MATRICES
USING VARIOUS CROSSLINKING AGENTS**

**D-GLİKOZUN
FARKLI ÇAPRAZ BAĞLAYICILAR KULLANILARAK
POLİ(2-HİDROKSİETİL METAKRİLAT) MATRİKS ÜZERİNE
RADYASYONLA BAŞLATILAN
MOLEKÜLER BASKILANMASI**

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Zeliha ATEŞ

ABSTRACT

Molecular imprinting is a rapidly developing technique where the creation of complimentary binding receptors can be uniquely tailored within a porous network. These macromolecular binding sites provide a wide variety of uses ranging from catalysis to chromatographic separation. To prepare molecularly imprinted polymers (MIPs), a functional monomer and crosslinking agent are polymerized in the presence of a template molecule. Following process is extraction of the template molecule with leaving sites which are complementary in both shape and chemical functionality to those of the template. Remaining crosslinked network becomes capable of selectively binding the template molecule.

Radiation induced molecular imprinting of D-Glucose onto poly (2-hydroxyethyl methacrylate) matrix was achieved to create three-dimensional cavities to recognize and bind glucose. MIPs were synthesized with different crosslinkers and varying amounts of the template molecule in an attempt to elucidate the impact of imprint quantities on the effectiveness of imprinting technique. The crosslinking agents used in this study were in the order of increasing chain length; diethylene glycol diacrylate (DEGDA), diethylene glycol dimethacrylate (DEGDMA), triethylene glycol dimethacrylate (TEGDMA), polyethylene glycol dimethacrylate (PEGDMA) and polypropylene glycol dimethacrylate (PPGDMA). Crosslinking agent concentration in the polymerization mixture (monomer, crosslinking agent and template) covered a range of 10, 15, 20, 30, 50 and 70 % by mole. The mole ratio of functional monomer, HEMA to template molecule, D-glucose was kept either as 3:1 or 6:1. The absorbed dose varied from 1 to 15 kGy. Control polymers were synthesized with exactly the same composition in the absence of D-glucose.

IR and swelling studies were achieved to understand the effect of systematically varying ratio of the involved components. The effect of crosslinking agent type and amount, monomer: template ratio and irradiation dose were also investigated by IR spectroscopy, swelling and PAL experiments

Cavity sizes of MIPs were measured by positron annihilation lifetime (PAL) experiments. PAL experiments were carried out by using a conventional fast-fast coincidence system having a time resolution (FWHM) of about 280 ps. Following positron annihilation experiments of the dry samples all of the samples were investigated in their full swollen state after which surface water had been removed by tissue paper. In the high concentration range of crosslinking agents, the change of free volume hole radius with crosslinking agent is obvious that free volume hole radius steadily increases by going from diethylene glycol diacrylate (DEGDA) to Poly(propylene glycol dimethacrylate) (PPGDMA Mn=560) with increasing chain length 0.26, 0.28, 0.30 nm, respectively.

In order to investigate the efficiency of imprinting system, MIP-based stationary phases were packed through stainless-steel HPLC column. Chromatographic constants; selectivity, efficiency, capacity factor and retention times were calculated via HPLC studies. Noticeable separations were accomplished on DEGDA containing MIPs which have highest efficiency value and capacity factor for glucose molecule; 21.67 and 3.92, respectively.

Keywords: (+)D-Glucose, molecular imprinting, 2-Hydroxyethyl methacrylate, positron annihilation lifetime spectroscopy, HPLC, free volume hole radius, column packing material

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D-GLİKOZUN FARKLI ÇAPRAZ BAĞLAYICILAR KULLANILARAK POLİ(2-HİDROKSİETİL METAKRİLAT) MATRİKS ÜZERİNE RADYASYONLA BAŞLATILAN MOLEKÜLER BASKILANMASI

Zeliha ATEŞ

ÖZET

Moleküler baskılama, gözenekli bir ağ yapı içerisinde kendine özgü bir biçimde birbirini tamamlayan reseptörlerin oluşturulduğu hızla gelişmekte olan bir tekniktir. Bu makromoleküler bağlanma bölgeleri katalizörlerden kromatografik ayırma yöntemlerine kadar geniş bir alanda kullanılır. Moleküler baskılı polimerleri hazırlamak için hedef molekül varlığında fonksiyonel monomer ve çapraz bağlayıcı polimerleştirilir. Bundan sonraki işlem, hedef molekülün hem boyut hem de kimyasal işlevsellik olarak tanımlayıcı bölgeler bırakarak yapıdan uzaklaştırılmasıdır. Elde edilen çapraz bağlı ağ yapı hedef moleküle seçici olarak bağlanabilir hale gelir.

D-Glikozun, poli (2-hidroksietil metakrilat) yapı üzerine radyasyonla başlatılmış moleküler baskılanması ile glikozu tanıyan ve tutabilen üç boyutlu kafesler oluşturulmuştur. Moleküler baskılama tekniğine baskılama bileşenlerinin etkisini açıklamak amacıyla değişik çapraz bağlayıcılar ve hedef molekül çeşitli miktarlarda kullanılarak MIP yapılar sentezlenmiştir. Bu çalışmada kullanılan dietilen glikol diakrilat (DEGDA), dietilen glikol dimetakrilat (DEGDMA), trietilen glikol diakrilat (TEGDMA), polietilen glikol diakrilat (PEGDMA) and polipropilen glikol diakrilat (PPGDMA) çapraz bağlayıcılar artan zincir uzunluğundadır. Polimerizasyon karışımında (monomer, çapraz bağlayıcı, hedef molekül) çapraz bağlayıcı miktarı mol cinsinden % 10, 15, 20, 30, 50 ve 70 aralığındadır. Fonksiyonel monomer HEMA'nın hedef molekül D-Glikoza mol oranı 3:1 ya da 6:1 olarak kullanılmıştır. Işınlama dozu 1 ile 15 kGy aralığında değişmiştir. Kontrol polimerleri D-Glikoz olmaksızın tamamen aynı bileşimde sentezlenmiştir.

Kullanılan bileşenlerin sistematik değişim oranlarının etkilerini anlamak için IR ve şişme çalışmaları yapılmıştır. Çapraz bağlayıcı cinsi ve miktarı, monomer: hedef molekül mol oranı ve ışınlama dozunun etkisi IR spektroskopisi, şişme ve PAL deneyleri ile incelenmiştir.

MIP'lerin kafes boyutları PAL deneyleri ile incelenmiştir. PAL deneylerinde geleneksel FWHM (fast-fast coincidence system) yöntemi yaklaşık 280 ps zaman ayıricılığı ile kullanılmıştır. Kuru örneklerin PAL deneylerini takiben tüm örnekler yüzeydeki suyun süzgeç kağıdı ile uzaklaştırmasından sonra tamamen şişmiş halde incelenmiştir. Yüksek çapraz bağlayıcı içeren bölgede, serbest hacim boyutlarının değişimi açık olarak görülmektedir ki serbest hacim boyutu dietilen glikol diakrilat (DEGDA)'dan polipropilen glikol diakrilat (PPGDMA $M_n=560$)'a doğru çapraz bağlayıcı uzunluğu arttıkça artmaktadır; 0.26, 0.28, 0.30 nm, sırasıyla.

Baskılama sisteminin seçiciliğini incelemek için MIP bazlı durgun faz dolgu maddeleri paslanmaz çelik HPLC kolonlarına doldurulmuştur. Kromatografik sabitler; verimlilik, kapasite faktör, seçicilik ve alıkonma süreleri HPLC çalışmaları ile hesaplanmıştır. DEGDA içeren moleküler baskılı yapılarda glikoz molekülü için en yüksek verimlilik ve kapasite faktörü değerleri sırasıyla 21.67 ve 3.92 değerleriyle en etkin ayırma elde edilmiştir.

Anahtar Sözcükler: (+)D-Glikoz, moleküler baskılama, 2-Hidroksietil metakrilat, PAL spektroskopisi, HPLC, serbest hacim boyutu, kolon dolgu maddesi

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ABBREVIATIONS

MIP	Molecularly imprinted polymer
NIP	Non-imprinted polymer
NA	Sample prepared without crosslinking agent
HEMA	2-hydroxyethyl methacrylate
DEGDA	Diethylene glycol diacrylate
DEGDMA	Diethylene glycol dimethacrylate
TEGDMA	Triethylene glycol dimethacrylate
PPGDMA	Polypropylene glycol dimethacrylate
PEGDMA	Polyethylene glycol dimethacrylate
DMSO	Dimethyl sulfoxide
PALS	Positron annihilation lifetime spectroscopy
PAL	Positron annihilation lifetime
TAC	Time-to-amplitude converter
MCA	Multi channel analyzer
ACAR	The Angular Correlation of Annihilation Radiation
SPSS	Sample-Positron source sandwich
PDF	Probability density function
FWHM	Full-Width Half-Maximum
MELT	Maximum Entropy for Life Time analysis
FTIR	Fourier transform infrared
HPLC	High Performance Liquid Chromatography

1. INTRODUCTION

Within last decade, interest in **M**olecularly **I**mprinted **P**olymers (MIPs) has strongly increased, with potential applications ranging from solid phase extraction materials to antibody-like sorbent assays and selective recognition layers in sensing devices. Advantages such as chemical, mechanical and thermal stability together with high selectivity for the templated analyte render MIPs interesting alternatives to routinely applied separation materials or antibodies. While non-covalently imprinted MIPs have been extensively characterized and their viability in real world applications demonstrated, only recently the need of relating the MIPs binding site heterogeneity to the basic mechanism during MIPs synthesis has been emphasized for the development of optimized recognition materials (Sellergren, 1989; Sellergren, 1997; Mosbach and Haupt, 1998). It is assumed that complex formation based on non-covalent interactions between the template analyte, the functional monomer, and the crosslinker is ultimately responsible for a successful imprint. However, confirmation of the proposed mechanisms is essential for the development of optimized MIPs.

The most important problems to be solved in the design of synthetic recognition-based networks are (Sellergren and Shea, 1993; Wang et. al., 1997; Lin et. al., 1997; Yilmaz et. al., 1999; Svenson and Nicholls, 2001):

- 1- to obtain reproducible interactions between ligand molecule and network, and an ability to select between the ligand and similar compounds
- 2- to create a network compatible with aqueous solutions
- 3- to overcome the diffusional limitations of ligand into bulk network
- 4- to establish a platform by which recognition-based polymers can be used as biomimetic systems.

MIPs have been employed as stationary phases for column chromatography since the development of the first truly functional silica-derived imprinted polymer systems by Curti et. al., who built upon the earlier work of Dickey and Pauling (Bernhard, 1952). By far the most significant objective for studies using MIP-based stationary phases has been to characterize the recognition characteristics of imprinted polymer systems. Although significant volumes of work have been aimed at the development of MIP-based stationary phases, (Sellergren, 1994; Kempe and Mosbach, 1995) the use of MIP-based HPLC stationary phases in commercial applications is still scarce. Nonetheless, the dramatic improvement in chromatographic performance was aimed in our study that has been achieved through the deeper insights into the cavity control of MIP system.

The expectation of creating synthetic networks that can be designed to recognize and bind any significant biological molecule holds great importance. These synthetic materials can then be used as unique systems or incorporated into systems that can aid in the removal or delivery of analytes so that the natural profiles of compounds in the body can be restored. Such developments will have a great importance on diseases, such as diabetes.

Diabetes mellitus is a disorder of metabolism that affects an estimated 100 million people worldwide, and 16 million in the United States alone. Because of the characteristic abnormally high blood glucose levels resulting from this disease, numerous complications in the endocrine, circulation and nervous system can occur in diabetics. Management of diabetes is a life-long and complicated task. D-Glucose was chosen as template molecule owing to the number of diabetes increasing rapidly.

On the other hand, 2-hydroxyethyl methacrylate (HEMA) was chosen as the functional monomer depending on the type and amount of solvent and amount of cross-linking agent, where poly (2-hydroxyethyl methacrylate) can form structures with different morphologies ranging from microporous to macroporous materials.

Furthermore, biocompatibility of poly (2-hydroxyethyl methacrylate) with blood and tissues is also an important property.

Crosslinking agents used in this study were chosen according to their chain lengths and functional groups. Imprinting procedure is directly related with the size-dependent affinity and interactions between functional groups of network and template molecule through created cavities. The control of cavity size can be provided by controlling crosslinking agent's size. Efficiency of imprinting system can also be controlled by interactions of functional groups onto functional monomer and crosslinking agent through target molecule.

Our aim is to optimize the identification conditions of D-glucose in the medium containing D-galactose and D-fructose. Success of imprinting system depends on control of affinity and cavity size properties of highly crosslinked network because of fact that imprinting process leaves sites in the polymer network which are complementary in both shape and chemical functionality to those of template molecules. As a result of the investigation of size-dependent affinity properties of MIP network, according to systematically varying ratio of the involved components; 15 % diethylene glycol diacrylate (DEGDA) containing network with 3:1 HEMA:glucose ratio was the optimized network which was obtained at 15 kGy dose.

The research described in this dissertation contributes to the development of a column support material, which is compatible with aqueous solutions, by molecular imprinting technique. The use of different kinds of crosslinking agent and solvent, different amounts of ingredients and irradiation dose effects related with material properties were investigated. IR and swelling studies were also carried out to understand the effect of systematically varying ratio of the involved components.

Pore size characterization was performed by positron annihilation life time spectroscopy studies. Positron annihilation lifetime (PAL) experiments presented in this study are the first attempt at molecular imprinting to analyze cavity size in nano

scale. TEM, AFM and SEM methods were also used to investigate pore size and surface properties of imprinted polymers with physical conformation of either irregular particles or microspheres (Sellergren et. al., 1998; Yoshimi et. al., 2001; Ye et. al., 2001; Piscopo et. al., 2002). However, these techniques cannot give precise results about the cavity sizes of MIPs. A sandwich arrangement was used for PAL experiments which were carried out using a conventional fast-fast coincidence system. In the high concentration range of crosslinking agents, the change of free volume hole radius with crosslinking agent is obvious that free volume hole radius steadily increases by going from diethylene glycol diacrylate (DEGDA) to Poly(propylene glycol dimethacrylate) (PPGDMA Mn=560) with increasing chain length 0.26, 0.28, 0.30, respectively.

HPLC characterization of synthesized MIPs and control polymers were studied in order to check the affinity efficiency. Irregular-shaped MIP and NIP polymers were packed to stainless-steel HPLC column with 25 mm length and 4.6 mm inner diameter in swollen state. D(+)-Glucose, D(-)-Fructose and D(+)-Galactose were used to determine affinity properties of synthesized MIPs. Chromatographic constants were calculated based on obtained chromatograms.

2. THEORY

2.1. Molecular Recognition

Scientists have discovered that biological recognition is very specific and selective; this specific recognition ability is a key factor for all life processes, which require molecules to recognize each other and bind each other in order to react. For example, the receptors in the cell membranes bind only to a specific hormone; each enzyme exclusively chooses a specific substrate; some amino acid residues of antibodies are oriented complementarily to the functional groups of the target antigens, etc. Likewise; enzymes can bind to the proteins in our food and form complexes that catalyze their decomposition (Lehninger and Nelson, 1993). On the other hand; our biological defense system can form antibodies that can recognize the corresponding antigens and disarm them by forming harmless complexes (Janeway and Travers, 1994). It has been found that in the substrate-binding sites of enzymes, several functional groups like -OH, -NH₂, -COOH, main chain amide groups, etc., precisely interact with the complementary functional groups of a specific substrate (Strayer, 1988).

Although such biological recognition involves simple interactions; they are always in short supply and also unstable when they are not in their native environment. So, the challenge of synthesizing “artificial receptors” that are capable of molecular recognition has drawn special attention. Artificial receptors can be designed freely according to any molecule of interest; they generally possess excellent stability, flexibility and other properties according to need.

In one remarkable example, Pederson discovered the crown ethers and did groundbreaking work in the creation of artificial compounds (Pedersen, 1988). By extending the work of Pederson, Cram designed and synthesized three-dimensional molecules that could mimic the functioning of natural molecules (Cram, 1988). Lehn synthesized a three-dimensional molecule that combined with a neurotransmitter in

the brain, opening the possibility of creating artificial enzymes (Lehn, 1988). For their development and use of molecules with structure-specific interactions of high selectivity, these three scientists shared the Nobel Prize in chemistry in 1987. Despite the successful design and synthesis of those “small-molecule” receptors, there remain challenges for designing and constructing synthetic macromolecular receptors. One promising approach for preparing macromolecular receptors is molecular imprinting.

2.2. Molecular Imprinting

Molecular imprinting is a rapidly developing technique for the preparation of polymeric scaffolds that provide an alternate pathway to overcome the synthetic barriers associated with constructing synthetic molecular receptors while still encompassing an analytical range capable of competing with biological designs which are capable of molecular recognition for selective separation and chemical identification. The concept of molecular imprinting can be traced back to the early

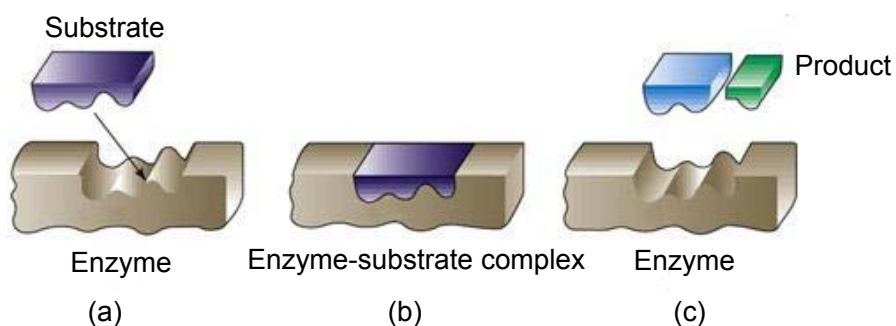


Figure 2.1. Representation of natural molecular recognition via lock and key mechanism.

1890's when Emil Fischer postulated the “lock and key” mechanism to represent natural molecular recognition processes (Dickey, 1949). It was not until years later in 1949 that Frank Dickey further elaborated on Fischer and Pauling's theories to design experiments toward the development of synthetic antibodies (Dickey, 1955). Since

that time numerous advancements have been made, it will be from their work and various others that the applications presented below are made possible. These macromolecular binding sites provide a wide variety of uses ranging from catalysis to chromatographic separation

2.2.1. Contributions of Polyakov, Pauling and Dickey

Polyakov prepared silica for chromatographic applications by acidification of sodium silicate solutions; he reported in 1931 that the addition of benzene, toluene, or xylene as a modifier during the preparation of silica gel results in the enhanced adsorption of the modifier on the silica gel (Polyakov, 1931). In other words, the “modifier” used as the template, which directly affected the surface properties of the resultant silica gel. Nine years later in 1940, Pauling proposed the theory of the antibody; he thought that antibody formation occurred in the presence of an antigen, which played a role as template during the antibody formation (Pauling, 1940). Although this antibody-antigen theory was later proven to be incorrect, this idea inspired the basic concept of molecular imprinting (Edelman, 2004). Chemists found this idea to be useful by forming a 3-D structure around the template to prepare synthetic antibody analogs that recognize the templates with similar selectivity as their biological counterparts. Dickey reported the first experiment to exploit this idea in 1949, and his method described the preparation of silica gel via polymerization of sodium silicate in the presence of the dye (Dickey, 1949).

An outstanding breakthrough in molecular imprinting was achieved in 1972 when the groups of Takagishi and Klotz and Wulff and Sarhan independently reported the first examples of molecular imprinting in synthetic organic polymers (Takagishi and Klotz, 1972; Wulff and Sarhan, 1972). Wulff first proposed a “covalent approach” for the synthesis of **Molecular Imprinted Polymers (MIPs)**. In later work, Mosbach and co-workers developed a non-covalent interaction (e.g., hydrogen bonding, dipolar interactions, electrostatic interactions etc.) approach to construct MIPs that eventually

became widely used because of its simplicity (Sellergren et. al., 1988; Mosbach et. al., 1996).

2.3. Preparation of MIPs

Molecular imprinting involves the synthesis of highly crosslinked, polymeric network in the presence of a template molecule(s) (Haupt, 2001). In most cases, the template is a small molecule, a biological macromolecule, or a microorganism (Perez et. al., 2001). By removing the template molecule after polymerization, a 3-D cavity remains in the network; the cavity has similar size dimensions and shape to the template, and the functional groups are oriented well to complement the template molecule or analogue structure. These characteristics enable the network material to selectively recognize or “remember” the template in subsequent applications. An excess of crosslinking monomer is ordinarily used to preserve and stabilize the specific cavities (Wulff, 1995).

There are two basic approaches to make MIPs based on interactions between the template molecules and the functional monomers. One is the (pre-organized) covalent approach mainly developed by Wulff and co-workers (Wulff et. al., 1977). The template and monomer species are bonded together covalently and this template-monomer complex remains interacting during the polymerization. Such covalent bonds need to be reversible in order to control the template in and out of MIPs.

Another approach is the self-assembly (non-covalent) approach mainly developed by Mosbach and co-workers (Arshday and Mosbach, 1981; Vlatakis et. al., 1993). Instead of covalent bonds, non-covalent bonds are used, such as hydrogen bonds, hydrophobic interactions, van der Waals forces, and Coulomb interactions between ionized groups (; Sellergren et. al., 1985; Andersson and Mosbach, 1990; Dunkin et. al., 1993; Fu et. al., 2003). In these cases, the template-monomer complex can be obtained simply by adding the components to a reaction mixture.

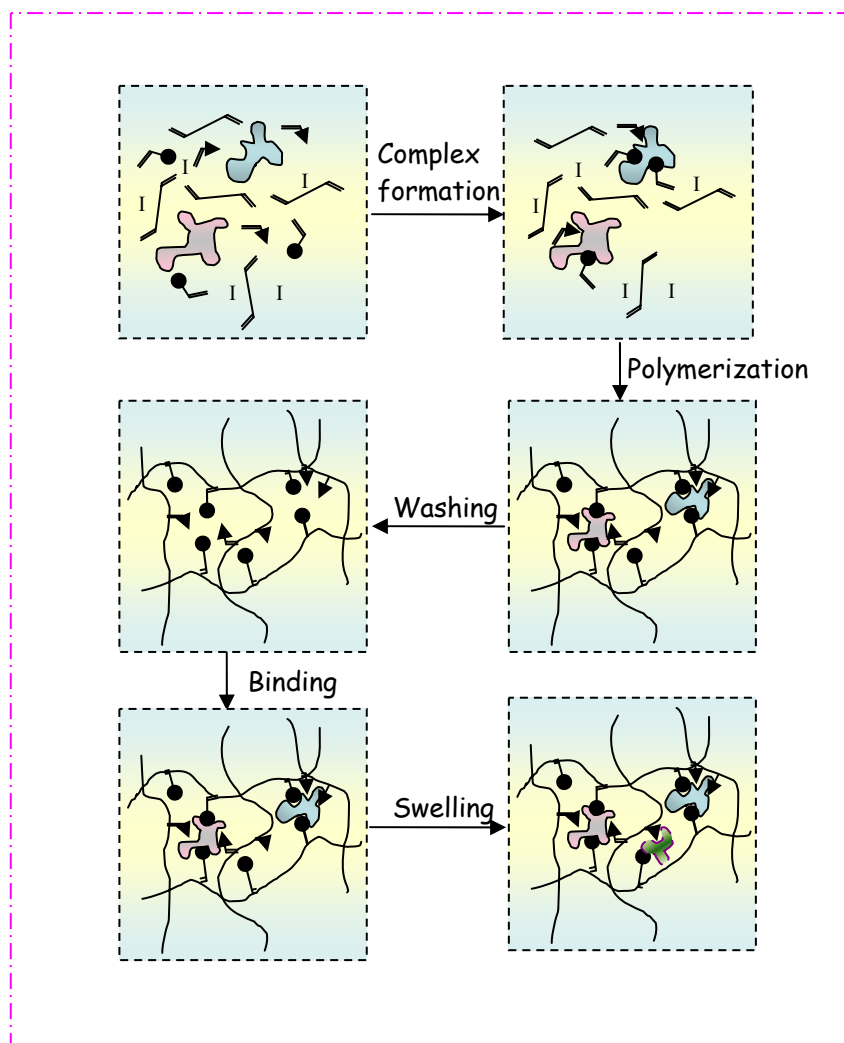


Figure 2.2. Schematic representation of molecular imprinting process. (I) represents initiator, irregular closed shapes represents target molecule, lines with circles and triangle represents functional monomer and lines ends up with double line represent crosslinking agent.

The above two approaches can be combined to take advantage of the favorable aspects of both as shown below in Figure 2.3. (Whitcombe et.al., 1995; Sellergren, 1997; Sellergren, 1998). In this “hybrid approach”, the MIPs are designed by covalent imprinting but the ligand binding takes place by non-covalent interactions (Whitcombe et.al., 1995).

The advantages of non-covalent imprinting methodology significantly broaden the scope of molecular imprinting, and restrictions of covalent approach will be discussed in the following sections briefly.

