PREPARATION OF MOLECULAR IMPRINTED POLYMERS AGAINST MODEL MOLECULES

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
DAIRY CHEMISTRY

BY
DIVYA M.P.

DIVISION OF DAIRY CHEMISTRY
NATIONAL DAIRY RESEARCH INSTITUTE
(I. C. A. R.)
KARNAL - 132001 (HARYANA), INDIA

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Approved by:

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(T.M. Santra)
EXTERNAL EXAMINER

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(Y.S. RAJPUT)
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CERTIFICATE

This is to certify that the thesis entitled, **"PREPARATION OF MOLECULAR IMPRINTED POLYMERS AGAINST MODEL MOLECULES"** submitted by **Ms. DIVYA M.P.** towards the partial fulfilment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY** in **DAIRYING (DAIRY CHEMISTRY)** of the **NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)**, Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

24/2/07

(Y.S. RAJPUT)
MAJOR ADVISOR & CHAIRMAN
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Dedicated to my

beloved Parents

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Date: February 24, 2007

(DIVYA M.P.)

सारांश

पिछले कुछ सालों में व्यह्याणिक छाप पुरूभाजें ने अपनी अर्क निकालने की ठोस पदार्थ अवस्था अन्तर्द्रव्य की क्षमता के कारण काफी ध्यान अर्जित किया है क्योंकि यह सक्षमता से पूर्व गाढ़े नमूने पर विशलेषण कर सकती है। इनकी उच्च तापमान और दबाव पर उच्च बाधा और विशलेष के लिए उच्च विशिष्टता के कारण ये प्राकृतिक गृहणकर्त्ता जैसे प्रतिकायें से ज्यादा बेहतर है। इस प्रस्तावित कार्य में छोटे व्याह्याणु जैसे टेट्रासाइक्लिन, सिपरोक्लोक्सासिन, रेटीनाइल एंसीटेट, एसकोरबिक ऐसीड और लेक्टिक ऐसीड जोकि बड़े व्यूह्याणु जैसे लाइसोजाइन है, व्यूह्याणिक छाप पुरूभाजें इनके विपरीत बनाए गया। पोली मेथाक्रिलेट व्यवस्था और पोली एलाईलअमीन हाइडरोक्लोराइड को छाप पुरूभाजें के बनाने में प्रयोग किया गया। टेट्रासाइक्लिन, रेटिनाइल एसीटेट और लेक्टि ऐसीड के विपरीत व्यूह्याणिक छाप पुरूभाजें सफलता से बनाए गये। बनाए गये पुरूभाज में छाप का समन्वय का अवलोकन विलायकों में रूपद के पुरूभाज बंधन की (i) पोलरीटी (ii) अयनिक शक्ति और (iii) पी एच की विभिन्नता से किया गया। वण्टन-गुणक अनुपात, छापे और ना-छापे पुरूभाज और विशिष्टता सूचक का आंकन किया गया। टेट्रासाइक्लिन छापे पुरूभाज की विभिन्न बंधन परिस्थितियों में 1.8 से 3.9 की सीमाओं के बीच विशिष्टता मूल्याँकन प्राप्त किया गया। इसी तरह से विशिष्टता मूल्याँकन 1.5 से 7.7 और 1.3 से 4. 6 सीमाओं के बीच लेक्टिक ऐसीड और रेटीनाइल ऐसीटेट (विटामिन ए) अपने-अपने निजी के विपरीत छापे पुरूभाज के लिए मूल्याँकण प्राप्त किया गया। विशिष्टता सीपराक्लोक्ससिज या लाइसोजाइन छापे पुरूभाज में व्यवस्थापित नही की जा सकी। सिपरोक्लोक्ससिज, टेट्रासाइक्लिन छापे पुरूभाज के साथ बंधन किया । करोमटोगरकिक कॉलम में छापे पुरूभाजें की उपलब्धि को आँका गया। विभिन्न परिस्थितियों में बंधन और प्रोद्वावज विद्या से यह सिद्ध हुआ है कि छापे पुरूभाजें के पास रूपद बंधन की जगह है। टेट्रासाइक्लिन छापे पुरूभाज का दूध में उपस्थित टेट्रासाइक्लिन से पारस्परिक कर सकी। अपने-अपने निजी पुरूभाज के साथ टेट्रासाइक्लिन और रेटिनाइल ऐसीटेट का स्वाभाव जल भीत था जबकि लेक्टिक ऐसीड का उसके पुरूभाज से बंधन अयजिक था। बचाए गए पुरूभाजें अपनी बंधन विशेषताओं को संवयज में 9 महीने तक सामान्य तापमान में रोक रखती है जो पुरूभाज बनाने के गुहा जाल में स्थिरता का सूचक है। एक बहुउद्देश्यीय यन्त्र जो प्रोटीन सकेन्द्रण, डाईलेसिस और प्रत्योराघ-साघज विनिमय के लिए बचाया गया तथा उसके लिए पेटेन्ट भी फाइल किया गया।

ABSTRACT

Molecular imprinted polymers have gained a lot of attention in recent years because of their ability to form a solid phase extraction matrix, which can be effectively used for the pre-concentration of samples to be analyzed. The higher specificity for the analyte and the higher physical resistance to extreme temperature and pressure makes them superior to natural receptors like antibodies. In the proposed work, attempts were made to prepare molecular imprinted polymers against small molecules like tetracycline, ciprofloxacin, retinyl acetate, ascorbic acid and lactic acid as well as lysozyme, a macromolecule. Poly-methacrylate system and poly-allylamine hydrochloride were used for the preparation of imprinted polymers. Molecular imprinted polymers against three molecules, viz., tetracycline, retinyl acetate and lactic acid were successfully prepared. The presences of imprints in prepared polymers were evaluated by measuring binding of template to polymer in solvents differing in (i) polarity, (ii) ionic strength, and (iii) pH. The ratio of partition coefficients for imprinted to non-imprinted polymer, an index of selectivity, was calculated. The selectivity value greater than one suggested presence of imprints in polymer. The selectivity values ranging from 1.8 to 3.9 in different binding conditions were obtained for tetracycline-imprinted polymer. Similarly, the selectivity values ranging from 1.5 to 7.7 and from 1.3 to 4.6 were obtained for imprinted polymers against lactic acid and retinyl acetate (vitamin A), respectively. The selectivity could not be established in ciprofloxacin or lysozyme imprinted polymer. Ciprofloxacin did bind to tetracycline-imprinted polymer. The imprinted polymers were evaluated for their performance in chromatographic column. The binding and elution pattern of template (analyte) under different conditions proved that imprinted polymers have binding sites for template. Tetracycline-imprinted polymer interacted with tetracycline present in milk. The interaction of tetracycline and retinyl acetate to their respective polymer was hydrophobic in nature, whereas binding of lactic acid to its polymer was ionic. The prepared polymers retained their binding characteristics up to 9 months of storage at