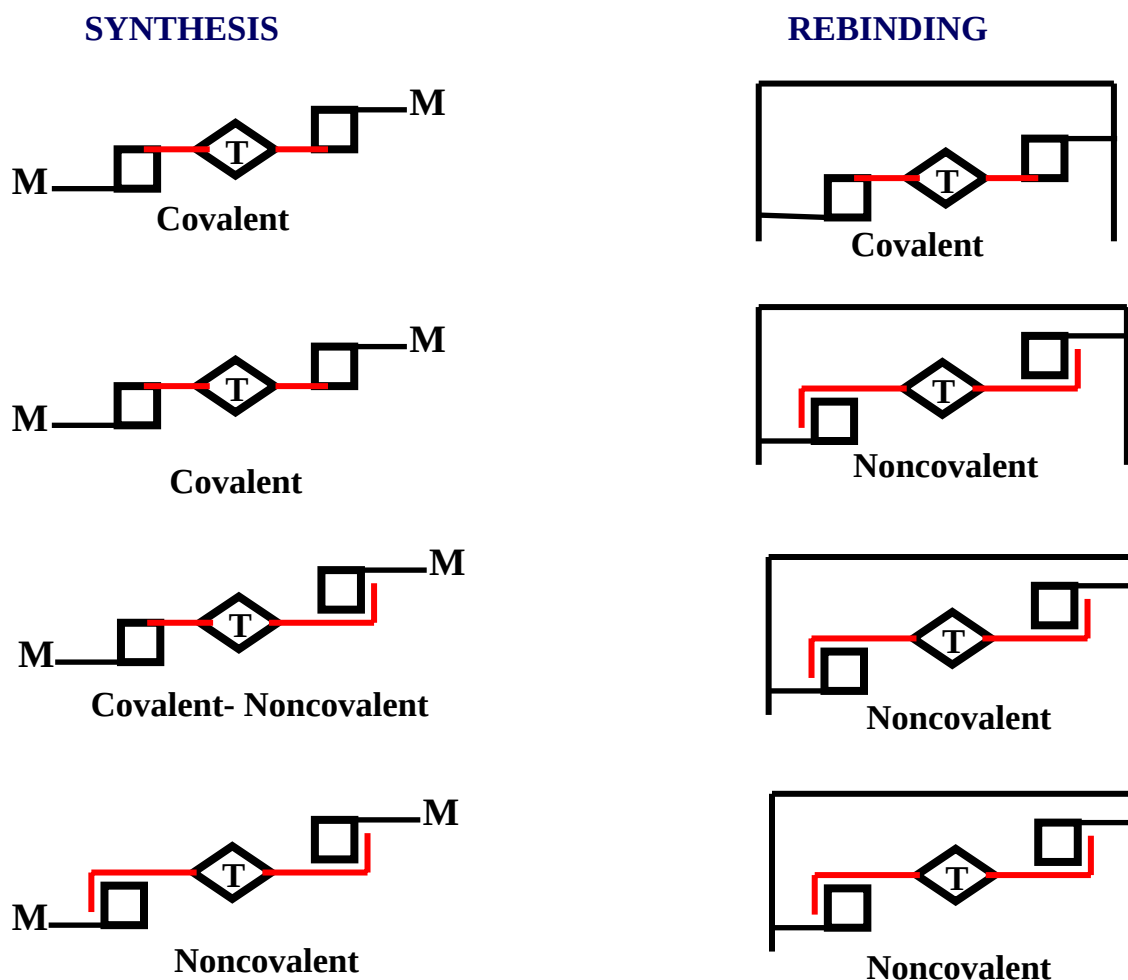


Figure 2.3. Hybrid approach of covalent and non-covalent imprinting techniques. (T) represents target molecule, (M) represents functional monomer, squares represent functional groups and red lines represent interaction between template and functional monomer.

2.3.1. Covalent Imprinting

In covalent imprinting, although the polymerization conditions are not restricted and the structures of imprinted sites can be probed in detail, the synthesis of a monomer-template complex is necessary. In this way, the binding monomer(s) in an imprint cavity can be employed in exact stoichiometric ratio to the template. After polymerization, the covalent bond is cleaved chemically, and resultant cavities can rebind the template covalently (or non-covalently). The ligand and release are relatively slow due to the formation and cleavage of the covalent linkage (e.g., to form a boronic acid ester or Schiff bases) (Bergold and Scouten, 1983; Wulff et. al., 1986). The resulting covalent system lacks flexibility because of the limits of the choices of monomer and templates.

The advantage of this approach is that the functional groups are only associated with the template site; however, only a limited number of compounds (alcohols (diols), aldehydes, ketones, amines and carboxylic acids) can be imprinted with this approach.

2.3.2. Non-Covalent Imprinting

In non-covalent imprinting, the interactions between functional monomer and template during polymerization are the same as those between polymer and template in the rebinding step. These are based on non-covalent forces such as H-bonding, ion-pairing and dipole-dipole interactions. This method was first introduced in organic polymers by Mosbach (Mosbach and Haupt, 2000). Due to its simplicity this method is the most widely used to create imprinted polymers. Many functional monomers are commercially available and a number of custom-designed functional monomers have also been reported (Miyata et. al., 1999; Matsui et. al., 2000). To date the most common functional monomer employed is methacrylic acid, first reported by Mosbach et al (Vlatakis et. al., 1993).

Most MIPs are prepared by free radical polymerization of functional monomers and crosslinking agents which have vinyl or acrylic groups (Alexendar et. al., 2005). Depending on the template, the functional monomers can be complementarily designed to have appropriate functional groups, such as carboxyl, amino, hydroxyl or amide groups, etc., for the non-covalent linkage.

To preserve the unity and rigidity of the cavity in the resultant MIPs, a very high degree of crosslinking is necessary. Ethylene glycol dimethacrylate (EGDMA) is one of the most widely used crosslinking agents (Alexendar et. al., 2005). The reactivity of the crosslinking agent should be similar to that of the functional monomer to ensure the functional monomer and the crosslinking agent's sufficient copolymerization. In addition, by choosing an appropriate crosslinking agent and the suitable mole ratio of crosslinking agent to functional monomer, the functional groups, which are responsible for the monomer-template complex formation, can be distributed in the polymer network (Yilmaz et. al., 1999).

The solvent (also called the porogen) plays an important role in the molecular imprinting especially in non-covalent (self-assembly) approach. Besides helping to dissolve the components for polymerization and dispersing the heat generated during the reaction, most importantly, the solvent provides a macroporous structure to determine the polymer morphology and affects the strength of non-covalent interactions. Since all non-covalent interactions are influenced by the properties of the solvent, non-polar solvents normally lead to the best recognition (Haupt et. al., 1998; Baggiani et. al., 2000). On the other hand, solvent affects the network morphology as the swelling of the polymer is dependent on the medium (Sellergren and Shea, 1993). This swelling behavior may lead to changes in the three-dimensional configuration of the functional groups taking part in the recognition in the sites resulting in poorer binding capability. In order to avoid any swelling problems, the best choice of recognition solvent is generally either the imprinting solvent or similar one.

3. BACKGROUND

3.1. Perspective through Imprinting System

Several variables are considered during the optimization of the imprinting process from polymerization components, to their ratios, polymerization temperatures and times since all of these can affect the resultant polymeric network and its properties. Understanding the effect of these variables for the imprinting system is important to get optimized MIPs and not waste time.

The success of imprinting process is governed by fundamentally five components;

- 1) The template is used to provide a mold for which the polymeric network can be constructed around.
- 2) The functional monomer is used to bind to template through a variety of interactions and usually contains a specific moiety so that it can be incorporated into the polymeric network by polymerization.
- 3) The crosslinker can be altered to provide a hydrophobic or hydrophilic matrix as it provides the network that locks the template and functional monomer into position.
- 4) The solvent (porogen) can affect the swelling behaviour of the polymer as well as the density of the crosslinked matrix.

Each of the variables will be discussed in details in the sections below.

3.1.1. Monomer and Crosslinking Agent Selection

Optimization of synthesis conditions for making MIPs is complicated and time consuming because several parameters are involved. Questions which must be answered are these: What is the best functional monomer and crosslinking agent for a specific template molecule? What is the optimum ratio between the functional monomer/crosslinking agent/template? What is the best solvent (porogen)? What temperature should be used for polymerization?

Although the preparation of MIPs by the non-covalent imprinting approach is technically straightforward, it relies on the successful stabilization of the weak interactions between the functional monomer(s) and template. An extra caution should be taken when choosing the monomer(s), crosslinking agent(s) and the solvent. Typical functional monomers used are carboxylic acids (acrylic acid, methacrylic acid, vinylbenzoic acid) and heteroaromatic (weak) bases (vinylpyridine, vinylimidazole). Since methacrylic acid can work as both a Lewis acid and base, it can be applied effectively for any template molecule with hydrogen donating or accepting functional groups.

Most commonly, the functional monomer of choice is methacrylic acid (MAA), acrylamides, itaconic acid, 4-vinyl pyridine and some specially designed monomers (Fischer et. al., 1991). The most commonly used crosslinking agent was the ethylene glycol dimethacrylate (EGDMA). When the polymerization was performed by free-radical reactions, 2,2'-azobis(2-isobutyronitrile) (AIBN) was the most common initiator, and it was initiated either by thermal methods or photochemical homolysis by exposure to UV light.

High percentage of crosslinking agent (70-90 %) is required in order to achieve rigid MIPs, the selection of crosslinking agent(s) is limited by the solubility of crosslinker itself in the polymerization mixture. An optimized degree of crosslinking exists in many imprinting systems. A more rigid polymer framework can be achieved with

higher crosslinker percentages, which often enhances the imprinting effect because the shape of the imprint cavities has less flexibility. Whereas, at lower crosslinker percentages, more functional monomer can be built into the polymer matrix to provide more specific interactions with the template.

One interesting approach to simplify MIP formation has been reported recently by Spivak and Sibrian-Vazquez, who used a single crosslinking agent with built in functionality (Spivak and Sibrian-Vazquez, 2003; Spivak and Sibrian-Vazquez, 2004). While investigating new cross-linkers for molecular imprinting, they have recently discovered a much simpler approach to MIP formation which utilizes a single cross-linking monomer, N,O-bismethacryloyl ethanolamine (NOBE, 1), in addition to template, solvent, and initiator. They reported that the amide functionality in the cross-linker NOBE interacts sufficiently with most templates, with the only exception of amines, to afford molecular recognition without the need of introducing any other functional monomer. They reported that utilization of the crosslinking agent alone often provides imprinted polymers with higher performance than MIPs incorporating functional monomer. Their results pointed out that the origin of the improved molecular recognition observed in organic media was determined to be the result of hydrogen-bonding interactions, whereas in aqueous media moderate hydrophobic interactions were responsible for the selectivity observed.

3.1.1.1. Poly (2-Hydroxyethyl Methacrylate) (PHEMA)

HEMA-based hydrogels are the most widely used biomaterial hydrogels owing to the biocompatibility of Poly (HEMA) with blood and biological tissues; it is a constituent of many recent devices that are designed to come in contact with blood. For more than forty years, it has been a part of most contact lens formulations. A few of recent applications have included the design of surgical adhesives as copolymer with PEG, delivery vehicle for cancer drug 5-fluorouracil as a copolymer with acrylamide, encapsulation of cells in the treatment of central nervous system diseases and as cell

adhesion surface as copolymers with ethyl methacrylate (Berkowicz and Peppas, 1995; Burmeister et. al., 1999; Garcia et. al., 2000; Mokry et. al., 2000).

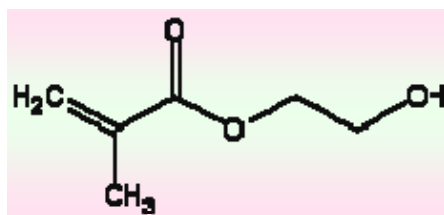


Figure 3.1. Structure of 2-hydroxyethyl methacrylate.

Structurally, PHEMA polymers have a lot of varieties. Depending on the type and amount of solvent and type and amount of cross-linking agent, PHEMA can form structures with different morphologies ranging from microporous to macroporous materials (Baker et. al., 1995; Okay, 2000).

As a functional monomer in imprinting applications, Sreenivasan investigated the feasibility of HEMA networks for imprinting testosterone (Sreenivasan, 1997; Sreenivasan, 1998). The material showed promise; however, the polymerization was performed in chloroform. Kugimiya et al. investigated the effect of HEMA content in a copolymer of HEMA with MAA, which was imprinted with indoleacetic acid in chloroform (Kugimiya and Takeuchi, 1999). Incorporation of HEMA in methacrylate-based MIPs makes the polymers less hydrophobic which helps the diffusion of hydrophilic templates. The results showed that higher HEMA contents led to better resolution of template and structurally similar compounds.

3.1.2. Solvent Selection

Solvent plays an important role in molecular imprinting. It facilitates mass transfer of templates and also creates pores by phase separation during polymerization. Thus, solvent parameters such as polarity, hydrogen bonding and acidity/alkalinity help to determine the final morphology of the polymer matrix and its porosity. Generally, the

best recognition properties of MIPs occur when the same solvent is used during the synthesis and rebinding process (David et. al., 1997; Sharma and Borovik, 2000). Furthermore, the solvating ability of the solvent for the polymers influences the shape of the imprint cavities and may reorganize the functional groups inside the binding sites. Thus, selective rebinding of template molecules is typically achieved when the difference in the solvating ability is minimized between the solvent used for preparing the MIP and the solvent used for rebinding (David et. al., 1997). Nonpolar, aprotic solvents are used generally, while attempts have been made to achieve noncovalent imprinting in an aqueous system (Turkewitsch et. al., 1998; Gore et. al., 2004).

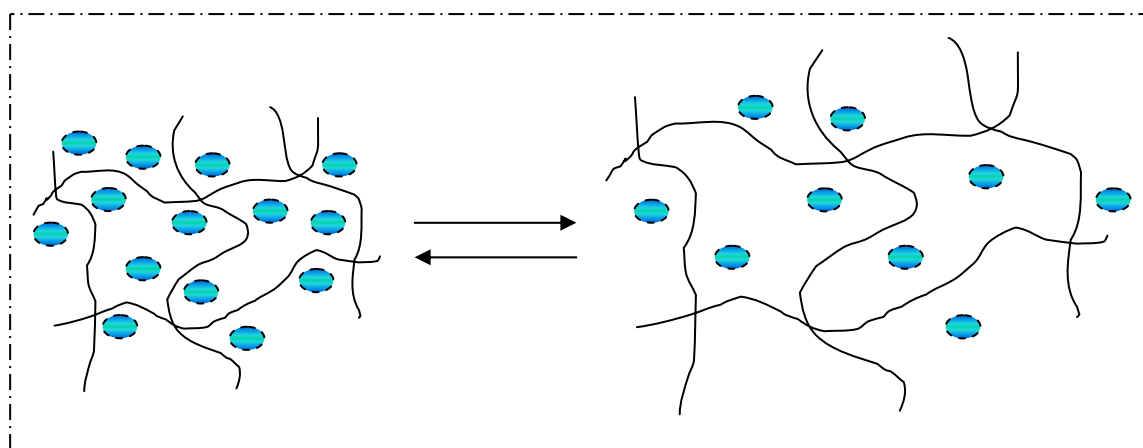


Figure 3.2. Binding and re-binding steps involves swelling process of molecularly imprinted network concerning which imprinting efficiency extremely depends on solvent.

In addition to the polymer synthesis approach to optimize MIP performance, Pilesky and Goto have tried to optimize selection of monomers and solvent using spectroscopy and molecular modeling to measure complexation constants for template to monomers(s) (Yoshida et. al., 2000; Subrahmanyam et. al., 2001; Pilatska et. al., 2004; Turner et. al., 2004). In their studies on making imprinting polymers specific for creatinine, biotin and ochratoxin-A, a computationally designed polymer demonstrated higher selectivity in comparison to the polymers prepared synthetically (non-optimized). By applying this computational simulation method for

designing MIPs for a broad range of template molecules such as chiral drugs, steroids, peptides, enzymes, proteins, cells, and antibodies, preliminary simulation results may provide useful information for rational selection of monomers, solvents, and molar ratio between monomer and template (Fischer et. al., 1991; Vlatakis et. al., 1993; Kempe and Mosbach, 1994; Piletsky et. al., 1994; Whitcombe et. al., 1995; Andersson et. al., 1995; Ansell and Mosbach, 1996; Mosbach and Ramstrom, 1996; Cheong et. al., 1997; Spivak and Shea, 1998; Rachkov et. al., 1998; Hishiya et. al., 1999; Lubke et. al., 2000; Martin et. al., 2000; Ye et. al., 2000; Matsui et. al., 2000; Yoshida et. al., 2000; Alveraz-Lorenza et. al., 2001).

3.2. Methods for Preparing MIPs

Free radical polymerization is most commonly used in the preparation of MIPs not only because of its versatile applicability and experimental use, but also economic reasons. The polymerization can be initiated by several factors; thermo or photolytic initiations comprising the most widely used. K. Sreenivasan and R. Sivakumar have also used radiation induced polymerization technique for imprinting process (Sreenivasan and Sivakumar, 1999).

3.3. Applications of MIPs

MIP technology is now in the process of maturing after a slow start because of difficulties in resolving problems associated with poor performance of MIPs in aqueous environments and because of the absence of any general procedure for MIP design.

Molecular imprinting is a versatile method for creating macromolecular matrices that display selective molecular recognition behaviour. These imprinted networks have high affinity and selectivity, which are similar to those of natural receptors, also have unique stability which is superior to that demonstrated by natural biomolecules, and the simplicity of their preparation and the ease of adaptation to different practical

applications. A great variety of chemical compounds have been imprinted successfully. The resulting imprinted networks are robust, inexpensive and, in many cases, possess an affinity and specificity that is suitable for industrial application. In recent times, four main areas of application for molecularly imprinted polymers have been pursued: 1) Affinity Separation, 2) Antibody Binding Mimics, 3) Enzyme Mimics (catalytic/synthetic applications), 4) Bio-Mimetic Sensors (Sellergren et. al., 1989; Vlatakis et. al., 1993; Kempe and Mosbach, 1995; Haupt et. al., 1999; Ye and Mosbach, 2001; Hilt and Byrne, 2004). A few developing applications will also be discussed shortly in below sections.

3.3.1. Affinity Separation

Affinity separation is the single 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 (Yoshida et. al., 2000; Fujimoto, 2002). Imprinted polymers as stationary phases in HPLC will be discussed in detail in the following sections.

Besides liquid chromatography, it is very noticeable that imprinted polymers as selective solid-phase extraction media are another main application area (Olsen et. al., 1998; Sellergren, 1999; Stevenson, 1999). It is highly probable that this is the area in which the first commercial application of these materials will be seen. Other key lines of research in the affinity separation area include membranes and capillary electrophoresis (Scheweitz et. al., 1998; Vallona and Remcho, 2000; Nilsson et. al., 2000).

3.3.2. Antibody Receptor Binding Mimics

Molecularly imprinted polymers for use as artificial antibodies/receptors have potential for rapid development, notably in immuno-assay-like diagnostic techniques (Murray and Uy, 2001). The physical and chemical resistance of imprinted polymers together with the possibility of sterilizing the polymers, the stability of the recognition properties, the low cost of preparation and obviation of the need for host animals are all obvious advantages of imprinted polymers in comparison to natural antibodies and receptors (Rachkov et. al., 1998; Herm and Schrader, 2000). In addition to immunoassays, screening applications may be a future prospect for imprinted polymers. In combination with combinatorial chemistry techniques, such polymers may be used as tools for the selection of lead molecules from large libraries of compounds. In particular, if a nature receptor desired for screening is difficult to isolate, initial screening may be accomplished by the use of molecularly imprinted receptor mimics.

3.3.3. Catalytic/Synthetic Applications

This is an area in which considerable effort has been invested in recent years. Although some intriguing results have been presented, the area still awaits a real breakthrough. For industrial synthetic schemes, though, polymeric catalysts have the potential to become a real complement to natural catalysts. The cost-efficiency with which such materials may be produced is a great advantage for catalytic applications.

In addition to 'real' catalysis, synthetic schemes where molecularly imprinted polymers are used to guide the product formation, either directly or indirectly may become interesting. Condensation of specific substrates in imprinted polymer sites is one approach which may produce very efficient synthesis. The use of molecularly imprinted polymers for the control or elimination of by-products, or for the shifting of unfavorable equilibria is also future applications of these materials.

Several different organic reactions have been successfully catalyzed with molecularly imprinted polymers, including aldol condensations, ester hydrolysis, Diels-Alder reactions and β -eliminations (Liu and Mosbach, 1997; Yu et. al., 2001; Ohkubo et. al., 2001). While molecularly imprinted polymers may not yet be able to compete with enzymes in terms of catalytic rate enhancements, they offer properties that are quite distinct from those of enzymes, because of their ready compatibility with organic solvents and their high temperature stability (Ramström and Mosbach, 1999; Whitcombe et. al., 2000; Motherwell et. al., 2001; Wulff, 2002; Alexander et. al., 2003). Thus they are more useful as a complement to enzymes rather than as a replacement, at least now.

3.3.4. Biosensor like Devices

The use of molecularly imprinted polymers in biosensor devices seems fairly close at hand. In the short term, effort should be devoted to obtaining imprinted structures that can be placed in direct contact with the transducer. The chances are good that biosensor-like devices can be obtained using imprints that are robust and which have binding specificities not found with biological molecules (Aboul-Enein and Stefan, 1998; Yano and Karube, 1999). Furthermore, the development of molecularly imprinted polymers capable of recognizing analytes carrying auxiliary markers, such as fluorescence labels, similarly to the non-labelled species will evidently lead to a broadening of this area.

3.3.5. Controlled Release Matrices

Depending on the conditions, the binding affinities for the imprint antigens exhibited by the molecularly imprinted polymers can be reasonably high (Allender et. al., 2000; Suedee et. al., 2002). This renders such polymers potentially useful as controlled release matrices. If the binding strength of the compounds may be sufficiently regulated in an on-off-manner, such that the binding can be reduced, either totally in one step or gradually, by means of simple external influences such as pH, light, or

heat, then polymers can be made to release a certain compound at a certain position and time. Thus, if the ligand bound is a drug compound and the polymeric matrix is biocompatible, such matrices may be employed to control the drug delivery in vivo. Other applications of this technique could be controlled release of antibiotics in solutions sensitive to bacterial degradation. By addition of targeting elements to such molecularly imprinted controlled-release matrices, control of the release position may also be achieved.

3.4. Imprinting with Glucose and Other Sugars

During the earlier stages of the molecular imprinting technique including which first attempts to sugar imprinting were done by the Wulff group concerning that a technique used to imprint some monosaccharide derivatives (Wulff and Vietmeier, 1987; Wulff and Schauhoff, 1991; Wulf, 1995). The monomer used 4-vinylphenylboronic acid, was able to form reversible covalent bonds with sugars. Even though very selective sites were obtained using this technique, polymerization was not performed in aqueous solution and required establishing covalent bonds upon rebinding. This type of imprinting technique would not be feasible in contact with biological fluids or in the body in view of the requirement of chemical reaction.

Two types of polymers based on non-covalent bonding have shown promise for keeping strong interactions between template and monomers. The first is polymers prepared by the coordination of metals to monomers. An example of this material was TACN-Cu(II) imprinted with glucose (Chen et. al, 1996). These polymers were able to recognize and connect with their templates in water as well. Another example of this precise complex formation was observed between the monomers and cobalt without water in an effort to create the binding site of chymotrysin synthetically (Lele et. al., 1999). The second type of polymers are β -cyclodextrin-based materials, where the hydrophobic cleft inside the cyclodextrin allows for efficient hydrophobic interactions and recognition (Asanuma et. al., 1999; Hishiya et. al., 1999). However, the recognition has been limited to very hydrophobic templates such as steroids.

Mayes et al. imprinted methacrylic acid monomers cross-linked by ethylene glycol dimethacrylate for α and β anomeric forms of p-nitrophenol-D-galactoside and p-nitrophenyl-D-fucoside (Mayes et. al., 1994). The solvent used was acetonitrile or chloroform and polymerization took place with thermal initiation of 2-2'-azobis(2,4-dimethylvaleronitrile) (ABDV) or AIBN for 16 hours at 45°C. Although hydrogen bonding between template and monomer was observed, the preparation solvent was not aqueous. Therefore, the performance of the MIP in water was substantially worse than organic solvents when resolving the anomeric forms of the sugars.

3.5. Diabetes Mellitus

Diabetes mellitus is a group of metabolic diseases characterized by high blood sugar (glucose) levels, which result from defects in insulin secretion, or action, or both. Diabetes mellitus, commonly referred as diabetes, was first identified as a disease associated with “sweet urine” and excessive muscle loss in the ancient world. Elevated levels of blood glucose (hyperglycemia) lead to spillage of glucose into the urine, hence the term sweet urine. Normally, blood glucose levels are tightly controlled by insulin, a hormone produced by pancreas. Insulin lowers the blood glucose level. When the blood glucose elevates (for example, after eating food), insulin is released from the pancreas to normalize the glucose level. In patients with diabetes, the absence or insufficient production of insulin causes hyperglycemia. Diabetes is a chronic medical condition, meaning although it can be controlled, it lasts for a lifetime.

3.5.1. The Symptoms Of Diabetes

Insufficient production of insulin (either absolutely or relative necessity of the body), production of defective insulin (which is uncommon), or the inability of cells to use insulin properly and efficiently leads to hyperglycemia and diabetes. This latter condition affects mostly the cells of muscle and fat tissues, and results in a condition

known as “insulin resistance”. This is the primary problem in type 2 diabetes (Lebovitz, 1999). The absolute lack of insulin, usually secondary to destructive process affecting the insulin producing beta cells in the pancreas, is the main disorder in type 1 diabetes (Lernmark, 1999). In type 2 diabetes, there also is a steady decline of beta cells that adds to the process of elevated blood sugars.

3.5.2. The Importance of Glucose

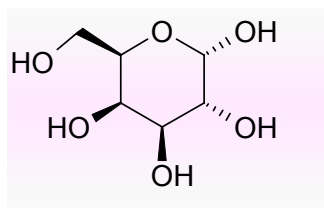


Figure 3.3. Structure of (+)D-Glucose.

Glucose is a simple sugar found in food as an essential nutrient provides energy for the proper functioning of the body cells. Carbohydrates are broken down in the small intestine and the glucose in digested food is then absorbed by the intestinal cells into the bloodstream, and is carried by the bloodstream to all the cells in the body where it is utilized. However, glucose cannot enter the cells alone and needs insulin to aid in its transport into the cells. Without insulin, the cells become starved of glucose energy despite the presence of abundant glucose in the bloodstream. In certain types of diabetes, the cells' inability to utilize glucose gives rise to the ironic situation of starvation in the midst of plenty. The abundant, unutilized glucose is wastefully excreted in the urine.

3.5.3. The Impact of Insulin

Insulin is a hormone that is produced by specialized cells (beta cells) of the pancreas. Insulin regulates the level of glucose in the blood and also important for helping the glucose for entering to the cells. After a meal, the blood glucose level rises. In

response to the increases in glucose level, the pancreas normally releases more insulin into the bloodstream to help the glucose to enter the cells and lower the blood glucose levels after a meal. When the blood glucose levels are lowered, the insulin release from the pancreas is turned down. It is important to note that even in the fasting state there is a low steady release of insulin which fluctuates a bit and helps to maintain a steady blood level during fasting. In normal individuals, such a regulatory system helps to keep blood glucose levels in tightly controlled range. As outlined above, in patients with diabetes, the insulin is either absent, relatively insufficient for the body's needs, or not used properly by the body (Davidson, 1998). All of these factors cause elevated levels of blood glucose.

3.5.4. Different Types of Diabetes

There are two major types of diabetes, called type 1 and type 2. Type 1 diabetes was also called insulin dependent diabetes mellitus (IDDM), or juvenile onset diabetes mellitus. In type 1 diabetes, the pancreas undergoes an autoimmune attack by the body itself, and is rendered incapable of making insulin. Abnormal antibodies have been found in the majority of patients with type 1 diabetes. Antibodies are proteins in the blood that are part of the body's immune system. The patient with type 1 diabetes must rely on insulin medication for survival.

Type 2 diabetes was also referred to as non-insulin dependent diabetes mellitus (NIDDM), or adult onset diabetes mellitus (AODM). In type 2 diabetes, patients can still produce insulin, but do so relatively inadequately for their body's needs, particularly in the face of insulin resistance. In many cases this actually means that the pancreas produces larger than normal quantities of insulin. A major feature of type 2 diabetes is a lack of sensitivity to insulin by the cells of body (particularly fat and muscle cells). Thus, larger quantities of insulin are produced as an attempt to get these cells to recognize that insulin is, in fact, present. In addition to the problems with an increase in insuline resistance, the release of insulin by the pancreas may also be defective and suboptimal. In fact, there is a known steady decline in beta cell

production of insulin in type 2 diabetes that contributes to worsening glucose control. This is a major factor for many patients with type 2 diabetes who ultimately require insulin therapy. Finally, the liver in these patients continues to produce glucose through a process called gluconeogenesis despite elevated glucose levels.

3.5.5. The Treatment of Diabetes

The major goal in treating diabetes is controlling elevated blood sugars (glucose) without causing abnormally low levels of blood sugar. Type 1 diabetes is treated with insulin, exercise, and a diabetic diet. Type 2 diabetes is first treated with weight reduction, a diabetic diet, and exercise. When these measures fail to control the elevated blood sugars, oral medications are used. If oral medications are still insufficient, insulin medications are considered (Davidson, 1998).

Based on what is known, medications for type 2 diabetes are designed to:

1. Increase the insulin output by the pancreas.
2. Decrease the amount of glucose released from the liver.
3. Increase the sensitivity (response) of cells to insulin.
4. Decrease absorption of carbohydrates from the intestine.
5. Slow gastric emptying to delay the presentation of carbohydrates for absorption in the small intestine.

Current treatments for diabetes are via oral glucose-lowering agents, which are used to supplement diet and exercise for mild cases of the disease, where there is no absolute insulin loss. These include insulin secretagogues (sulphonylurea), which stimulate the pancreatic cells to produce insulin, metformin and phenformin, which slow the rate of glucose absorption from the intestine, α -glucosidase inhibitors and

thiazolidinediones. Most of these drugs have short lives (4-12 hrs); thus, the effective doses are limited.

The ones that act over a longer period of time sometimes cause hypoglycemia. For those patients, who require insulin and who have had acidosis or ketosis, insulin is required. Insulin is delivered by subcutaneous injections before meals. The oral drugs are not effective on a large percentage of patients and insulin injection is a very inconvenient way of treating the disease, patient compliance is low.

Many researchers have tried to make injection of insulin less painful and more comfortable by developing delivery devices other than syringes such as jet-injectors, external pumps and patches. Also, new delivery ways are researched such as rectal, pulmonary, oral or ultrasound delivery. These devices are promising in keeping more accurate track of insulin delivered and providing quick and efficient delivery. However, the delivery of insulin at regular times during the day is not enough to keep sustained levels of glucose concentrations that mimic the natural control in the body. Despite such efforts, the bioavailability of the insulin delivered by these methods is still much lower than subcutaneous injection. Most of the patients suffer from mild to debilitating conditions caused by hyperglycemia and inefficient control of blood glucose levels.

3.6. Experimental Methods for the Characterization of MIPs

The purpose and glamour of molecule imprinting technique are that after imprinting, the formed polymeric materials contain recognition sites of predetermined specificity. With hundreds of reports of specific rebinding of template in MIPs, the properties of binding sites are worthy of investigations. Basically, there are two experimental methods to study the ligand binding activity of MIPs, liquid chromatography and batch rebinding experiments.

In the chromatographic method, bulk MIPs are prepared, ground and size selected. Then, the MIP particles are packed into a stainless steel column and used as the

stationary phase for high performance liquid chromatography (HPLC). The binding strength and selectivity of MIPs toward ligand molecules are analyzed in terms of their chromatographic behavior. Successful imprinting selectivity promotes the binding of template molecules, increasing their retention time. The benefit of this technique is that a high degree of sensitivity can be obtained if a small quantity of analyte is injected in to a large chromatography column. Good resolutions result from the large number of theoretical plates. The selectivities of the imprinted matrices may then be expressed by the retention volumes of different ligands. Another advantage with this technique is that the binding performance may be estimated by frontal zone analysis from which apparent affinity constants (K_D) and site population densities (B_{max}) may be obtained. However, although chromatographic analysis is a useful and reliable tool for the evaluation of recognition properties, the (flow) kinetics of the process may interfere with the interpretation of the results. As a consequence of the mobile phase flow through the matrix, only the sites with fast binding kinetics are detected and evaluated. This is clearly demonstrated when comparing the affinity constants determined with either frontal zone chromatography or radioligand batch analysis.

In the batch method, the ligand-binding activity of MIPs can be directly determined in terms of amount of ligand bound by MIPs. After the extraction of the templates from MIPs, some amount of MIPs is incubated in a ligand solution at various concentrations until the binding reaches equilibrium. After filtration, the concentration of ligand in the liquid phase can be determined by chromatographic, spectroscopic or other analytical methods. By comparing the amounts of different ligands bound in the polymer, the selectivity of the MIPs can be studied.

For studies of the recognition process occurring under equilibrium conditions, all the sites in the matrix are taken into consideration and no external kinetic processes have to be regarded. However, the sensitivity enhancement given by the chromatographic process is not available since only one equilibrium step is involved. Several different approaches may be used in equilibrium studies depending on the level of sensitivity

required. To observe small differences in binding, or binding of very small quantities of ligand, methods with very low detection limits are required. In recent years, radioligand binding assays have been used extensively due to the very high sensitivity of this method, which makes it possible to study the very small population of sites with strongest binding. The recognition properties are estimated either by monitoring the direct binding of the ligands of interest to the imprinted polymers, or by using a competition assay technique, where the ligands compete for the binding sites with the (radiolabelled) imprint antigen. By these techniques, the apparent affinity constants and binding site populations, may be obtained.

High performance liquid chromatography (HPLC) is one mode of chromatography, the most widely used analytical technique. Chromatography processes can be defined as separation techniques involving mass-transfer between stationary and mobile phases.

HPLC utilizes a liquid mobile phase to separate the components of a mixture. These components (or analytes) are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture is resolved into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures.

Characterization of structure and morphology of molecularly imprinted polymers together with characterization of the imprinting effect by HPLC are necessary to determine whether imprinting was successful and to assess the effect of the different factors related to MIP synthesis on polymer recognition properties. Factors such as accessibility of binding sites within the polymer network will be seen in

chromatographic studies since they affect mass transfer kinetics and load capacity of the polymeric separation material.

Distribution between a stationary and a mobile phase and adsorption/retention of analytes within the stationary phase both contribute analyte separation in high performance liquid chromatography (HPLC). A stronger interaction with the stationary phase will result in a longer residence time within the separation column.

3.6.1. Classic Chromatographic Theory

The classic chromatographic theory from Martin and Synge can be viewed as the series of discrete partitioning steps translated into a continuous procedure in a separation column (Martin and Synge, 1941). The plate height H (cm) is the height equivalent to a theoretical plate connected to the number of theoretical plates N by;

$$N = \frac{L}{H} \quad (3.1)$$

with L (cm) corresponding to the length of the column. It is assumed that within every theoretical plate equilibrium conditions for each analyte are achieved for partitioning between the stationary and the mobile phase. H and N characterize the efficiency of a column; N can be determined directly determined from a chromatogram with

$$N = 5.54 \left[\frac{t_R}{b_{1/2}} \right] \quad (3.2)$$

with t_R as the total retention time and $b_{1/2}$ the width at half-maximum of a (ideally) Gaussian peak.

3.6.2. Kinetic Chromatography Theory

Nevertheless, the classic chromatography theory has limitations. It does not explain peak broadening effects, which rely on the limited rate at which mass transfer processes occur between the stationary and the mobile phase. The plate height H is a function of the linear flow rate $\bar{u}$ (cm.s^{-1}) of the mobile phase, as shown below in the modified van Deemter equation (van Deemter et. al., 1956; van Deemter et. al., 1995):

$$H = A + \frac{B}{\bar{u}} + C_S \bar{u} + C_M \bar{u} \quad (3.3)$$

with A Eddy diffusion

$B / \bar{u}$ Longitudinal diffusion

$C_S \bar{u}$ Mass transfer coefficient to and from the solid stationary phase

$C_M \bar{u}$ Transfer coefficient to and from the mobile phase

The modified van Deemter equation results in a minimum for H at an optimum mobile phase flow rate $\bar{u}$.

3.6.2.1. Characteristic Values of a Chromatogram

Following parameters are used to characterize the efficiency of the chromatographic separation (Kellner et. al., 1998):

 Total retention time, t_R

Time required (at peak maximum) for an analyte in the mobile phase to pass through the column (under defined chromatographic conditions)

✚ Hold-up time, t_M

Time required for an analyte in the mobile phase, which does not interact with the stationary phase (void maker), to pass through the column. t_M is also called as dead time.

✚ Capacity factor, k'

$$k' = K \frac{V_S}{V_M} = \frac{K}{\beta} = \frac{t_R - t_M}{t_M} \quad (3.4)$$

with K Partition coefficient

V_S, V_M Volume of stationary and mobile phase respectively

β Phase ratio

✚ Chromatographic resolutions, R

$$R = \frac{\sqrt{N}}{4} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{1 + k'_B}{k'_B} \right) \quad (3.5)$$

The base width w is determined from the intersection of the tangents of the inflection points with the baseline of the Gaussian peak. R describes the selectivity of the system.

3.7. MIPs as Stationary Phase for HPLC

HPLC is a suitable method evaluating the properties of a stationary phase comprising a molecularly imprinted polymer with respect to its selective recognition/retention properties for the template analyte in contrast to structural analogues. A control polymer (non-imprinted polymer, NIP) stationary phase prepared at identical conditions but in absence of the template molecule is used as a reference. Solvent serves as the void marker. The recognition properties of the polymers are assessed

by comparing the retention times t and capacity factors k' . In equations given below; k' represents retention time of the analyte molecule and t_0 represents retention time of the solvent (corresponding to void volume). For a given MIP and NIP polymer system, the separation factor α and the retention index RI of the analyzed analytes are calculated as follows:

$$\text{Capacity factor: } k' = \frac{t' - t_0}{t_0} \quad (3.6)$$

$$\text{Separation factor: } \alpha = \frac{k'_{\text{print molecule}}}{k'_{\text{test molecule}}} \quad (3.7)$$

$$\text{Retention index: } RI = \frac{\alpha_{NIP}}{\alpha_{MIP}} \quad (3.8)$$

3.8. Positron Annihilation Spectroscopy

3.8.1. The Formation of Positronium

Positrons (e^+) are the antiparticles of electrons (e^-), having the same mass as an electron but opposite charge. Occasionally a positron may bind with an electron forming a semi-stable species called positronium (Ps). Positronium (Ps) represents a bound state between a positron and an electron, which can be treated formally as hydrogen atom. There are several models of positronium formation, which were reviewed in a monograph by Mogensen, 1995. The first general theory was published by Ore, 1949. He assumed that positronium is formed during slowing down of positrons at energies E_+ in the "Ore gap", $E_{\text{ion}} - 6.8 \text{ eV} < E_+ < E_{\text{ion}}$. E_{ion} is the ionization energy of molecules or atoms in the crystal, i.e. the energy necessary to release the electron for the Ps formation. However, this model can only be applied for solids.

The bubble model of the Ps states in liquids was introduced by Ferrell, 1958. The most accepted model nowadays is the "spur model" by Mogensen, 1974. Positronium is formed mainly by the reaction between the positron and an excess electron in the spur. The positron spur is the terminal track of the positron, formed when it loses the last part of its kinetic energy. The positronium yield can be quantitatively treated with reaction kinetics of positron spur reactions (Mogensen, 1995).

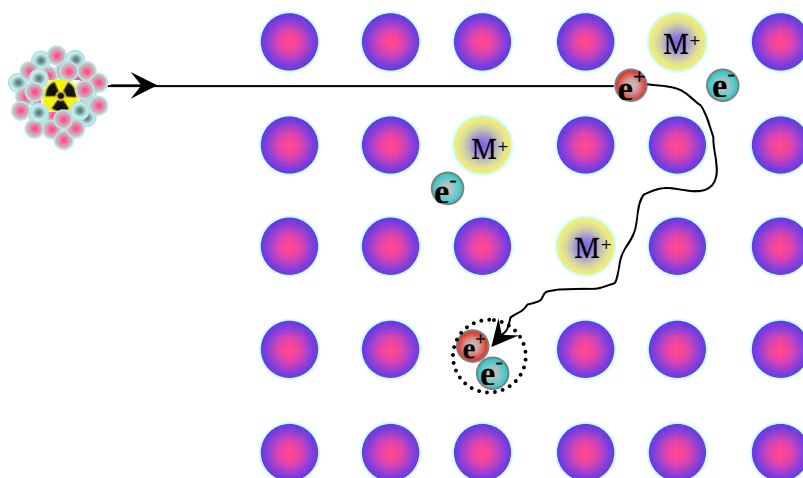


Figure 3.4. Interaction of a positron with matter showing the thermalisation process causing a "spur", diffusion of the thermalised positron and positronium formation.

3.8.2. Types of Positronium

Positronium is basically formed in two states, 75 % as ortho-positronium with parallel spins (triplet state) and 25 % as para-positronium with antiparallel spins (singlet state). The energy difference between these spin states (hyperfine splitting) is only 8.4×10^{-4} eV . The electron density in matter must be sufficiently low in order to allow for the formation of positronium. The lifetime of para-Ps was theoretically calculated

to be 123.91 ps. The best theoretical value for ortho-Ps is 142.08 ns. Experimentally, it was found to be 141.88 ns.

Para-positronium annihilates intrinsically (i.e. annihilation between the particles forming the Ps) mainly into two γ -rays of 511 keV. However, ortho-positronium annihilates in vacuum into three γ -rays in order to conserve spin. In matter, the positron wave function overlaps with electrons outside the positronium. The annihilation with such electrons having an antiparallel spin decreases the lifetime by about two orders of magnitude and amounts thus to about 1 to 5 ns. This process is called pick-off annihilation and results in two γ -rays. The lifetime of the positron is equal to the reciprocal of the pickoff rate.

The occurrence of positronium has been extensively studied in ice. This material is one of the rare cases where Ps is delocalized in a Bloch state leading to a peak in angular correlation of annihilation radiation with a negligible width. A corresponding experiment is shown in Figure 3.5.

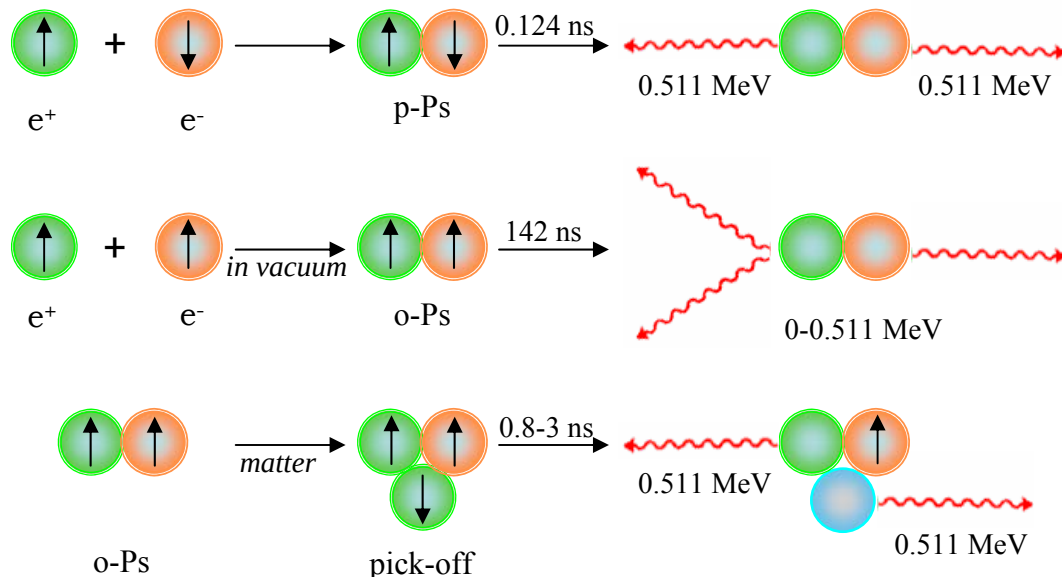


Figure 3.5. The two possible positronium states and their annihilation processes.

3.8.3. Positron Sources

Positrons can be obtained from the β^+ -decay of radioactive isotopes, e.g. from ^{22}Na according to the decay reaction $^{22}\text{Na} \rightarrow ^{22}\text{Ne} + \beta^+ + \nu_e + \gamma$. The ^{22}Na isotope gives a relatively high positron yield of 90.4 % (Figure 3.6.) and has several other advantages. First of all, the appearance of a 1.27-MeV γ -quantum almost simultaneously with the positron enables positron lifetime measurement by a start–stop coincidence g-spectrometer. Moreover, the manufacture of laboratory sources is simple, due to the easy handling of different sodium salts in aqueous solution, such as sodium chloride or sodium acetate. The half-life of 2.6 years and a reasonable price make this isotope the most used source material in positron research. The accidental contamination of laboratory personnel is less harmful, since the biological half-life is only a few days. In addition to ^{22}Na , other isotopes (^{64}Cu , ^{58}Co , etc.) can be used, but are less common. The generation of electron–positron pairs from high-energy photons is an alternative way to obtain positrons for use in beam systems. No broad application has been reported so far for applications in semiconductor research due to the need for appropriate accelerators.

The positrons generated in the β^+ -decay reaction mentioned above exhibit a broad energy distribution up to an energy of 540 keV and can penetrate deep into a sample. In electron-irradiation experiments, a particle energy of 0.5 MeV may be sufficient to generate irradiation defects such as Frenkel pairs in many materials. This should be similar for positrons. However, the dose amounts only to $\sim 1 \times 10^{11} \text{ cm}^{-2}$ for a rather long positron experiment of several days. Supposing a defect introduction rate of 1 cm^{-1} , the density of introduced defects is $\sim 1 \times 10^{11} \text{ cm}^{-3}$ and thus several orders of magnitude lower than the sensitivity limit of positron annihilation.

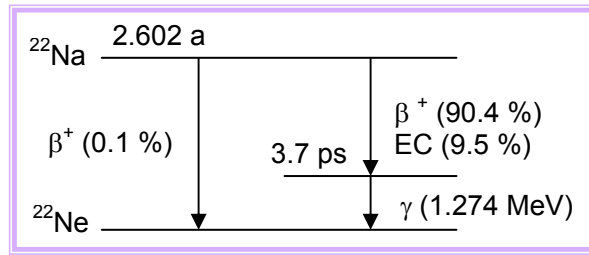


Figure 3.6. Decay scheme of the radioactive isotope ^{22}Na . 90.4 % decays by emission of a positron and an electron neutrino to the excited state of ^{22}Ne . The ground state is reached after 3.7 ps by emission of a γ -quantum of 1.274 MeV. Competitive processes with lower probabilities are electron capture (EC) and direct transition to the Ne ground state.

3.8.4. Methods of Measurements

The different positron techniques are based on the analysis of the annihilation radiation. It appears through the mass-energy transformation of electron-positron pairs, mostly into two g-quanta of 511 keV. In special cases, e.g. at surfaces, a positron may form a bound state with an electron, which is called positronium (Ps). The annihilation of Ps can lead to two or three annihilaton γ -quanta.

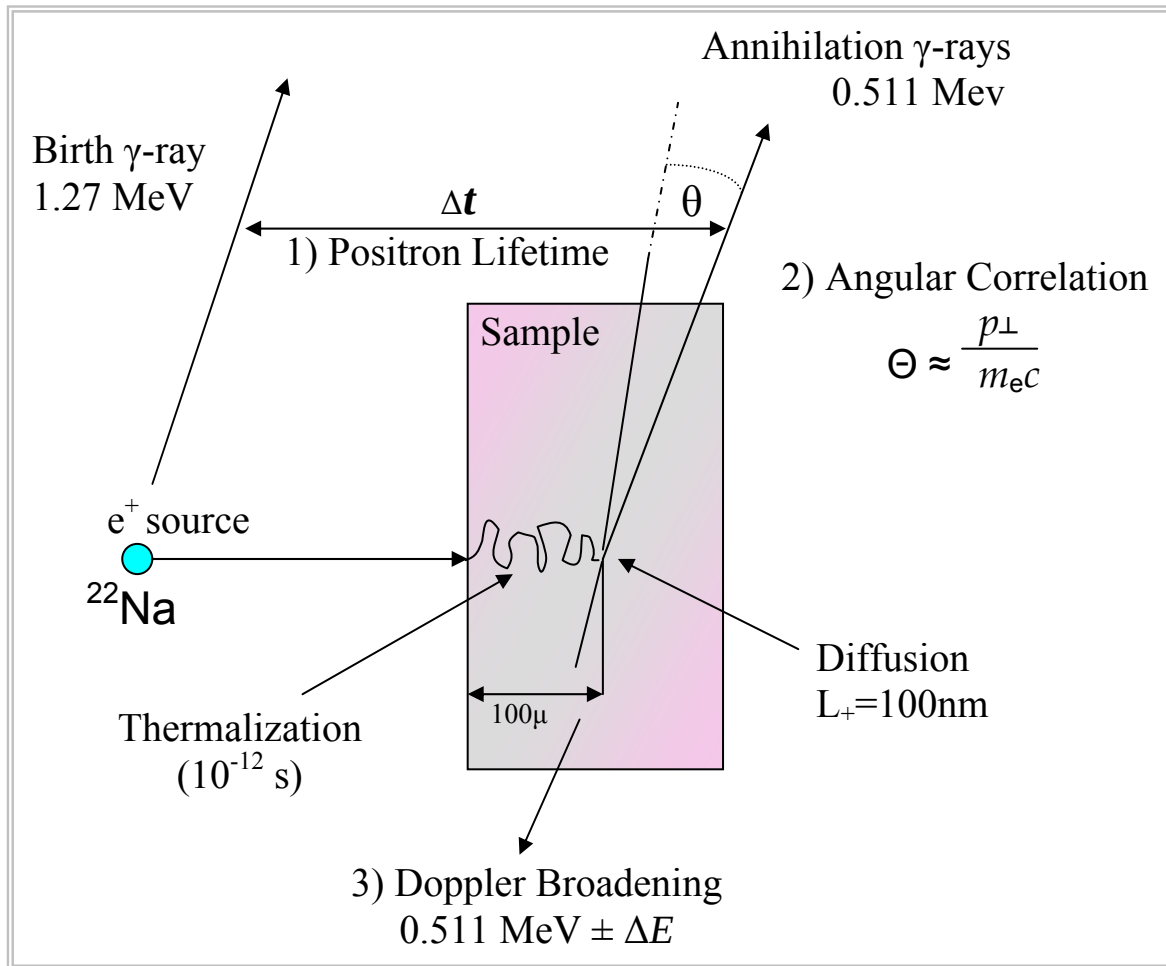


Figure 3.7. Schematic representation of positron annihilation indicating the basis for the three experimental techniques of the positron annihilation spectroscopy: lifetime, angular correlation and Doppler broadening.

They can be classified into two principal groups which are distinguished by the sensitivity of positrons to the electron density (positron lifetime measurement) and to the electron momentum distribution in the sample (Doppler-broadening spectroscopy and angular correlation of annihilation radiation).

The positron lifetime is registered as the time difference between the emission of the 1.27-MeV γ -quantum generated almost simultaneously with the positron in a ^{22}Na isotope, which is the most commonly used positron source, and one of the 0.51-MeV annihilation γ -quanta. These two techniques are based on the principle of momentum conservation in the annihilation process. The momentum distribution of the electron-positron pair is conserved in the annihilation radiation. The momentum component in the γ -ray propagation direction leads to a small Doppler shift of the annihilation energy. Several million annihilation events are summed up as a Doppler-broadened spectrum of the 0.51-MeV annihilation line in an energy-dispersive spectrometer. This method is called Doppler-broadening spectroscopy. The momentum components in the other two space directions result in the deviation Q from collinearity of the two 0.51-MeV annihilation γ -quanta. This deviation can be measured in a γ - γ -coincidence arrangement in one or two dimensions. These setups are called one- and two-dimensional angular correlation of annihilation radiation.

3.8.4.1. Positron Annihilation Lifetime Spectrometer

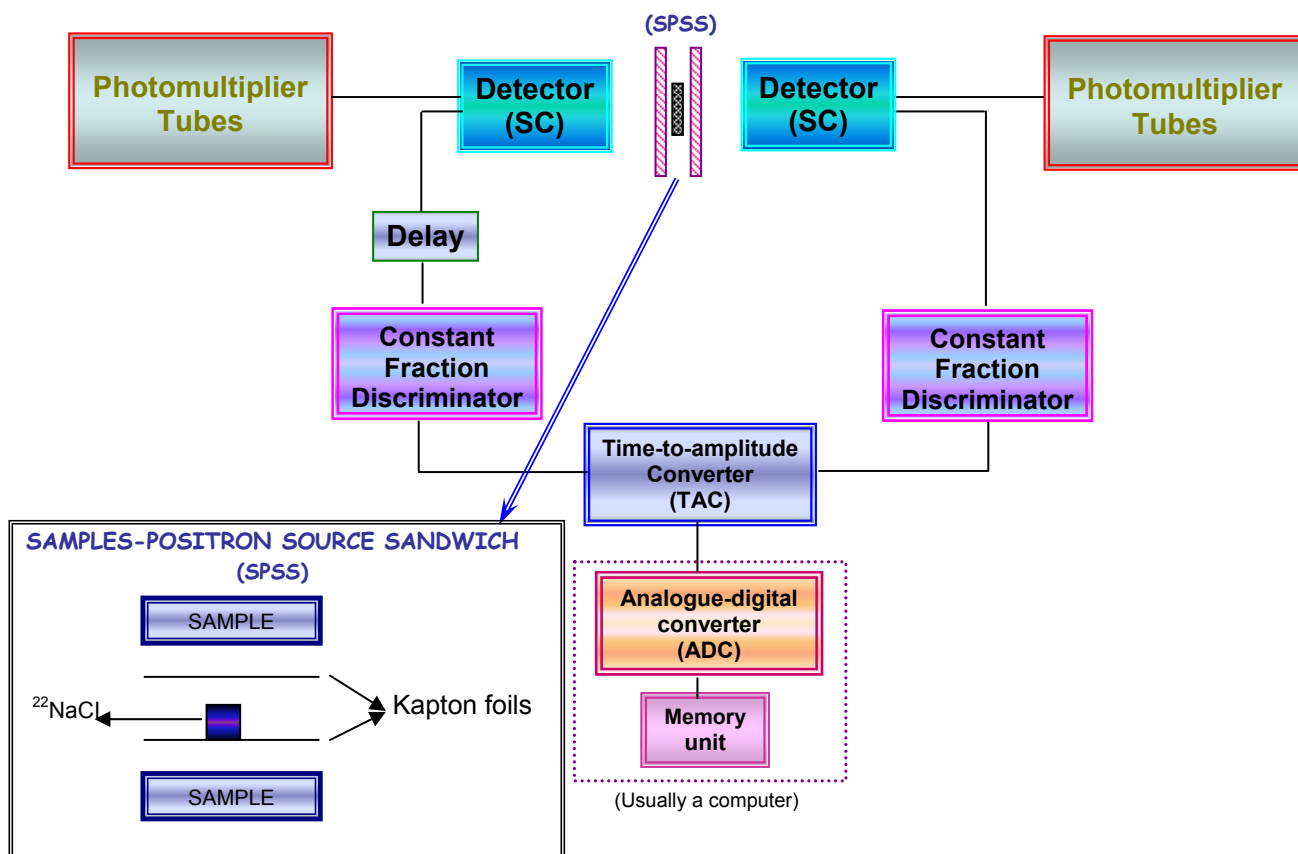


Figure 3.8. Experimental setup for fast-fast coincidence systems.

Figure 3.8. outlines a general scheme of fast-fast coincidence systems by which almost all spectra are obtained. PALS measurements involve placing the sample with the positron source between two fast scintillation detectors (SC) attached to photomultiplier tubes. The detectors convert the measured radiation into electrical pulses: one detecting the birth quantum of 1.27 MeV, the other one the death quanta of 0.511 MeV. The resolution of the detectors is usually around 250 ps. The pulses are processed by constant fraction discriminators (CF DISC), their outputs starting and stopping a time-to-amplitude converter (TAC).

The amplitude of the output of the TAC converter is proportional to the time difference between the birth and death annihilation quanta, and thus represents the positron

lifetime. The amplitude of each event (count) is sorted and stored into a channel of a multi channel analyser (MCA). Each channel represents a specific time interval and all counts are stored and added up into a particular channel according to their amplitudes. A positron lifetime spectrum is obtained by plotting the number of counts against the channel number and generally represents approximately a total of one million counts.

3.8.4.2. Angular Correlation of Annihilation Radiation

The momentum distribution of annihilation γ photons are measured by one-or-two-dimensional angular correlation spectrometers. The Angular Correlation of Annihilation Radiation (ACAR) spectrum is determined by the momentum conservation law, i.e. the initial momentum of the annihilating positron-electron pair should be reflected in the angular distribution of γ photons. For a zero-momentum pair, in the case of 2γ -annihilation, the angle between the directions of photons is 2π radians (i.e. 180°). For realistic electron energies this angle is modified by several milliradians. This modification is approximately:

$$\Theta \approx \frac{p_{\perp}}{m_e c} \quad (3.9)$$

where $p_{\perp}$ indicates the momentum component perpendicular to the axis of the spectrometer, m_e is the electron mass, and c is the speed of light.

3.8.4.3. Doppler-Broadening Spectroscopy

The basics of this method are very similar to that of ACAR spectroscopy. For an electron-positron pair of zero kinetic energy, 2γ -annihilation results in two identical photons of 0.511 MeV propagating into opposite directions. If the pair has a momentum component parallel with the direction of photons, the 0.511 MeV energy of photons deviates a little up or down. The phenomenon is called Doppler-broadening of annihilation radiation.

The evaluation of Doppler-broadening spectra is a rough method. Usually only the central region of the annihilation peak is compared with the total area of the peak, which indicates that low-kinetic-energy part of electrons and is called as S-parameter. Sometimes another parameter, w , is also determined to describe the high-momentum regions of the spectrum.

3.8.5. Sample Preparation

Sources of weak activity are needed for conventional positron lifetime and Doppler-broadening measurements. Even activities as low as 8×10^5 Bq (20 μ Ci) are sufficient. The sources are usually prepared by evaporating a solution of a ^{22}Na salt on a thin metal or polymer foil. The most common foil materials are Al, Ni, and mylar or kapton. In order to ensure the almost complete annihilation of positrons in the specimen volume, the foil source is placed between two identical samples (“sandwich arrangement”, Figure 3.9.). A minimum sample thickness is required to ensure that the essential fraction of positrons annihilates in the sample pair. The scintillators should be placed as close as possible to the sample–source sandwich. If cooling of the sample is required, the design of the cryostat should allow the separation between the scintillators to be as small as possible in order to avoid a considerable reduction in the counting rate. A small fraction of the positrons annihilates in the source. This amounts to 2 to 15 % depending on the foil thickness and the atomic number of the sample, which determines the backscattering and, thus, the multiple passing of positrons through the source. For the analysis of positron lifetime spectra, this fraction must be carefully determined and subtracted. The so-called source correction is an essential task of the lifetime spectra evaluation. Details can be found e.g. in the paper of Staab et al., 1996. To reduce the source contribution, the isotope solution can be directly deposited onto the sample surface. The disadvantage of this procedure is radioactive contamination of the samples, which can thus hardly be used for further investigations.

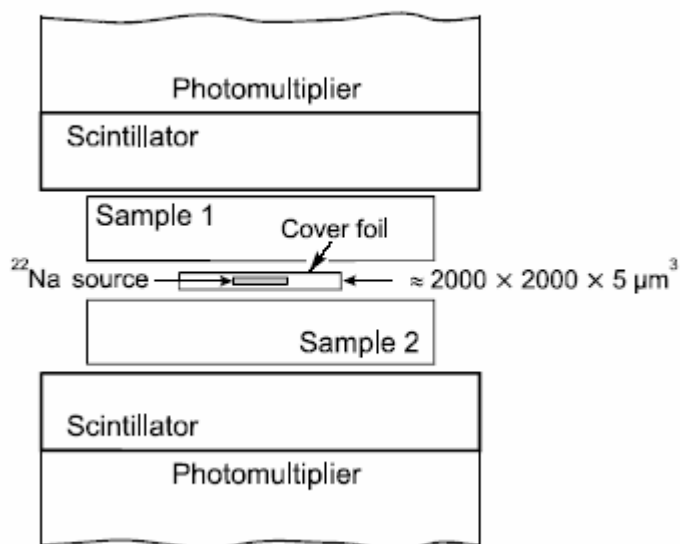


Figure 3.9. Schematic representation of Sandwich arrangement.

3.9. Positron Behaviour in Condensed Matter

Positrons diffusing through matter may be captured in special trapping sites. These trapping centers are crystal imperfections, e.g. vacancies and dislocations. The wave function of a positron captured in such a defect is localized until it annihilates with an electron of the immediate surrounding into γ -rays. Since the local electron density and the electron momentum distribution are changed with respect to the defect-free crystal, the annihilation radiation can be utilized to obtain information on the localization site. Hence, the character and the concentration of the trapping centers, i.e. lattice defects, can be investigated by positrons.

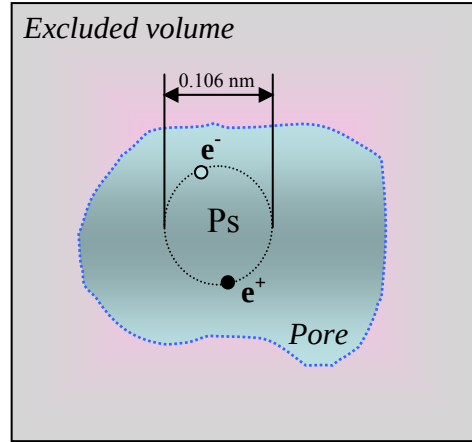


Figure 3.10. A cross-sectional view of in situ nature of the Ps as a probe for pores in porous material. Ps is preferentially localized in pore before the annihilation occurs.

To simplify, a positron enters the cave at the end of the thermalization process and bounds an electron creating inside the cave Ps, as shown in figure 3.10. Then the annihilation rate being the inverse the lifetime is proportional to the overlapping o-Ps wave function with the environment as it was postulated by Tao 1972 and put into shape by Eldrup et al. 1981. In the case of spherical cave of the radius R , the lifetime of o-Ps (τ) can be expressed as follows:

$$\tau = \frac{1}{2} \left(1 - \frac{R}{R + \Delta R} + \frac{1}{2\pi} \sin \frac{2\pi R}{R + \Delta R} \right)^{-1} \quad (3.10)$$

As given above Tao-Eldrup equation establishes the direct relation between the lifetime of o-Ps (τ) with the radius of the spherical cave (R). “ Δ ” denotes the environmental depth which Ps wave function penetrates.

3.9.1. Defects in Materials

Real-world materials do not have exactly uniform properties throughout; all materials contain tiny inhomogeneities (or defects) in which the properties are substantially different from that in the rest of the material.

Defects are central to any understanding of material characteristics. They determine a whole host of material properties, including strength, ductility, resistivity, and opacity. In some cases, defects can build up in a material until it fails, as has occurred with some aircraft components.

Defects in materials occur over a wide range of scales, as shown in figure 3.11. At the smallest scale, defects consist of single missing atoms (or vacancies) in the material. At higher defect concentrations, the vacancies may connect into dislocations, the first stage toward what may become a crack. Larger defects with no material in them are called voids. In polymers, which are made up of long molecules, defects may aggregate between molecules and form microscopic holes.

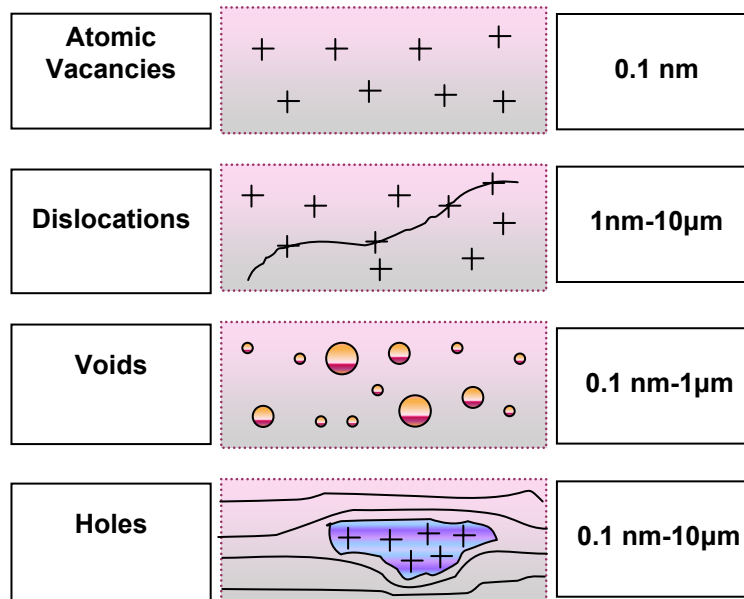


Figure 3.11. Defects range from single-atom vacancies through macroscopic voids and holes.

3.9.1.1. Semiconductors

The positron trapping behaviour in semiconductors is more complex compared with metals. This is mainly due to the fact that the positron as a charged particle is sensitive to the charge state of defects. The charge state of vacancies in semiconductors strongly influences the sensitivity range of positron annihilation. Positively charged vacancies repel positrons and are thus invisible to the different positron-related defect detection methods. The sensitivity range for neutral vacancies in semiconductors is comparable to that of metals and hence dislocations can be observed only after plastic deformation. Temperature-dependent lifetime measurements may distinguish between both defect types.

3.10. The Use of Positron Annihilation Lifetime Spectroscopy in Polymer Physics Research

The use of positron annihilation lifetime spectroscopy (PALS) of polymeric materials has become a useful method for probing the free volume on an angstrom size-scale, between polymer chains. Whilst the concept of free volume is complex, not least because of issues of static and dynamic free volume, it is able to give insights into chain packing and even into interfaces of multicomponent systems. It is a non-destructive, radioactive technique in which the anti-particle of an electron is ejected, from a radioactive source, into the polymeric material. Upon ejection, a characteristic energy is given off which is timed. Upon entering the solid sample, a number of different mechanisms exist by which the positron, or species of positrons + electrons, can decay. Regardless of which mechanism occurs, another characteristic energy is given out. The decay curve which results can be fitted to a range of components and, of particular interest to polymers, the lifetime of ortho-positronium (o-Ps) and its intensity can be determined. The lifetime, τ , is related to the size of the free volume, and the intensity to the number concentration of free volume sites, and sometimes also the chemistry of the functional groups in the polymer. By using various simple

models and assumptions, an approximate “size” of free volume can be determined and a measure of free volume fraction (which may be expected to be related, in some way, to macroscopic density) (Brandt et. al, 1960; Tao, 1972; Eldrup et. al., 1981). This type of properties that have been found to be related to these parameters include: thermal ageing (densification—mainly to the number concentration), temperature properties such as glass transition, gas permeability in glassy solids, etc.

4. EXPERIMENTAL

4.1. Materials

The template molecule, (+) D-glucose and functional monomer, 2- hydroxyethyl methacrylate (HEMA) were obtained from Fluka. The crosslinking agents; diethylene glycol diacrylate (DEGDA), diethylene glycol dimethacrylate (DEGDMA), triethylene glycol dimethacrylate (TEGDMA), polypropylene glycol dimethacrylate (PPGDMA with $M_n = 560$), polyethylene glycol dimethacrylate (PEGDMA with $M_n = 550$) were purchased from Aldrich. The solvents; dimethyl sulfoxide (DMSO) and ethanol (EtOH) were purchased from Merck. All chemicals were analytical grade and used as received. The materials and their chemical structures used in this study are given in Table 4.1.

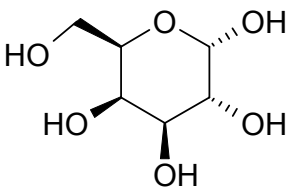
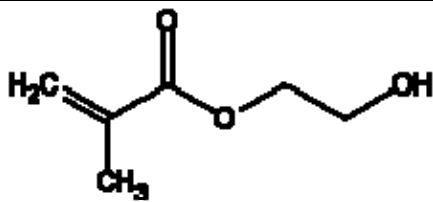
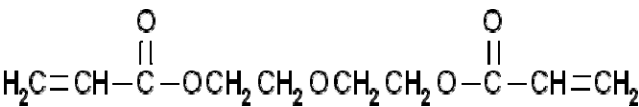
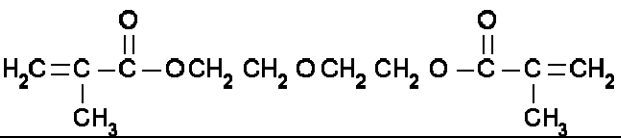
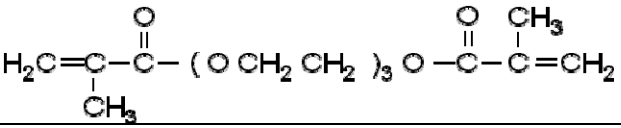
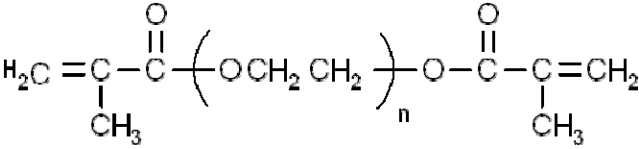
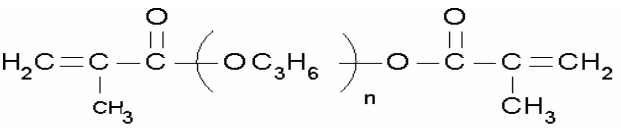
4.2. Method

4.2.1. Synthesis of D-Glucose Imprinted Network

All Molecularly Imprinted Polymer (MIP) systems with different compositions of template, crosslinking agent and functional monomer were synthesized in the presence of a DMSO-EtOH solvent mixture via radiation polymerization. In order to achieve pre-polymerization complex formation; template molecule, D-glucose was first mixed with the monomer and then the solvent and the crosslinking agent were added. The crosslinking agents used were DEGDA, DEGDMA, TEGDMA, PEGDMA, PPGDMA; in order of increasing chain length. Crosslinking agent concentration in the polymerization mixture (monomer, crosslinking agent and template) covered a range of 10, 15, 20, 30, 40, 50, 60 and 70 % by mole with respect to HEMA. The mole ratio of functional monomer, HEMA to D-glucose was kept either as 1:1, 3:1 or 6:1. Control polymers were synthesized with exactly the same compositions as MIPs in the absence of D-glucose. To investigate the crosslinking effect of radiation, HEMA networks were also prepared without crosslinking agent in the same manner. All MIP

systems were prepared in appropriate amount of DMSO: EtOH (3:1 v/v) solvent mixture. Once the mixtures mentioned above were prepared they were placed in between clamped glass slides (60 mm x 60 mm x 2 mm) separated with a natural rubber ring. Irradiations were carried out in air at ambient temperature in a Gamma cell 220, ^{60}Co - γ irradiator (Nordion, Canada) with an irradiated dose from 1 to 15 kGy. At the end of predetermined irradiation periods the glass slides were placed in deionized water for 6 h, crosslinked polymers were then carefully separated from the glass slides and cut into discs of 12 mm diameter. Discs were then placed into 200 mL of deionized water. The rinsing solutions were changed three times a day to remove the template and unpolymerized material if any. The resulting discs were then dried in air at room temperature and then placed in a vacuum oven ($T=40\text{ }^{\circ}\text{C}$, 100 mbar vacuum), until complete dryness.

Table 4.1. Chemical structures of materials used in this study.

Name of the compound	Chemical structure
(+) D-Glucose	
2-Hydroxyethyl methacrylate (HEMA)	
Diethylene glycol diacrylate (DEGDA)	
Diethylene glycol dimethacrylate (DEGDMA)	
Triethylene glycol dimethacrylate (TEGDMA)	
Poly(ethylene) glycol dimethacrylate (PEGDMA) (n=10)	
Poly(propylene) glycol dimethacrylate (PPGDMA) (n=8)	

4.2.1.1. Synthesis of D-Glucose Imprinted Network with 3:1 HEMA: D-Glucose Ratio Containing 15 % Crosslinking Agent

In a typical experiment, predetermined amount of glucose, HEMA were firstly mixed with appropriate amount of solvent (DMSO-EtOH mixture) with the addition of necessary amount of crosslinking agent into the mixture. All prepared solutions were mixed for 30 minutes. Once the solutions mentioned above were prepared they were placed in between clamped glass slides (60 mm x 60 mm x 2 mm) separated with natural rubber ring. Irradiations were carried out in air at ambient temperature in a Gamma cell 220, ^{60}Co - γ irradiator (Nordion, Canada) with an absorbed dose ranging from 1 to 15 kGy. Several different compositions of polymer are shown in Table 4.2.

Discs were then placed into 200 mL of deionized water. The rinsing solutions were changed three times a day to remove the template and unpolymerized material if any. Daily solutions were taken to control complete removal of glucose and unpolymerized material by HPLC and UV measurements, Figure 4.2. UV measurements were carried out by a Varian, Cary100 UV-visible spectrophotometer. The resulting discs were dried in air at room temperature and then placed in a vacuum oven ($T=40\text{ }^{\circ}\text{C}$, 100 mbar vacuum), until complete dryness.

In pre-polymerization step, possible interaction mechanism between D-Glucose and networks can be presented as shown below. Each $-\text{OH}$ groups of D-Glucose can interact with $-\text{OH}$ group and oxygen of carboxyl group of HEMA via hydrogen bonding.

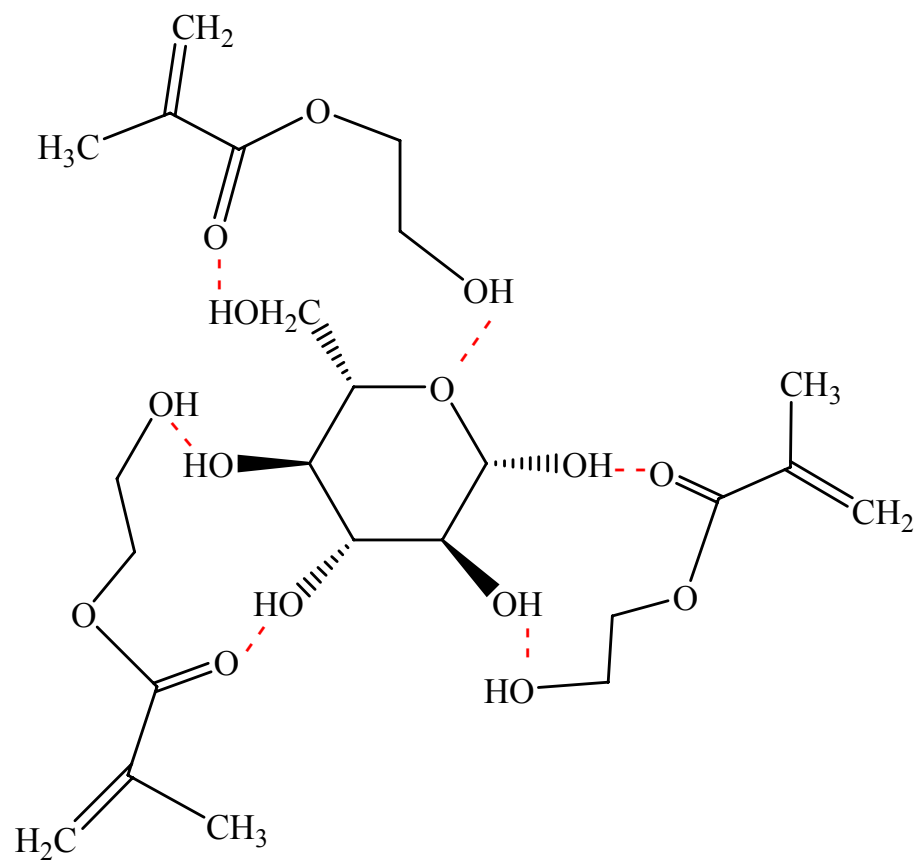


Figure 4.1. Possible interaction mechanism between D-Glucose and monomer units.

Table 4.2. Composition of synthesized MIP and NIP network which include 15 % crosslinking agent and 3:1 HEMA:glucose mole ratio. All of compositions were prepared with in 2 mL of DMSO-EtOH solvent mixture.

Type of MIP-NIP	Amount of Components (mole)						
	<i>D-Glucose</i>	<i>HEMA</i>	<i>DEGDA</i>	<i>DEGDMA</i>	<i>TEGDMA</i>	<i>PEGDMA</i>	<i>PPGDMA</i>
<i>Imprint</i>	4.00×10^{-3}	1.25×10^{-2}	2.21×10^{-3}	-	-	-	-
<i>Non-imprint</i>	-	1.25×10^{-2}	2.21×10^{-3}	-	-	-	-
<i>Imprint</i>	4.03×10^{-3}	1.21×10^{-2}	-	2.15×10^{-3}	-	-	-
<i>Non-imprint</i>	-	1.21×10^{-2}	-	2.15×10^{-3}	-	-	-
<i>Imprint</i>	3.87×10^{-3}	1.16×10^{-2}	-	-	2.05×10^{-3}	-	-
<i>Non-imprint</i>	-	1.16×10^{-2}	-	-	2.05×10^{-3}	-	-
<i>Imprint</i>	5.30×10^{-3}	1.59×10^{-2}	-	-	-	2.81×10^{-3}	-
<i>Non-imprint</i>	-	1.59×10^{-2}	-	-	-	2.81×10^{-3}	-
<i>Imprint</i>	2.98×10^{-3}	8.95×10^{-3}	-	-	-	-	1.58×10^{-3}
<i>Non-imprint</i>	-	8.95×10^{-3}	-	-	-	-	1.58×10^{-3}

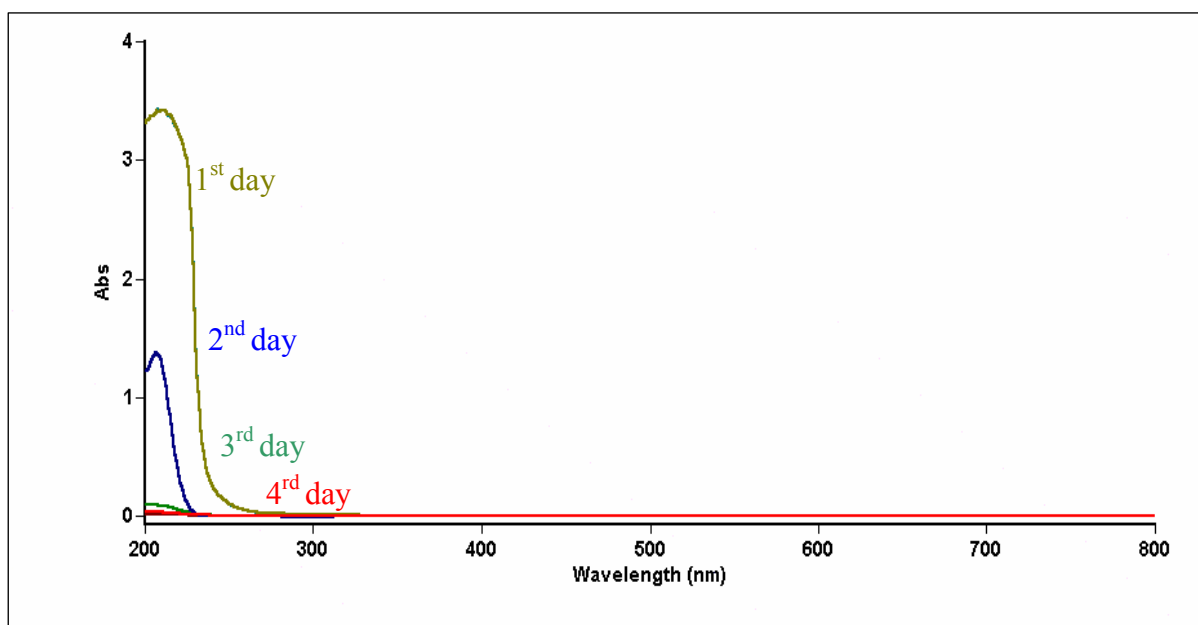


Figure 4.2. UV Spectrum of washing solution taken during daily wash of 15 % TEGDMA containing MIP with 3:1 HEMA:Glucose mole ratio, irradiated to 5 kGy.

4.3. Experimental Measurements

4.3.1. Spectroscopic Measurements

Spectroscopic characterizations of molecularly imprinted and non-imprinted polymers were carried out using a Nicolet 520 model FTIR spectrometer. FT-IR spectra of samples were obtained between 4000-400 cm^{-1} with 32 scans collected for each spectrum with a nominal 4 cm^{-1} resolution. Synthesized MIP-NIP samples were mixed with spectroscopic grade KBr, ground and prepared as proper pellets. Thin films were prepared over the KBr pellets to obtain good FT-IR spectra of liquid forms of crosslinking agents and functional monomer.

4.3.2. Swelling Studies

Determining the water content provides information on the degree of hydrophilicity of a polymer. On the other hand, swelling properties are directly related with the cavity size of crosslinked networks. In this study, it was seen that swelling measurements support PALS measurements.

Dynamic swelling experiments were performed on both deionized water and different glucose concentrations with dry discs. The water content of the MIPs-NIPs was determined by gravimetric measurements. Discs were taken from solutions in proper time intervals. Discs were dried with filter paper and the samples were quickly weighed in an aluminum weighing boat. Percent swelling data given here is the average of triplicate measurements and presented to give approximate values.

Swelling values were calculated by using equation 4.1. where w_t is the mass (g) of the swollen polymer at time t and w_0 is the mass (g) of the dry polymer at time 0.

$$\text{Swelling \%} = \frac{w_t - w_0}{w_0} \quad (4.1.)$$

4.3.3. Pal Experiments

For positron annihilation lifetime (PAL) experiments a positron source was prepared by depositing ca. 1.8 MBq of aqueous $^{22}\text{NaCl}$ on a 7 μm -thick Kapton foil having a $10 \times 10 \text{ mm}^2$ area. After drying, the foil was covered with foil of the same size, and the foil edges were glued with epoxy resin. A sandwich arrangement (sample-source-sample) was used. PAL experiments were carried out using a conventional fast-fast coincidence system having a time resolution (FWHM) of about 280 ps. The measurements were carried out in air at room temperature. The spectra were recorded at every 2 hours with total counts in each spectrum being $\sim 1.8 \times 10^6$. Then, for each type of sample, the first 5 spectra were summed together resulting in $\sim 9 \times 10^6$ counts. In a number of experiments on polypropylene and polyethylene, the exposure to the energetic positrons of the weak, radioactive source may lead to increased Ps formation inhibition with the elapsed time of the exposure what is known as source effect (Peng et. al., 1996). No such sequential change was observed over the time frame (for some of the samples more than 48 h) of the measurements.

Assuming that e^+ annihilates from states with definite discrete lifetimes, τ_i , the PAL spectrum can be written as

$$S(t) = R(t) \left\{ (N_t - B) \sum_{i=1}^n (I_i / \tau_i) \exp(-t/\tau_i) + B \right\} \quad (4.2)$$

where $R(t)$ is the apparatus time resolution function, n is the number of the different states, I_i ($\sum_{i=1}^n I_i = 1$) denotes the relative intensity of the corresponding component or probability of annihilation with lifetime τ_i , N_t is the total counts, and B is the background. For a continuous distribution of the lifetime, the sum in Equation 4.2. has to be replaced by the integral $\int_{\tau=0}^{\infty} (1/\tau) I(\tau) \exp(-t/\tau) d\tau$, where $\int_{\tau=0}^{\infty} I(\tau) d\tau = 1$. First of all the experimental spectra were analyzed by MELT program assuming continuous lifetime

distribution (Shukla et. al.,1997). As it was expected, for the short lifetime region (<0.5 ns), two sharp peaks of the lifetime distribution were observed (Figure 4.2). The mean lifetime of the first peak was close to 0.125 ns in good agreement with the p -Ps lifetime. The mean value of the second peak was ~ 0.380 ns, which is a value usually ascribed to annihilation of free-positrons. However, for the longer lifetime region (>0.5 ns), we observed different types of distributions (unimodal or bimodal) even for a single sample (see Figure 4.2).

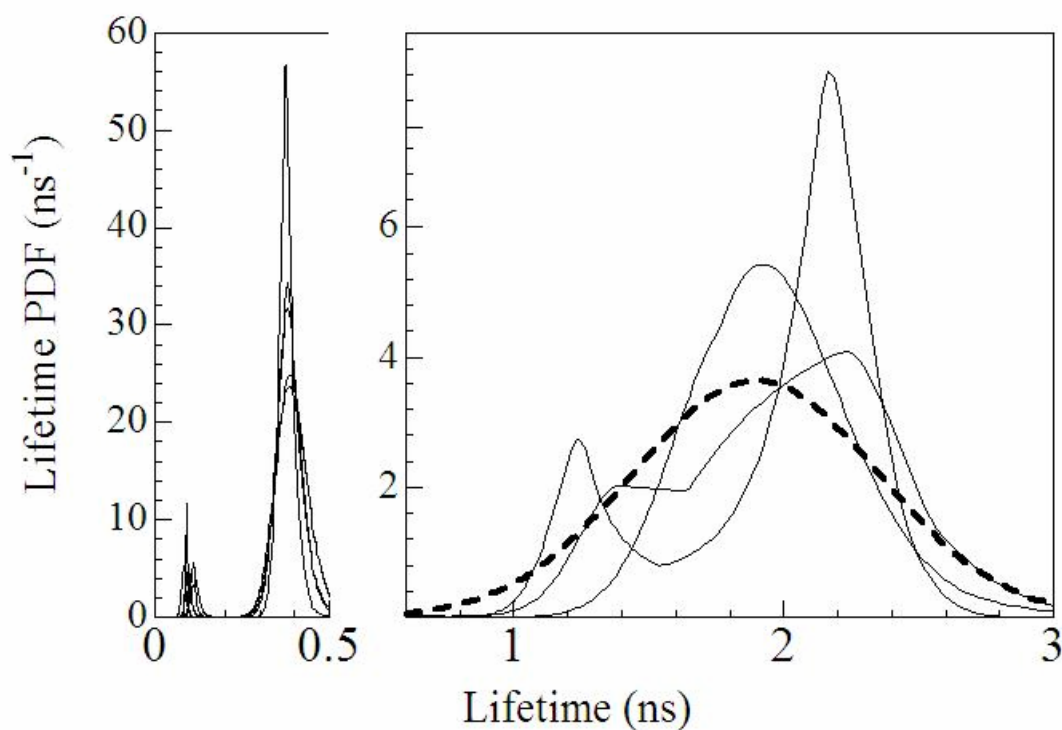


Figure 4.3. Example of PDFs of the lifetime for 30 % TEGDMA containing sample with 3:1 HEMA:glucose mole ratio and 5 kGy irradiation dose. The continuous lines represent MELT result for consecutive spectra for the same sample with statistics of $\sim 1.8 \times 10^6$ counts. The dashed line is plotted from the third component data obtained by LT from the summed spectrum $\sim 9 \times 10^6$ counts.

For the case of discrete lifetimes, the LT program was used, and the spectra were resolved first into four components (Kansy, 1996). The experimental errors of the two long lifetimes and their corresponding intensities were large which lead us to the conclusion that, actually, the two long-lived components represent one component but with a wide distribution. Therefore, for the final analysis, we used the feature of the LT program that allows mixed analysis: two short-lived components were assumed to be discrete and a long-lived components, due to pick-off *o*-Ps annihilation, and were assumed to have a Gaussian distribution. It should be mentioned that the LT results for the *o*-Ps component differ very little for the consequent spectra of the same sample. That is why; the *o*-Ps lifetime probability density function (PDF) only for the summed spectrum is plotted in Figure 4.2. For all of the samples FWHM of the Gaussian distribution of τ_3 obtained by LT program was in the range of 0.11-0.13 ns.

4.3.4. Doppler Broadening Of Annihilation Gamma-Line

For the measurements of the Doppler broadening of the annihilation line, a Ge detector with energy resolution of 1.2 keV at 662 keV line of ^{137}Cs was used. The measurements were performed simultaneously with the PAL measurements. The shape of the Doppler-broadened annihilation γ -line is usually characterized by the integral *S* and *W* parameters. The *S* parameter was defined as ratio of the sum of counts in the central region of the peak ($|\Delta E| < 0.9$ keV, where ΔE is the shift from the 511 keV peak maximum) relative to the total peak counts (N_{tot}) and the *W* parameter – as the ratio of the sum of counts in the peak wings ($2 < |\Delta E| < 7$ keV) to N_{tot} .

4.3.5. Microscopic Measurements

Microscopic studies were performed by fluorescence inverted microscope, Olympus IX70 (Japan) for dry and swollen samples. Microscopic studies were performed for all types of samples used for column packing. Samples were placed onto microscope

plate and several images were taken for each sample. Homogeneity of ground samples were determined within a range of μm scale.

4.3.6. High Performance Liquid Chromatography Studies

Characterization of the imprinting effect by HPLC is necessary to determine whether imprinting was successful and to assess the effect of the different factors related to MIP synthesis concerning with recognition properties. The recognition properties of synthesized MIPs were therefore tested by using them as HPLC column packing materials.

HPLC system was set up by Waters 1525 Binary HPLC pump, Waters inline degasser AF, Waters 2414 refractive index dedector and column heater module. 15 kGy irradiated MIPs with 3:1 HEMA:glucose mole ratio were chosen for HPLC studies. The MIPs were chosen according to their swelling properties and results related to PAL experiments. All MIPs were ground by food processor to size range of 750-1000 μm in swollen state.

After grinding and sieving, the MIP particles, in swollen state, were packed into stainless-steel HPLC column with 25mm length and 4.6mm inner diameter. Prepared columns were washed on-line with deionized water at 0.5 mL/min until a stable baseline was obtained. The imprinted and the control columns were equilibrated with deionized water as mobile phase. The elutions were performed at room temperature at a flow rate of 1.0 mL/min and monitored by refractive index dedection. The injection volume was 20 μL . Following analytes; D(+)-Glucose, D(-)-Fructose and D(+)-Galactose were prepared with deionized water at concentration of 10 ng/mL for each saccharide. These three sugar samples were injected to the system three times to determine affinity properties of synthesized MIPs. Relevant chromatographic constants were calculated based on obtained chromatograms.

5. RESULTS and DISCUSSIONS

5.1. Spectroscopic Measurements

Fourier transform infrared, FT-IR spectra were used to characterize HEMA based MIP and NIP networks with various crosslinking agents.

Strong absorbance observed in the region between $3400\text{--}3500\text{ cm}^{-1}$ was attributed to the --OH group of 2-hydroxyethyl methacrylate. The stronger absorbance that is observed for imprinted polymers containing DEGDA as crosslinker in this region can most probably be the result of an increase of the hydrogen bonding interactions, as seen from Figure 5.1.

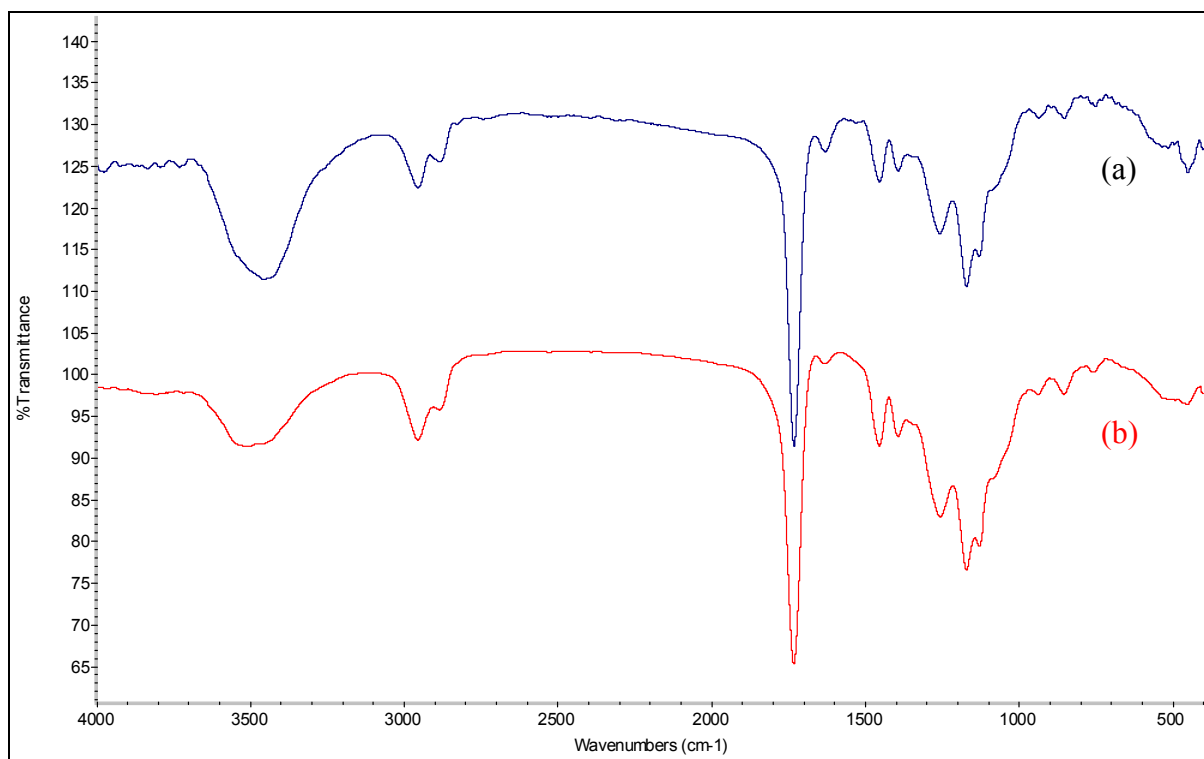


Figure 5.1. FT-IR spectra of 30 % DEGDA containing (a) MIP and (b) NIP with 3:1 HEMA:glucose mole ratio, irradiated to 5 kGy.

A qualitative estimation of the extent of unreacted double bonds considering the C-H out of plane bending at 950 cm^{-1} can be used to estimate the degree of crosslinking in the imprinted polymers. FT-IR analysis of the C-H out of plane bending at 950 cm^{-1} indicates complete reaction of the double bonds giving a result of a highly crosslinked structure. This result indicates that the reactivity of the double bonds were very high.

The carbonyl band in the FT-IR spectrum of each of MIPs at 1730 cm^{-1} was also observed. Intensity of carbonyl band for each sample was found to be nearly the same with each other. Absorption of medium intensity near 2950 cm^{-1} was accompanied by the asymmetrical stretching and symmetrical stretching of methylene groups, $-\text{CH}_2$ on the chains. Main difference between crosslinking agents was the number of methylene groups on the chains. Increasing band intensity was observed at 2950 cm^{-1} with increasing methylene group on the chain. Absorption arising from C-H bending vibrations was observed at the region of $1480\text{-}1400\text{ cm}^{-1}$.

On the other hand, $-\text{C-O-}$ stretching absorption band was observed at 1100 cm^{-1} . In our imprinting systems, number of $-\text{C-O-}$ groups on the chain increases as chain length increases. As seen from Figure 5.2, band intensity at 1100 cm^{-1} was properly increasing with increasing chain length consequently increasing the number of $-\text{C-O-}$ groups.

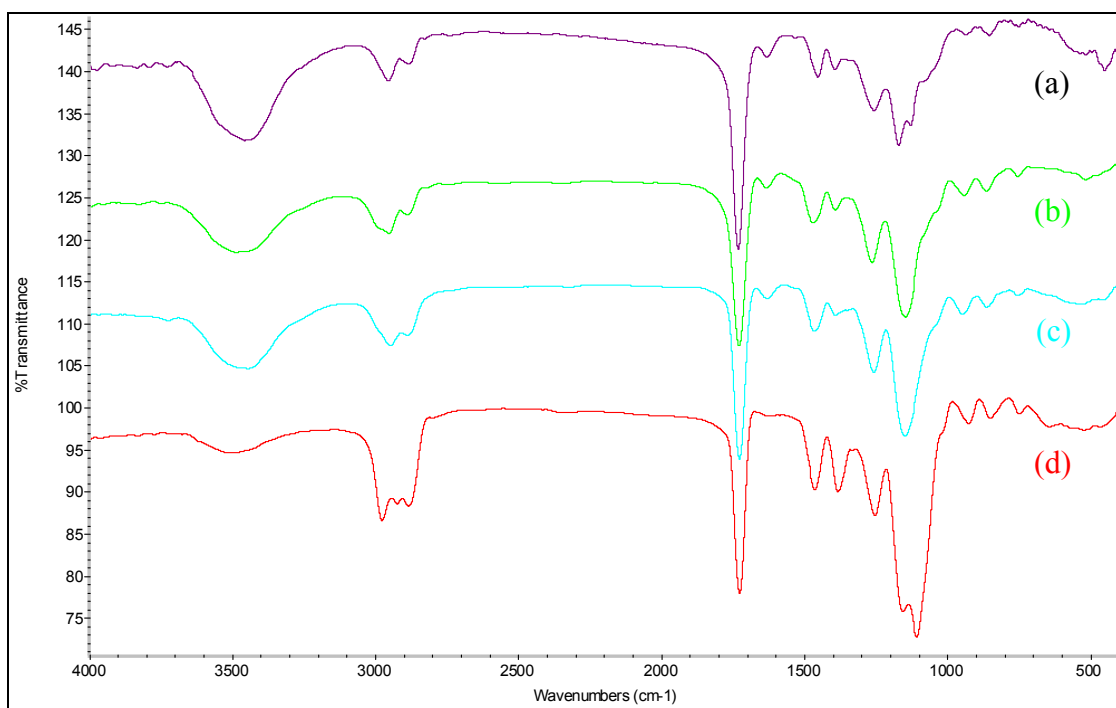


Figure 5.2. FT-IR spectra of samples containing various crosslinking agents. 30 % (a) DEGDA, (b) DEGDMA, (c) TEGDMA, (d) PPGDMA. Imprinted with 3:1 HEMA:glucose mole ratio, irradiated to 5 kGy.

As seen from Figure 5.2, band intensity at 2950 cm^{-1} for PPGDMA was the highest due to the highest number of $-\text{CH}_2$ group on the chain. It is also seen that band intensity at 1100 cm^{-1} for PPGDMA was the highest value due to the highest number of $-\text{C-O}-$ groups on the chain.

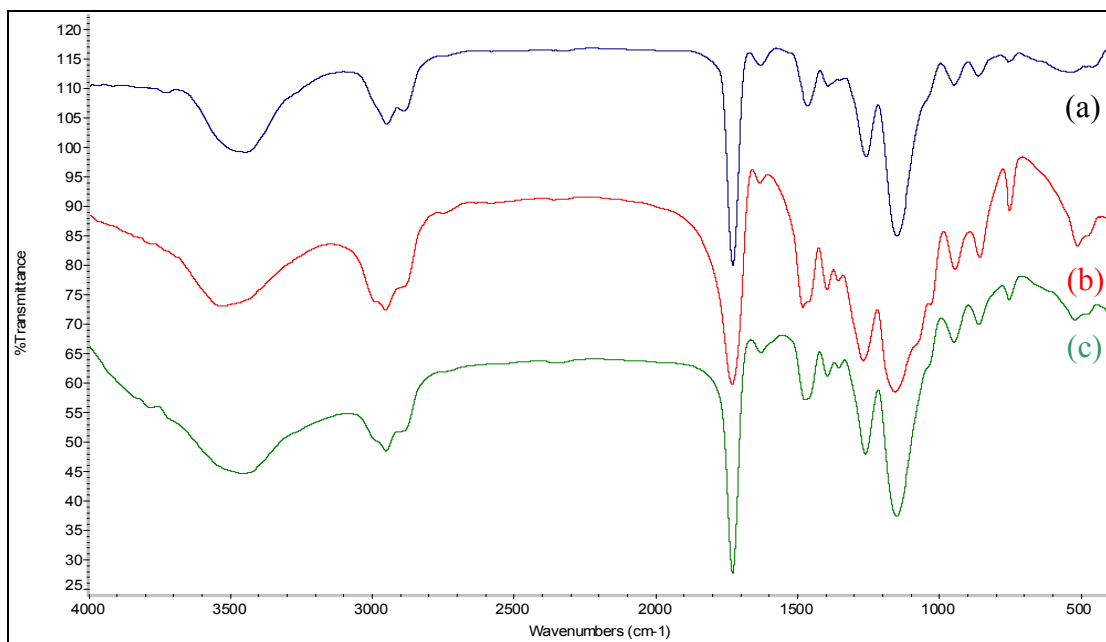


Figure 5.3. FT-IR spectra of samples containing (a) 30 %, (b) 50 %, (c) 70 % TEGDMA and imprinted with 3:1 HEMA:glucose mole ratio, irradiated to 5 kGy.

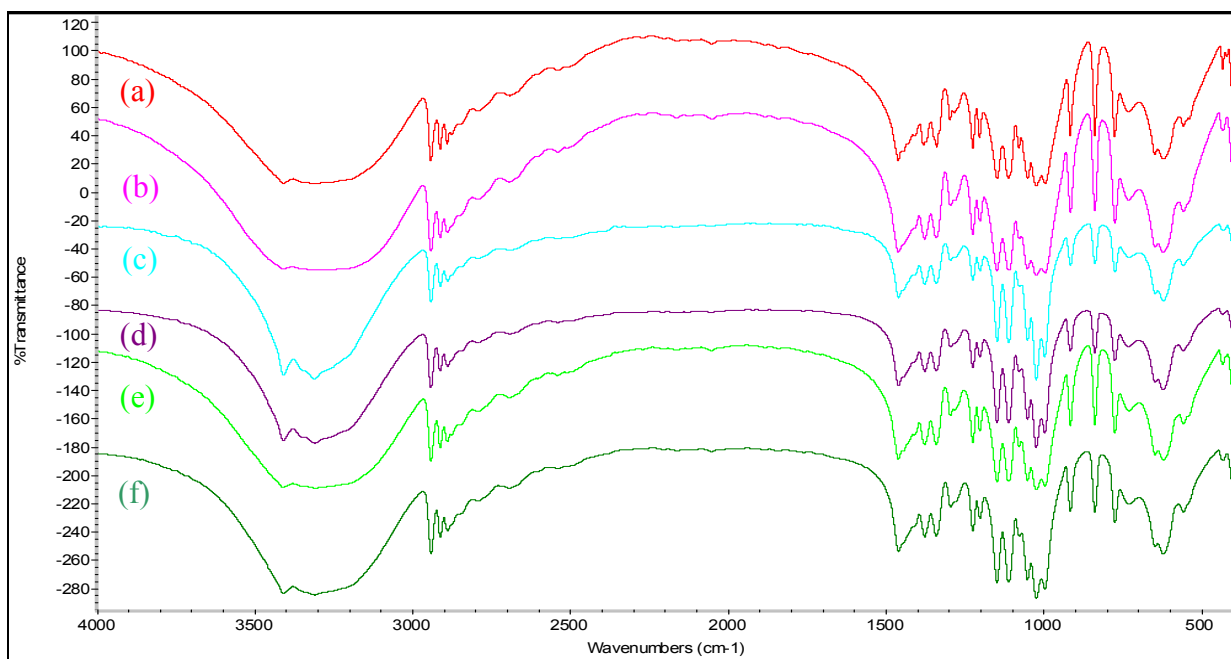


Figure 5.4. FT-IR spectra of D (+) Glucose (a) 0, (b) 1, (c) 3, (d) 5, (e) 7 and (f) 15 kGy irradiated, respectively.

During polymerization template glucose molecules were also involved in the polymerization solution hence dose effect on D-glucose was also investigated for five different dose values. Figure 5.4 show that radiation dose does not affect glucose structurally except for a little change of –OH band at the region of 3000-3500 cm^{-1} .

5.2. Swelling Studies

5.2.1. The Effect of Type and Amount of the Crosslinking Agent

In order to determine the relation between swelling property and cavity size, swelling studies were achieved as mentioned in experimental method section. In this study five different crosslinking agents (DEGDA, DEGDMA, TEGDMA, PEGDMA, and PPGDMA) were used to choose the appropriate crosslinker for optimization of MIP network structure. The difference between each crosslinker is the number of ethylene oxide groups in the chain. On the other hand, DEGDA is different from rest of it because of acrylate functionality. In our study, swelling behaviour of higher amount of crosslinker containing networks was stand in opposite of lower amount of crosslinker containing networks up to which contains 20 % crosslinker. The reason of radiation polymerization usage was also reducing the amount of crosslinker in the imprinting system besides getting rid of impurities.

In Figure 5.5, PPGDMA was used as the crosslinking agent with 3:1 HEMA:Glucose ratio with an irradiation dose of 5 kGy. Change of crosslinking agent amount varies as 10, 15, 20, 30, and 70 %. In fact, it was expected that increasing amount of crosslinking agent would increase the crosslink density consequently decreasing cavity size. As seen from Figure 5.5, the percent swelling value increases with increasing amount of crosslinking agent directly. The reason of this behaviour is that the crosslinker acts as monomer in the imprinting system by increasing amount of it. Behind 30 % cavity size was determined by the chain length of crosslinking agents used in the system.

This behaviour causes larger cavities than expected. Besides swelling experiments, PAL experiments were used to determine radius of free volume holes sensitively. The same behaviour was also observed from PAL studies. Those results will be discussed in PAL experiments section. The crosslinking agents behaving like a monomer was also observed in other MIP networks in which DEGDA, DEGDMA, TEGDMA and PEGDMA were used (Figure 5.6).

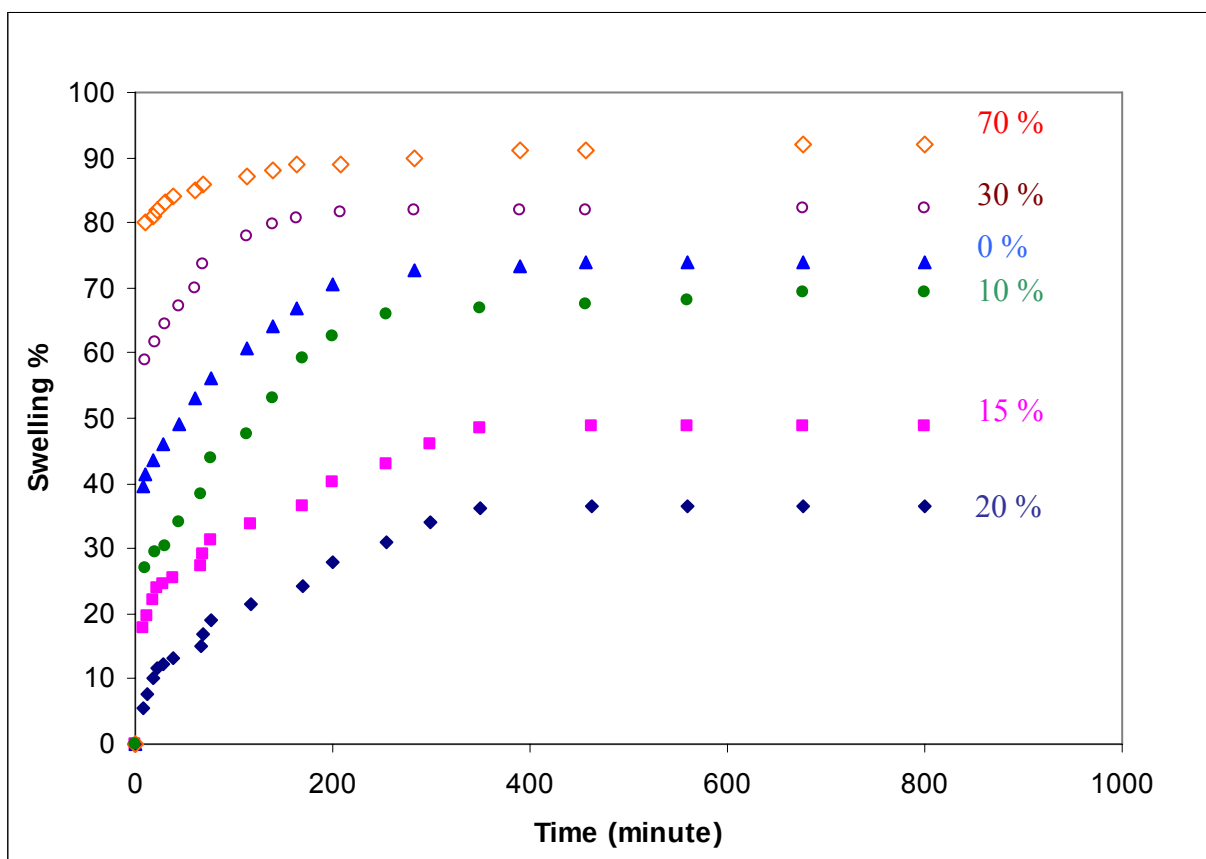


Figure 5.5. Percent swelling versus time graph for 0, 10, 15, 20, 30, 70 % PPGDMA containing MIP with 3:1 HEMA:glucose mole ratio, irradiated to 5 kGy.

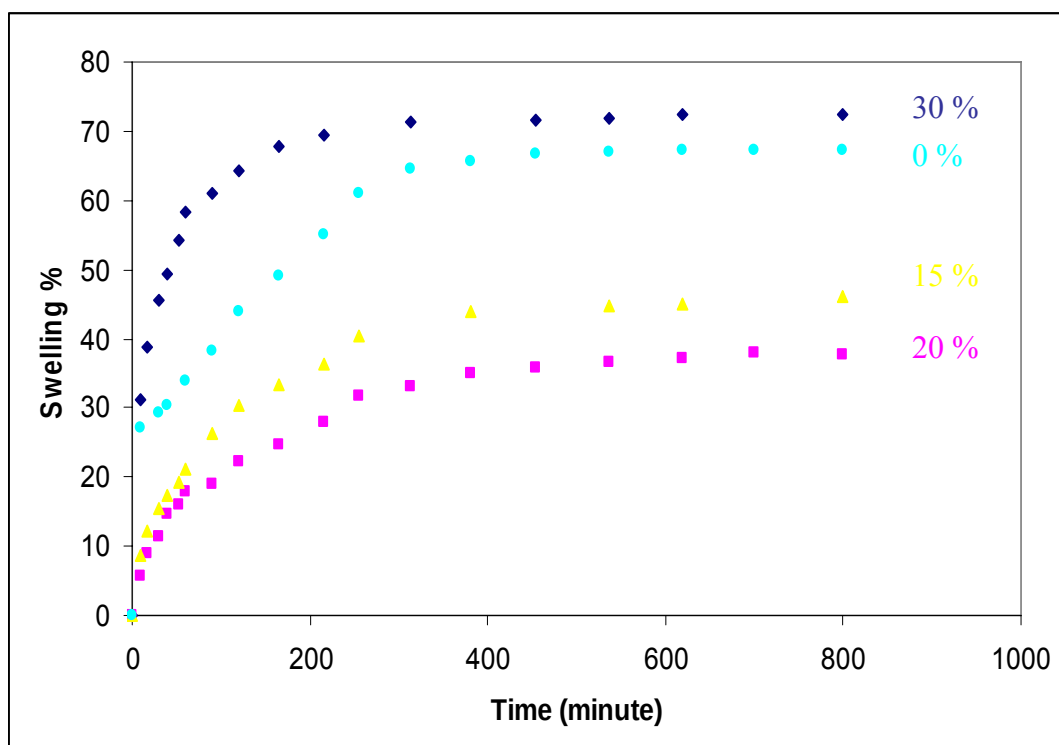


Figure 5.6. Percent swelling versus time graph for 0, 10, 20, 30, 70 % TEGDMA containing MIP with 3:1 HEMA:glucose mole ratio, irradiated to 5 kGy.

Another important factor is choosing the appropriate crosslinking agent for efficient imprinting system. As mentioned above five different crosslinkers with different chain lengths and two different functionalities. The general swelling behaviour observed was increasing chain length of the crosslinking agent increasing percent swelling value. Another remarkable point is that acrylate functionality containing DEGDA used MIPs shows higher response to swelling than methacrylate functionality containing DEGDA used MIPs but lower than TEGDMA containing MIPs, as seen from Figure 5.6.

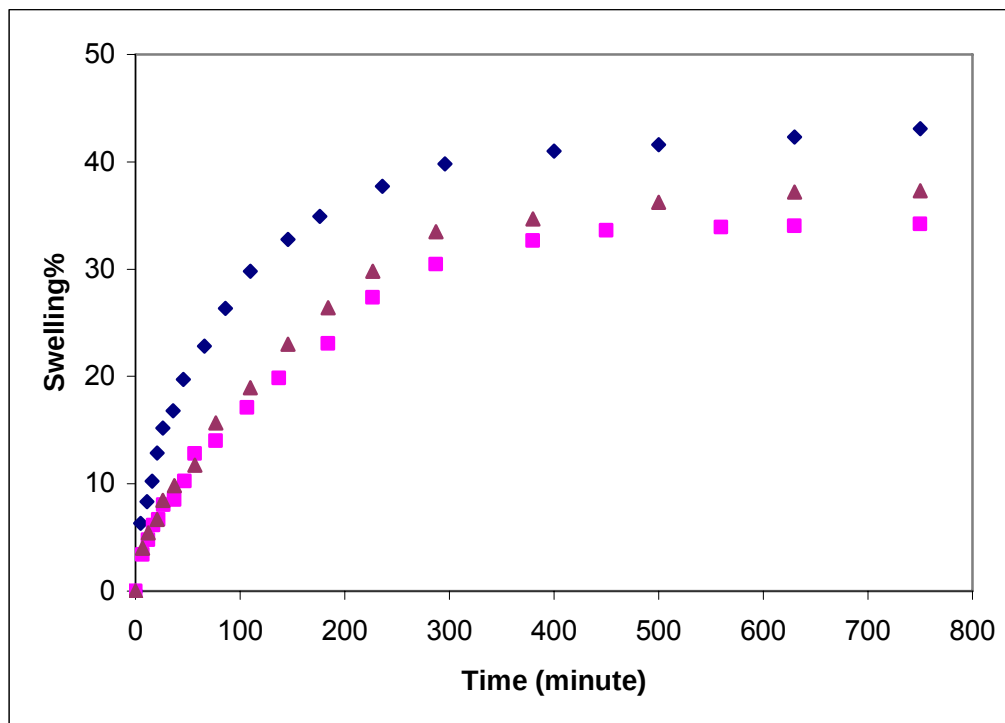


Figure 5.7. Percent swelling versus time graph for 15 % (◆) TEGDMA, (■) DEGDM, (▲) DEGDA containing MIP with 3:1 HEMA:glucose mole ratio, irradiated to 5 kGy.

5.2.1.1. Swelling Studies in Glucose Solutions

In order to determine the swelling response of MIPs in glucose solutions swelling studies were repeated by using glucose solutions instead of deionized water. Two different glucose concentrations, 100 mg/dL and 300 mg/dL, were studied.

Swelling behaviour of MIP and NIP networks was the same as in deionized water for different glucose concentrations studied. The effect of chain length of crosslinker was observed to be the same as the previous results showing that percent swelling increases with increasing chain length of crosslinking agent (see Figure 5.8.).

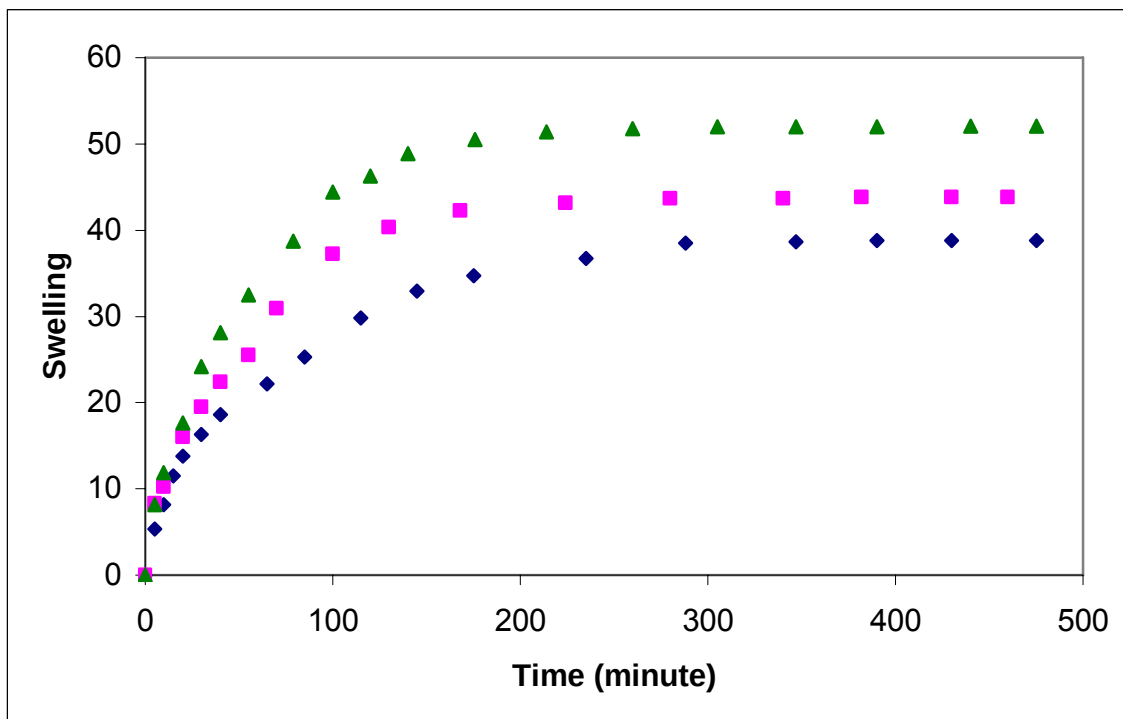


Figure 5.8. Percent swelling versus time graph for 10 % (◆) DEGDMA, (■) TEGDMA, (▲) PEGDMA containing MIP with 3:1 HEMA:glucose mole ratio, irradiated to 5 kGy, in glucose solution of 300 mg/dL.

5.2.2. The Effect of Radiation Dose

The effect of radiation dose on the imprinting system was also investigated due to the fact that radiation induced polymerization was used as the polymerization method which provides means for extra crosslinking of MIP network. The effect of radiation dose on MIP systems was studied at five different dose values; 1, 3, 5, 7, and 10 kGy.

Increasing radiation dose normally results in increasing crosslinking density consequently decreasing percent swelling value. In our study, this behaviour was observed in lower crosslinking agent containing systems. On the other hand, higher amount of crosslinking agent containing MIPs shows that increasing radiation dose

increases percent swelling. The increasing swelling can be explained by reduced crosslink density which may be due to the scissioning of some chains strained by crosslinks.

Consistency of PAL results with swelling test results will be discussed in next PAL's section. Shortly, the results obtained from swelling tests were in full agreement with the results of PAL experiments. In higher crosslinking agent containing networks, radius of free volume holes decreases with increasing radiation dose up to 7 kGy.

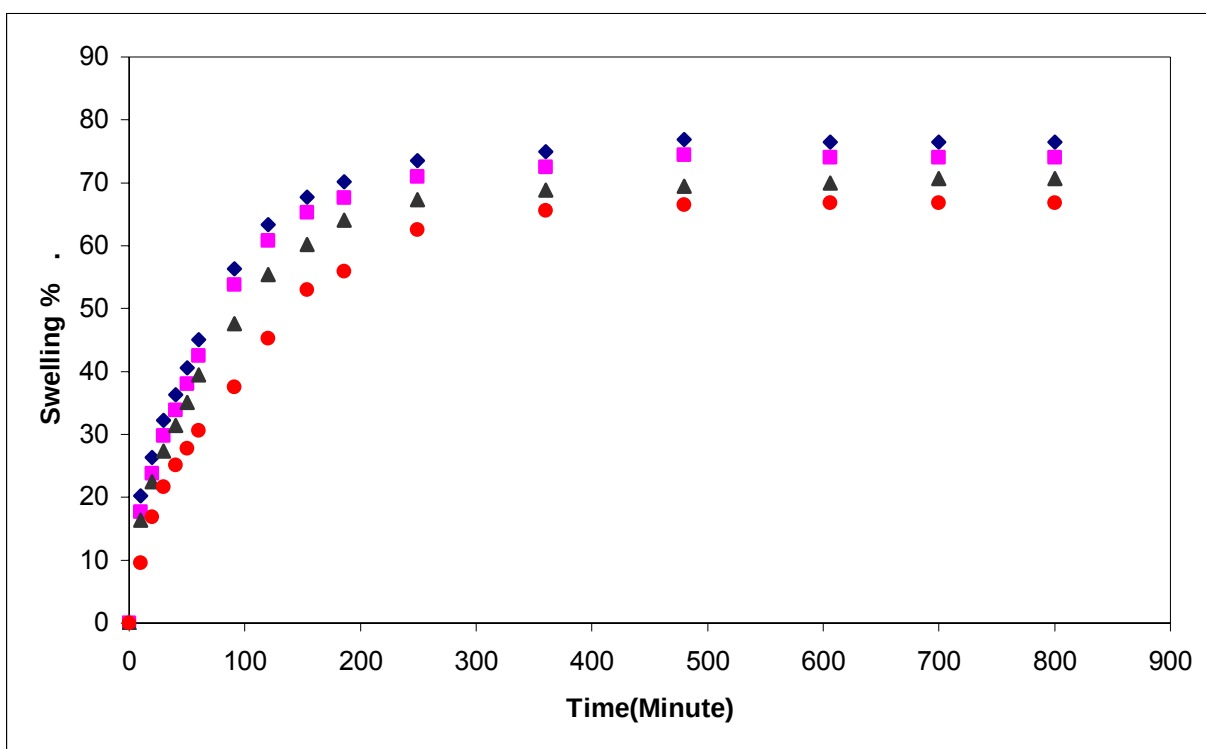


Figure 5.9. Percent swelling versus time graph for 30 % TEGDMA containing MIP with 3:1 HEMA:glucose mole ratio which were (♦) 10 kGy, (■) 7 kGy, (▲) 5 kGy, (●) 3 kGy irradiated.

5.2.3. The Effect of Template Concentration

The efficiency of imprinting system was closely related with the amount of template used during polymerization. Interaction between template molecules and monomer determines the cavity properties of the network. In our system, interaction of -OH group of D-glucose with -OH group of HEMA was supposed to occur during pre-polymerization step with the formation of a spatial arrangement. In order to choose proper monomer: template mole ratio, 1:1, 3:1 and 6:1 HEMA:glucose mole ratios were studied for MIP and NIP samples.

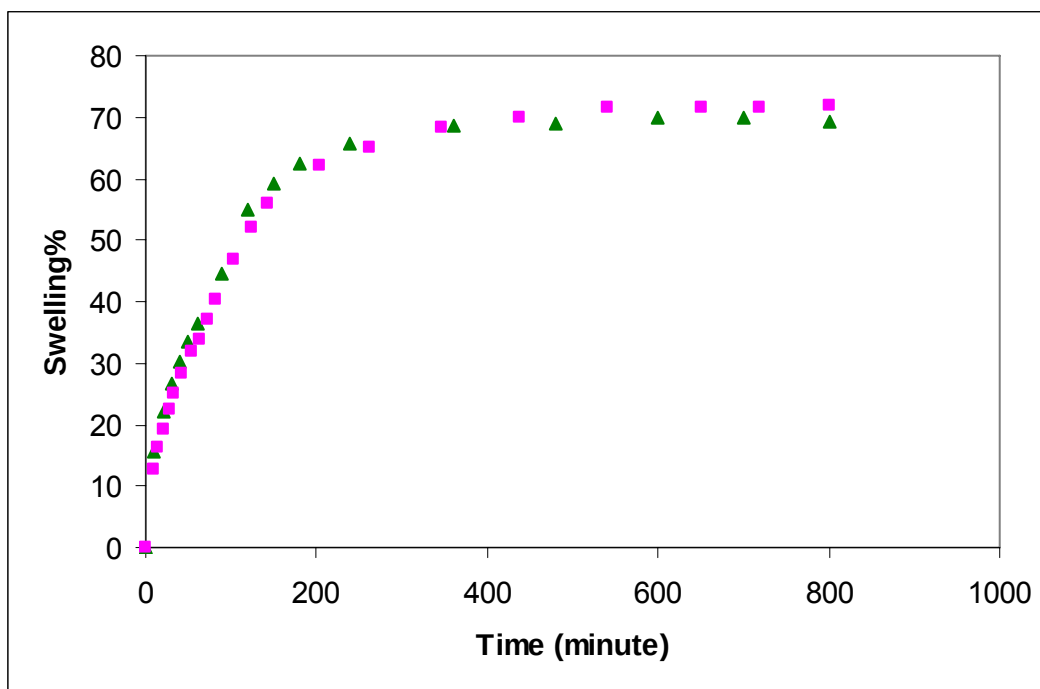


Figure 5.10. Percent swelling versus time graph for HEMA containing no crosslinking agent with 3:1 HEMA:glucose mole ratio, (■) MIP and 6:1 HEMA:glucose mole ratio, (▲) MIP, irradiated to 5 kGy.

As seen from Figure 5.10. the difference in swelling between the samples with 3:1 and 6:1 monomer: template ratio was not significant. Very small increase in swelling

value of 3:1 HEMA:glucose used samples was the result of higher amount of glucose in the imprinting system related with control of cavity interactions.

On the other hand, swelling results of imprinted and non-imprinted samples support this idea as seen from Figure 5.11. about 3% difference in the equilibrium swelling of imprinted polymers is clearly due to the volume of the template used in the preparation of MIP.

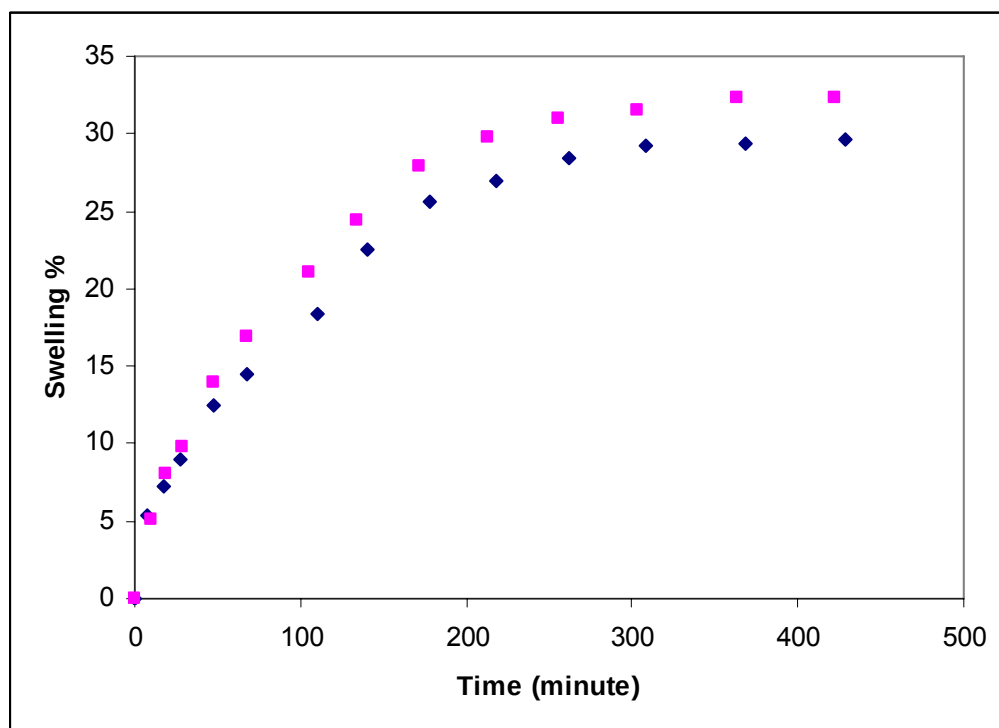


Figure 5.11. Percent swelling versus time graph for 15 % DEGDMA containing (■) MIP and (◆) NIP with 3:1 HEMA: glucose mole ratio, irradiated to 5 kGy.

5.3. Microscopic Evaluation

In any separation process, the factors affecting the separation are divided into the chemical factors, known as selectivity (α) and the capacity (κ') and physical factors, known as the efficiency, or theoretical plate count (N). Efficiency is directly related with the pore size of column support material and surface area via particle size. The sizes of ground particles before packing the column were investigated by an optical microscope to have a better idea on their effects in the HPLC system.

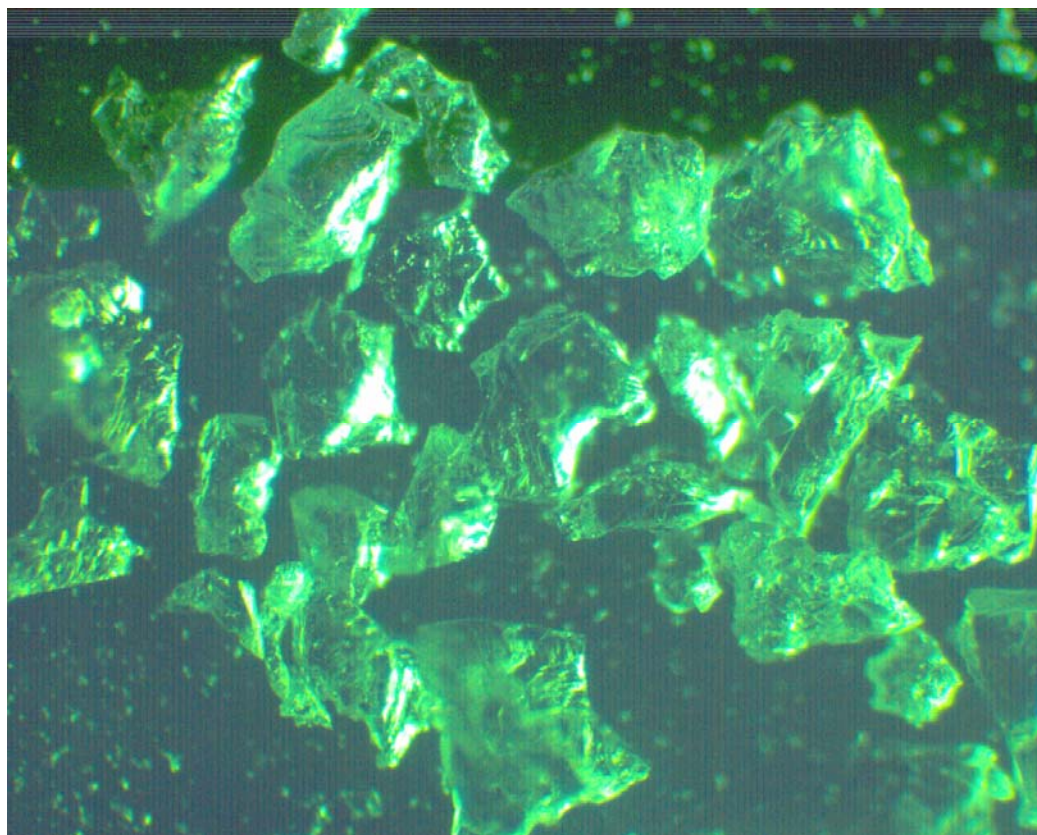


Figure 5.12. Micrograph of swollen MIP sample containing 15 % PPGDMA with 3:1 HEMA:glucose mole ratio, irradiated to 15 kGy. (4x magnification)

Microscopic studies were also performed in the swollen state of polymers like packing procedure owing to all polymeric packings exhibit swelling and shrinking with a change of the mobile phase. This penetration effect can cause low column efficiency due to the reduced mass transfer.

Irregular-shaped particles were obtained by our grinding procedure. The ranges of particle size were determined to be between 750-1000 μm . As a result, our irregular-shaped column packing material was considered to be practically homogenous.

5.4. PAL Experiments

5.4.1. The Effect of Type and Amount of the Crosslinking Agent

5.4.1.1. Dry Samples

Figure 5.13 shows the mean free-volume hole radius, R , and $o\text{-Ps}$ intensity versus the type of the crosslinking agent at different concentrations. The presented results are for dry samples with fixed HEMA/Glucose (H:G=3:1) ratio at a γ -irradiation dose of 5 kGy. The values for the radii are calculated by iteration procedure based on Equation 5.1.

$$\tau_{o\text{-Ps}} = 0.5 \left[1 - \frac{R}{R_0} + \frac{1}{2\pi} \sin \left(\frac{2\pi R}{R_0} \right) \right]^{-1} \quad (5.1)$$

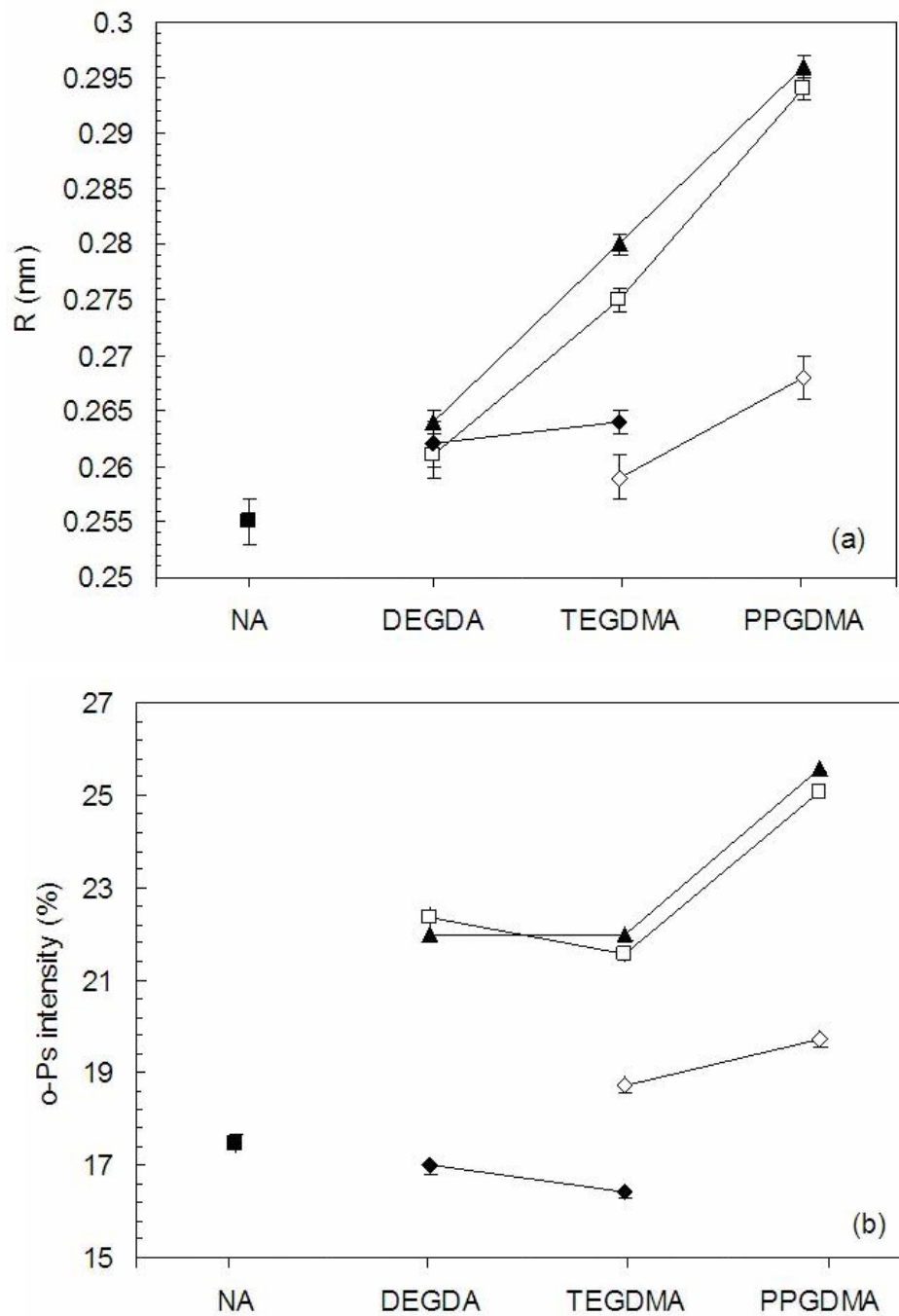


Figure 5.13. Free-volume hole radius (a) and *o*-Ps intensity (b) for dry samples versus the type of the crosslinking agent at different concentrations. 5 kGy irradiated samples with 3:1 HEMA: glucose mole ratio; symbols ▲, □, ◇, ◆, ■ indicate 70, 30, 20, 10 % and no crosslinking agent containing samples, respectively. NA indicates sample prepared without crosslinking agent.

The results shown in Figure 5.13 can be evaluated under two categories: low (with the crosslinking agent range of 10 and 20 %) and high (with the crosslinking agent range of 30 and 70 %) concentration of the crosslinking agent. When the concentration is low, R is slightly affected from the molecular size of the crosslinking agent, as we go from pure HEMA (denoted by NA on the figures) crosslinked by gamma rays only to HEMA crosslinked in the presence of DEGDA, there is an increase of about 0.1 nm in the radius of holes. We can explain this behavior with the purely random crosslinking of HEMA alone resulting in dense crosslinkings whereas in the presence of DEGDA the two functionality of the crosslinking agent imposes a certain limited mesh size on the crosslinked structure. In the same concentration range by increasing the size of the crosslinking agent from DEGDA to TEGDMA, R does not seem to be affected much if not for slightly decreasing. Although by going from DEGDA to TEGDMA there is an increase of one ethylene glycol unit on the chain, this small increase in size is compensated by the presence of methyl group of methacrylate. The increase in R in the presence of PPGDMA as compared to other crosslinking agents is clear from the figure, which is simply related to rather big difference in their respective chain lengths. But still the effect of PPGDMA is not as big as expected which can be related to dominating structure of randomly crosslinked HEMA in this range, comprising 80 and 90 % by mole of the total.

In the high concentration range of crosslinking agents (with the crosslinking agent range of 30 and 70 %) however, the change of R with crosslinking agent type is obvious as seen from Figure 5.13 (a). R steadily increases by going from DEGDA to PPGDMA from about 0.26 to 0.28 and 0.30 nm, respectively. In this crosslinking agent concentration range, HEMA is in rather low amounts as compared to the crosslinking agent and the overall structure of crosslinked system is dominated by the polymerization of crosslinking agent itself resulting in mesh sizes imposed by the lengths of crosslinking agents. A comparison of the relative sizes of free volumes in the swollen state of MIPs can be made by measuring the equilibrium swelling degrees of these crosslinked systems. In parallel with PALS studies, the swelling behaviors of MIPs were also investigated. The equilibrium swelling ratios obtained for HEMA

crosslinked in the presence of 30 % of DEGDA, TEGDMA and PPGDMA were 37, 43 and 82 %, respectively, which show a good correlation with free-volume hole radii of dry samples as given above.

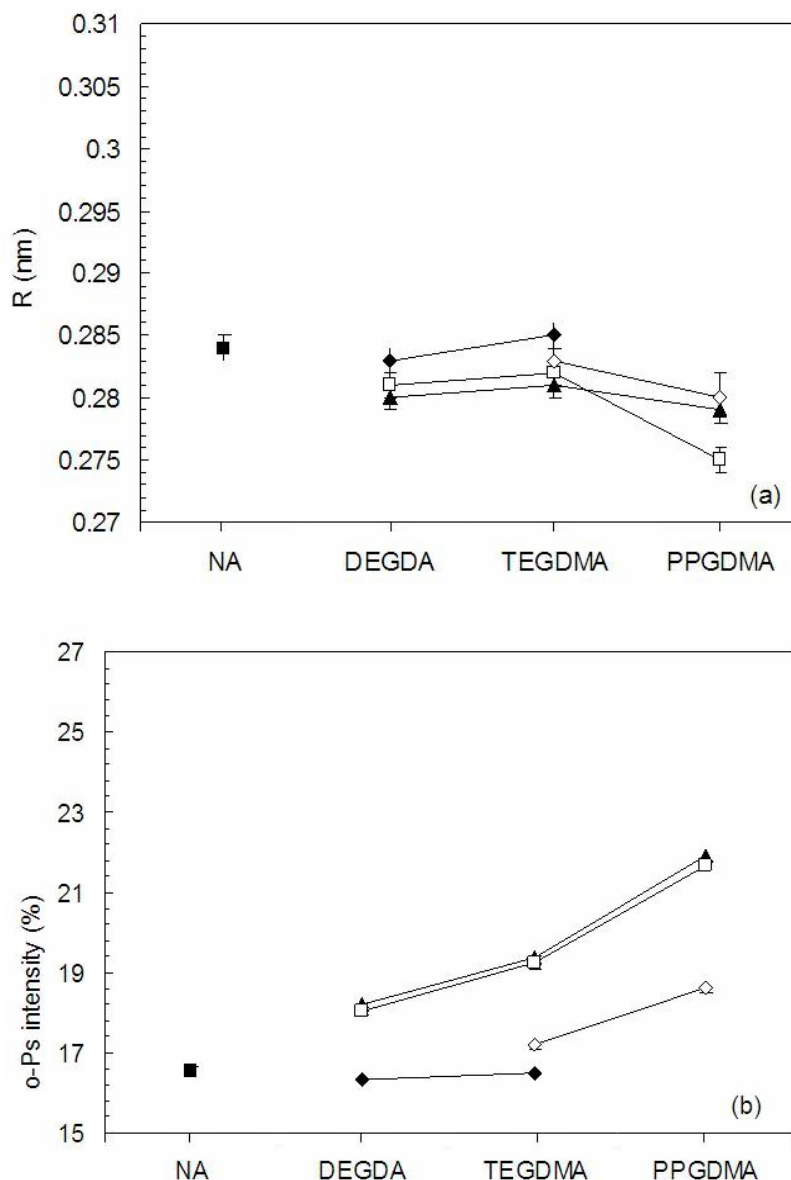


Figure 5.14. Free-volume hole radius (a) and *o*-Ps intensity (b) for water swollen samples versus the type of the crosslinking agent at different concentrations. 5 kGy irradiated samples with 3:1 HEMA: glucose mole ratio; symbols $\blacktriangle$, $\square$, $\diamond$, $\blacklozenge$, $\blacksquare$ indicate 70, 30, 20, 10 % and no crosslinking agent containing samples, respectively. NA indicates sample prepared without crosslinking agent.

5.4.1.2. Samples Swollen in Water

Figure 5.14 shows the PALS results for the same MIP samples used in Figure 5.13, this time fully swollen in water. R versus crosslinking agent type graph given in Figure 5.14 (a) reveals an interesting result. Irrespective of chain length of crosslinking agent and its concentration, the free-volume hole radius determined for these MIPs were found to be the same for all systems. Water absorption and consequent changes in the free volume characteristics in polymers are widely studied by PAL spectroscopy (Suzuki et. al., 1996; Ito et. al., 1996; Ito et. al., 1997; Hodge et. al., 1998; Dlubek et. al., 1999). With an increase of water content in the polymer an increase in the sizes of free volume holes has been observed. This has been explained by the plastification effect (enhanced polymer chain mobility) of the water molecules absorbed at the inner surfaces of free volume holes. What is measured by PAL spectroscopy in this case is the free volume not occupied by water. At higher water concentration, the o - Ps lifetime reaches a saturation value that corresponds to free volume hole radius of 0.28 nm. The last value is the Ps -bubble size in water at room temperature which means that Ps is formed in bulk water fully or partially occupying the free volume hole (Kenji et. al., 1999).

The decrease of free volume hole size after swelling for the samples prepared with PPGDMA, with the crosslinking agent range of 30 and 70 %, (Figure 5.14 (a)) indicates that the free-volume holes inside the swollen crosslinked polymeric system are larger than the Ps -bubble size and probably fully occupied with water. For the rest of the samples, an increase can be seen in the free volume size as compared to dry samples because of the swelling. Therefore for the last-mentioned samples plastification effect is the other possible explanation for the observed hole size enhancement. However, because of the almost constant R -values for the swollen samples, we are inclined to believe that what is measured for all of the swollen samples is the size of the Ps -bubble in water which occupies the free-volume holes. This conclusion has also lead us to propose that the pores created during formation of MIPs are not closed but all interconnected giving access to diffusion of water.

O-Ps intensity (Figures 5.13 (b) and 5.14 (b)) is a quantity directly connected to the *Ps* formation probability. Although *Ps* formation probability depends mostly on the available free-volume holes, it is affected by many additional factors as discussed in the Introduction section. For the studied samples, the main factor is the presence of polar groups in the chemical structure of the sample precursors. The electronegativity of oxygen atom is 3.5 P.u., while those of hydrogen and carbon are 2.1 and 2.5, respectively. Therefore, groups like C=O and COH express definite polarity, and oxygen is partially negatively charged. Thus, positrons are attracted by the oxygen of these groups and *Ps* formation is inhibited. If the difference can be neglected for the polarity of the different oxygen containing groups, estimation can be made roughly for the oxygen effect on the *o-Ps* intensity. That is why both effects of the actual change in the number of free-volume holes and *Ps* inhibition have to be considered carefully. If we consider only the oxygen effect because of the presence of DEGDA and TEGDMA crosslinking agents on the *o-Ps* intensity we should observe a decrease in the values for HEMA samples. However, the observed change is an increase (Figure 5.13 (b) and 5.14 (b)). So, it could be concluded that there is an increase in the free-volume hole number due to crosslinking which is evident for higher crosslinking agent concentrations (with the crosslinking agent range of 30 and 70 %), while for the lower concentrations (with the crosslinking agent range of 10 and 20 %) is not clear. The *o-Ps* intensity value for the PPGDMA crosslinking agent is higher compared with those for the other crosslinking agents but this does not mean surely higher number of free-volume holes. Most probably this comes from the lower oxygen weight in PPGDMA.

Additional confirmation of the last suggestion comes from the plot of *S* parameter as a function of *W* parameter (see Figure 5.15) obtained from DBAL experiments. *S* parameter depends mostly on the *Ps* yield, while *W* parameter is sensitive to the chemical structure of the sample. For our choice of the energy range for *W* parameter calculation, *W* parameter is highly sensitive to annihilation on oxygen (Kenji et. al., 1999). Although the experimental data scattering in Figure 5.15 is high, it can be seen that the trendlines of the sample groups prepared with PPGDMA (dry and swollen)

differ from the rest of the sample groups. The slopes of the trendlines for these two groups are ~ -1.70 , while those for the other groups lie in the range of -1.33 to -1.17 . This means that for the same decrease of the Ps yield (S parameter decrease) the increase in W parameter for PPGDMA containing samples is lower than that for the other group of samples. The reason is that fewer positrons are trapped and annihilate on oxygen in the samples prepared with PPGDMA than these in the samples prepared with TEGDMA and DEGDMA, and in pure HEMA, because of the relatively less oxygen content of propylene groups.

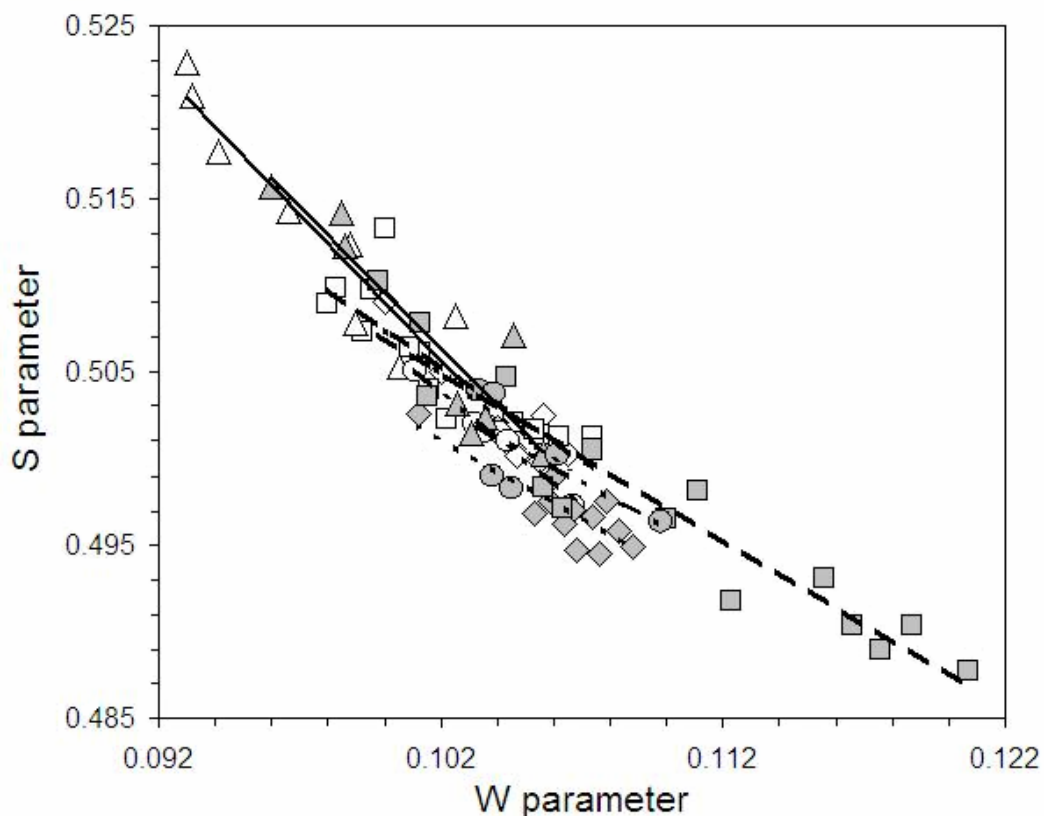


Figure 5.15. S parameter as a function of W parameter. Horizontal and vertical error bars (not drawn for clearness) for S and W are about twice the size of the points. The lines are linear fits of the corresponding groups of experimental points; ($\diamond$) NA, ($\blacklozenge$) NA swollen, ($\square$) TEGDMA, ($\blacksquare$) TEGDMA swollen, ($\triangle$) PPGDMA, ($\blacktriangle$) PPGDMA swollen, ($\circ$) DEGDA, ($\bullet$) DEGDA swollen, ($\cdots$) linear NA and linear NA swollen, ($- - - -$) linear TEGDMA and linear TEGDMA swollen, ($- - - -$) linear PPGDMA and linear PPGDMA swollen, ($- - - -$) linear DEGDA and linear DEGDA swollen.

5.4.2. The Effect of Template Concentration

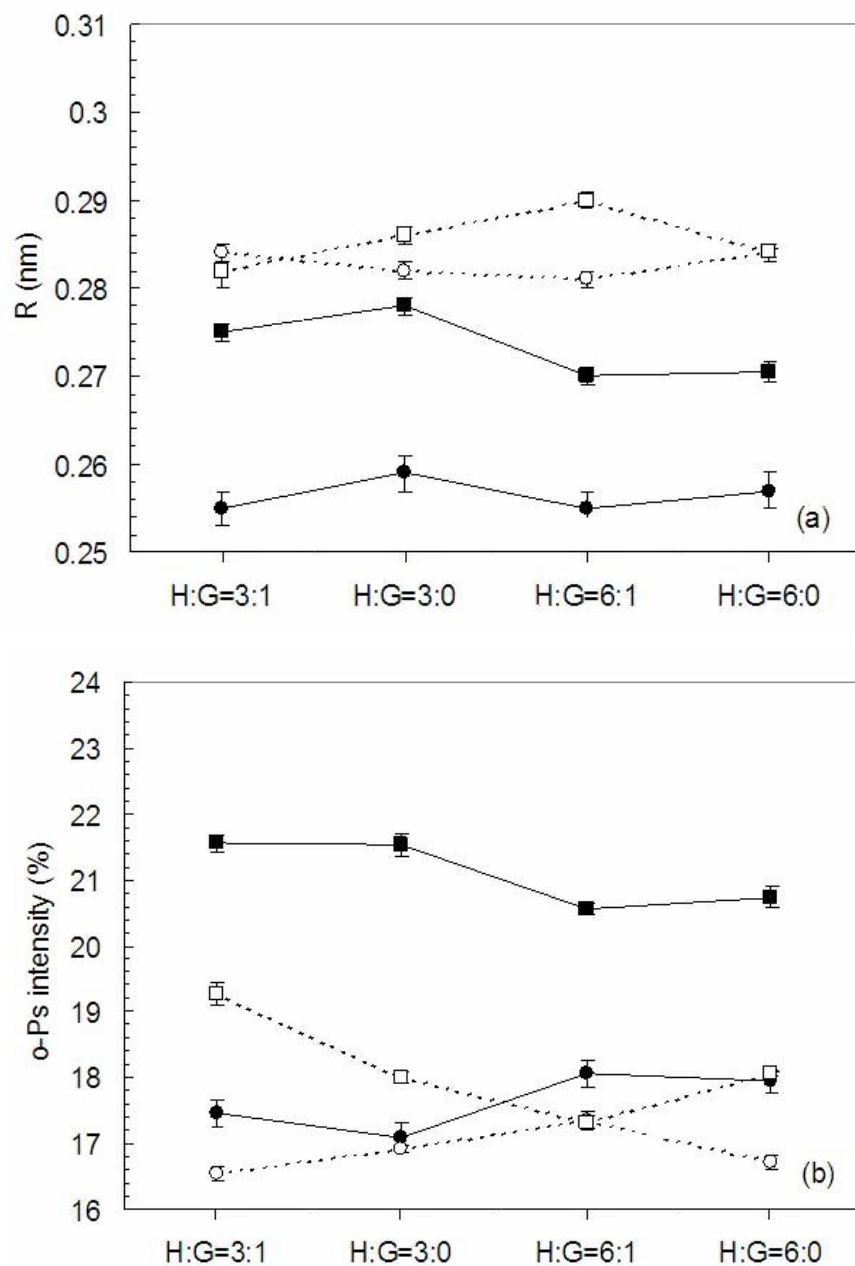


Figure 5.16. Free-volume hole radius, R , (a) and o -Ps intensity (b) versus HEMA/Glucose (H: G) mole ratio. NA indicates samples prepared without crosslinking agent. 5 kGy irradiated samples with 3:1 and 6:1 HEMA: glucose mole ratio; symbols ●, ○, ■, □ indicates NA in dry state, NA in swollen state, 30 % TEGDMA in dry state , 30 % TEGDMA in swollen state, respectively.

Figure 5.16 shows the effect of template concentration on the free-volume hole radius on different MIPs. Two classes of samples are considered, those prepared from pure HEMA only by radiation-induced crosslinking denoted by NA (see the caption of the figure) and those prepared from HEMA - TEGDMA (with crosslinking agent amount 30%) when HEMA to glucose concentrations were kept at 3:1 and 6:1. PAL spectra of all these samples were taken in both dry and swollen states. In dry state, although small, still a difference was observed for imprinted (those with the template, HEMA:glucose 6:1 and 3:1) and non-imprinted (HEMA:glucose 6:0 and 3:0) samples. The free-volume hole radii of imprinted samples were found to be smaller than that of non-imprinted ones which shows that the presence of template brings a control of cavity volume though small in magnitude. In swollen samples since what is measured is the dimension of the *Ps*-bubble in water filling the free-volume holes, the *R*-value does not change much with neither crosslinking agent nor template ratio. In fact, the equilibrium swelling ratios of non-imprinted HEMA samples with different template ratios were almost the same about, 69 and 71 %, indicating that even in the expanded state they do not differ much in volume.

5.4.3. The Effect of Radiation Dose

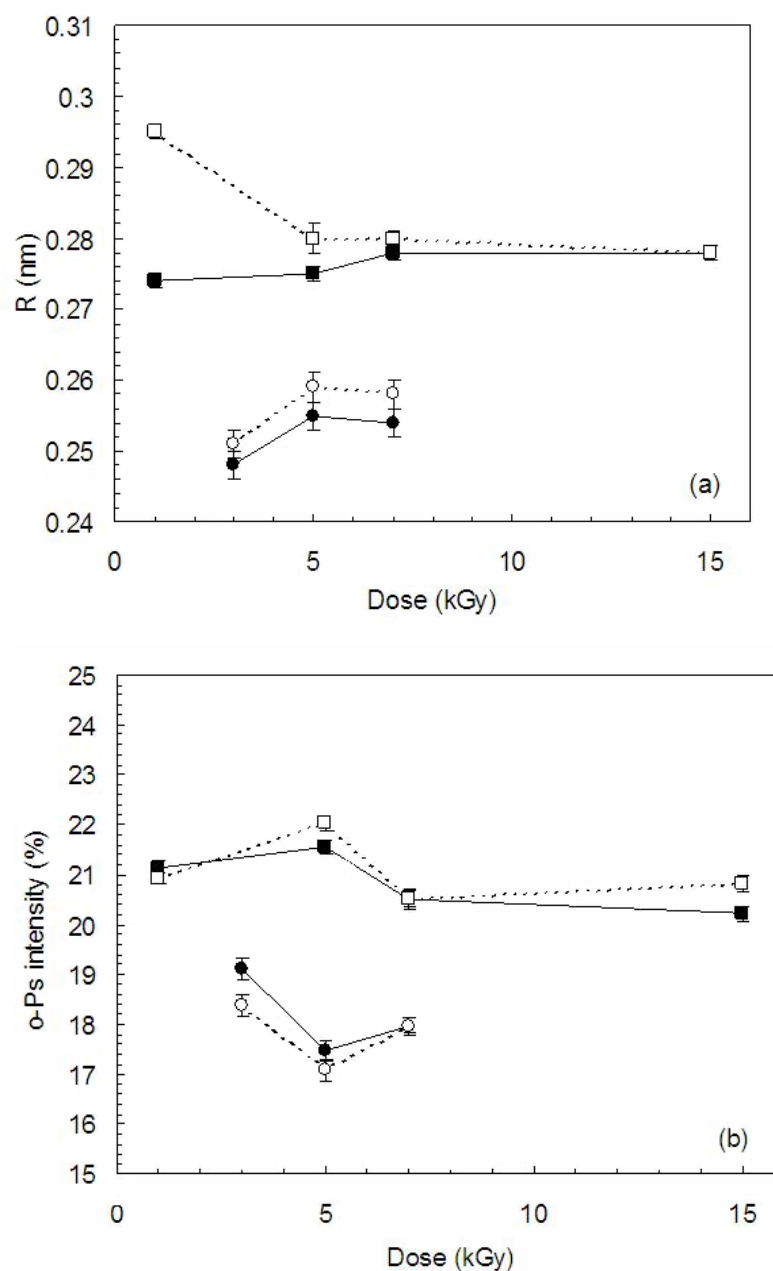


Figure 5.17. Free-volume hole radius, R , (a) and o -Ps intensity (b) versus irradiation dose. NA indicates samples prepared without crosslinking agent. Samples with 3:1 HEMA: glucose mole ratio; symbols ●, ○, ■, □ indicates Glucose imprinted NA, non-imprinted NA, glucose imprinted 30 % TEGDMA containing sample, non-imprinted 30 % TEGDMA containing sample with varying dose, respectively.

The change of R with dose in the above figure is given only for samples prepared in the presence of TEGDMA and shows that for non-imprinted samples R is smallest at the lowest dose and becomes independent of dose beyond 5 kGy. This is an expected result since the lower the dose the less will be the concentration of crosslinks thus wider will be the space between the chains. With increasing dose crosslink density will increase and beyond a certain dose, considering the chain scission effect of radiation as well, it will reach a saturated value.

5.5. High Performance Liquid Chromatography Studies

In the past few years, advances in base material technology have been made by molecularly imprinted polymeric packing materials in chromatography studies (Baggiani et. al., 2000; Schweitz et. al., 1998). This technology incorporates size selectivity of analyte molecules besides their affinity selectivity. The goals of analytical separation can be achieved by optimizing the elution conditions, the column packing and the column configuration.

The variables of the column material are the bonded phase (packing material), pore size, particle size and surface area. The variables in the column configuration are the length, internal diameter, and material of construction (stainless steel, plastic etc.).

A packed column can be characterized by the measurements of column efficiency, or theoretical plate count. However, plate count is a measure of the efficiency of the entire HPLC system, not just the efficiency of the column.

HPLC system and columns were prepared as described in the experimental method section. According to previous results of swelling and PAL experiments; DEGDA, DEGDMA, TEGDMA and PPGDMA with 3:1 HEMA:glucose ratio which were irradiated to 15 kGy were chosen for HPLC studies. 15 kGy was chosen as the irradiation dose owing to control swelling properties of the system. Previous experiments conclude that after 7 kGy cavity properties did not show appreciable change. Furthermore, packing the column material in swollen state aimed to prevent the column properties from swelling and shrinking effects.

In order to investigate the efficiency of imprinting process on the selective separation of D-Glucose; D-Galactose, D-Fructose and D-Glucose solutions were injected into columns filled with NIPs and MIPs. As it is clearly seen from Figure 5.18, columns filled with NIP as packing material did not show any separation for those three saccharides as seen from Figure 5.18. When the same test was made by using

columns filled with MIPs synthesized in this work a separation was achieved as seen from Figure 5.19. Efficiency and capacity factor values were calculated as described in section 3.7 and 3.6.2.1.

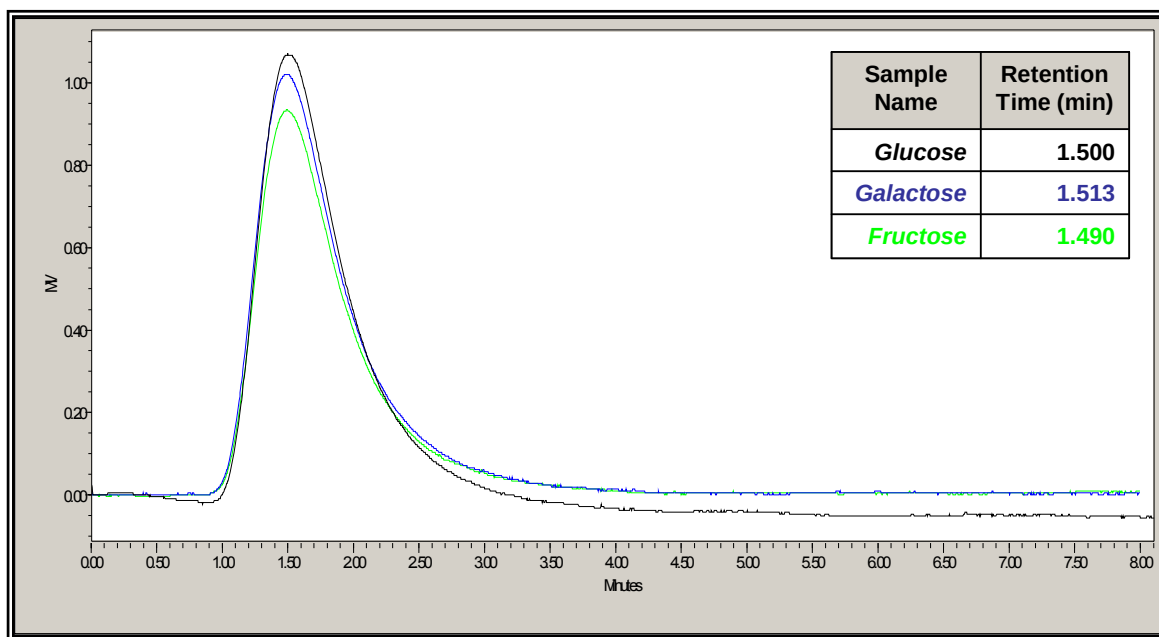


Figure 5.18. Chromatograms of D-glucose, D-galactose and D-fructose with column packing material consisting of NIPs crosslinked with 15 % DEGDA, containing 3:1 HEMA: glucose mole ratio, irradiated to 15 kGy.

Since the non-imprinted networks have not complementary cavities to glucose molecules, retention time of glucose in the column almost same for other two monosaccharide molecules as glucose. For each MIP and NIP packed columns; three monosaccharide molecules have almost same retention time values for NIP based packed columns as seen from Table 5.2.

On the other hand, the difference of retention times between MIP and NIP packed columns were clearly seen which result from MIP networks have specific interactions with template molecule. As the increasing interactions between the template molecule

and the MIP network, increasing retention time of analytes results in increasing efficiency values according to calculated values.

For each NIP and MIP packed columns efficiency values of NIPs were smaller than MIPs which show the success of our imprinting systems. As seen from Table 5.1, efficiency of D-Glucose for DEGDA containing MIP packed column was 21.7 whereas efficiency for NIP was 6.1.

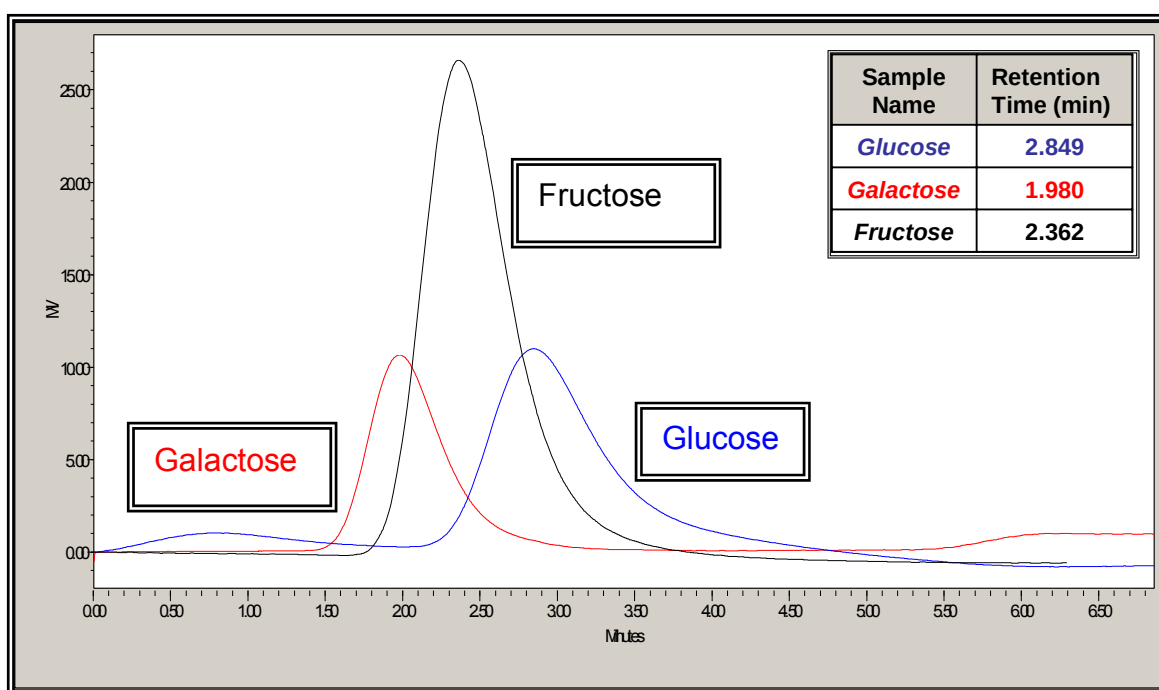


Figure 5.19. Chromatograms of D-glucose, D-galactose and D-fructose with column packing material consisting of MIPs crosslinked with 15 % DEGDA, containing 3:1 HEMA: glucose mole ratio, irradiated to 15 kGy.

Among various MIPs, prepared DEGDA containing MIPs showed the highest affinity toward D-Glucose as seen from Figure 5.19. D-glucose retained in the column longer than other monosaccharide owing to the specific interactions between D-Glucose imprinted cavities and D-Glucose. Contribution of this result may be the effect of

acrylate groups of DEGDA on the polymer chain. On the other hand, retention time of each monosaccharide molecule was almost the same for non-imprinted polymers which were supposed to occur.

Calculated selectivity and retention time values are given in Table 5.1 and 5.2, respectively. Capacity factor values were also calculated for each monosaccharide. However, the capacity factor is a measure of how well the sample molecule is retained by the column during an isocratic separation. The capacity factor is directly affected by the column packing material and the elution conditions. Typically, the capacity factor range between two and five represents a good balance between resolution and analysis time. Calculated capacity factor values are in the range between 3.92 and 8.47 for MIP and NIP polymers. Efficiency values of DEGDA and PPGDMA containing MIPs were calculated as 21.67, 16.66, respectively. The rest of MIPs had relatively poor values.

However, some of the prepared columns displayed either inadequate or rather strong retention, which may be caused by lower surface area or the presence of trace amount of cavities. Noticeable separations were accomplished on DEGDA containing MIPs which have highest efficiency value and capacity factor for glucose molecule; 21.67 and 3.92 respectively.

Table 5.1. Efficiency values of each monosaccharide for each MIP and NIP packed columns.

Type of sample	Efficiency of Glucose, N_{Glu}	Efficiency of Galactose, N_{Gal}	Efficiency of Fructose, N_{Fru}
DEGDA containing MIP	21,67	9,42	5,80
DEGDA containing NIP	6,05	5,27	4,30
DEGDMA containing MIP	4,10	5,60	4,20
DEGDMA containing NIP	2,90	4,70	3,10
TEGDMA containing MIP	4,50	6,25	4,55
TEGDMA containing NIP	3,19	5,40	3,15
PPGDMA containing MIP	16,66	11,58	8,31
PPGDMA containing NIP	3,66	2,89	2,33

On the other hand, selectivity is a measure of the relative retention of two components in a mixture. The selectivity of the column is a function of the column packing material and the elution conditions. Furthermore, the mixture of three monosaccharide was injected to the system. Obtained broad single peak area was the total peak area of three single peaks of monosaccharides. Direct relation with selectivity and retention time retention time values were given. As seen from Table 5.2. retention time values of galactose and fructose from glucose for DEGDA containing MIP packed columns were lowest; 1.98 and 2.36, respectively.

Table 5.2. Retention times (minute) of each monosaccharide for MIP and NIP samples used as column packing material.

Type of sample	Retention time of D-glucose	Retention time of D-galactose	Retention time of D- fructose
<i>DEGDA containing MIP</i>	2,84	1,98	2,36
<i>DEGDA containing NIP</i>	1,50	1,51	1,49
<i>DEGDMA containing MIP</i>	1,93	1,70	1,82
<i>DEGDMA containing NIP</i>	1,71	1,69	1,68
<i>TEGDMA containing MIP</i>	2,50	2,43	2,39
<i>TEGDMA containing NIP</i>	2,30	2,34	2,29
<i>PPGDMA containing MIP</i>	2,66	2,49	2,45
<i>PPGDMA containing NIP</i>	1,99	2,01	1,98

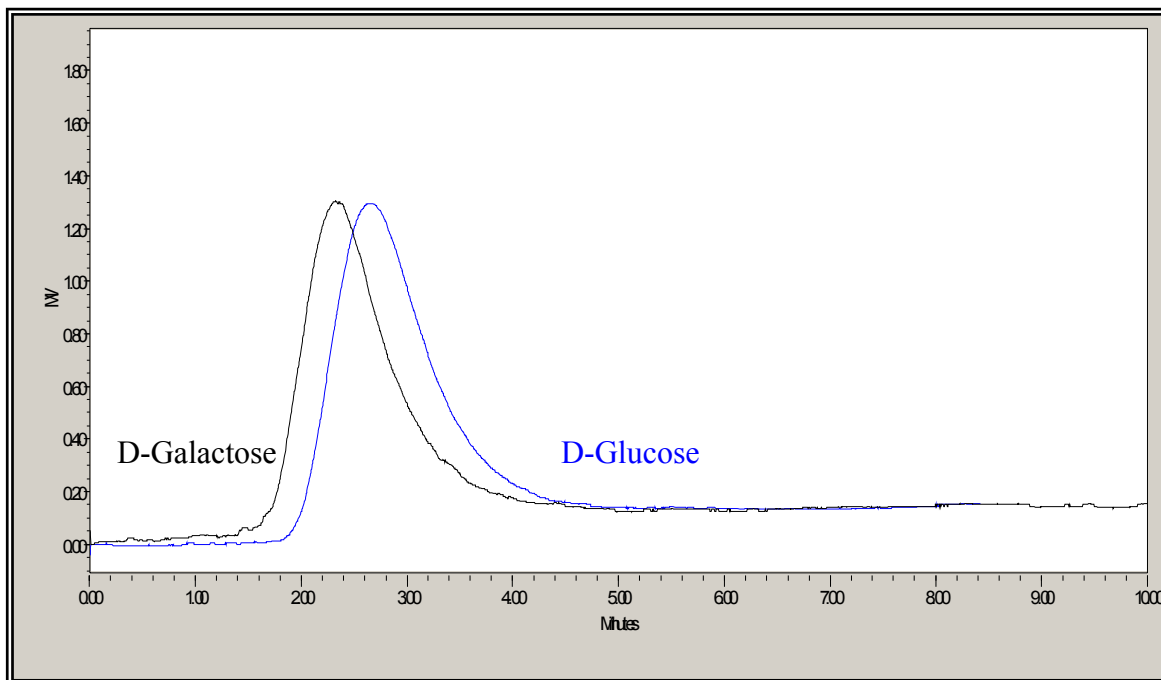


Figure 5.20. Chromatograms of D-glucose and D-galactose with column packing material consisting of MIPs crosslinked with 15 % PPGDMA, containing 3:1 HEMA: glucose mole ratio, irradiated to 15 kGy.

Besides specific bindings through system, nonspecific bindings also occur. This results in tailing of each peak. As the PPGDMA containing MIPs have higher efficiency, 16.7, nonspecific bindings are also higher (Figure 5.20).

6. CONCLUSION

This research demonstrated the successful formation of radiation induced non-covalent molecular imprinting of D-(+)-Glucose with different crosslinking agents. Considering crosslinking agents were chosen at different chain lengths of methacrylate functionality except for diethylene glycol diacrylate. The functional groups on the template molecule, 2-hydroxyethyl methacrylate was chosen to form the template-monomer complex by non-covalent imprinting.

D-Glucose imprinted networks were synthesized with different crosslinkers by using radiation induced polymerization technique. For the optimization of free volume holes in imprinted network; crosslinkers with different chain lengths and amounts, template-functional monomer ratio, radiation dose effects were investigated via PAL and swelling experiments. Characterization of synthesized MIP-NIP polymers were carried out by FT-IR, swelling and PAL experiments, which provided evidence for cavity size of MIP-NIP polymers.

Fourier transform infrared, FT-IR spectra were used to characterize HEMA based MIP-NIP networks with various crosslinking agents. Intensity of carbonyl band at 1730 cm^{-1} of each sample was found to be nearly the same with each other. Band intensity at 2950 cm^{-1} for PPGDMA was the highest due to the highest number of $-\text{CH}_2$ group on the chain. It is also seen that band intensity at 1100 cm^{-1} for PPGDMA reached the highest value due to the highest number of $-\text{C-O}-$ groups on the chain.

The swelling of higher amount of crosslinker containing networks was found to be higher than that of lower amount of crosslinker containing networks up to which containing 20% crosslinker. The reason for the usage of radiation polymerization was at the same time to reduce the amount of crosslinker in the imprinting system in addition to getting rid of impurities. In this study, these results demonstrate the feasibility of controlling cavity size based on the template molecule in the imprinting system.

Positron annihilation lifetime (PAL) experiments presented in this study are the first attempt at molecular imprinting to analyze cavity size in nano scale. The free volume hole control of D-glucose imprinted polymers via PAL spectroscopy demonstrated in this study is promising for effective molecularly imprinted polymeric systems. PAL experiments were performed in dry and swollen state. Crosslinking agent type and amount, monomer: template ratio and irradiation dose were also investigated by PAL experiments.

Furhermore, particle size directly affects the column properties. In our system irregular-shaped particles were used to pack the column. The particle size range of packed MIP-NIP polymers were determined via microscopic measurements.

The successful formation of the MIP and NIP polymers and binding studies were checked by HPLC studies. Calculated chromatographic values reported that diethylene glycol diacrylate (DEGDA) containing networks show the highest efficiency for glucose molecule 21.67.

Noticeable separations were accomplished on DEGDA containing MIPs which have highest efficiency value and capacity factor for glucose molecule; 21.67 and 3.92 respectively. For each NIP and MIP packed columns, selectivity and efficiency values of NIPs were smaller than MIPs which prove the success of our imprinting systems. The imprinted networks were utilized to achieve shape-selective recognition of D-glucose over its homologues, D-galactose and D-fructose, which differ only for conformation together with its affinity.

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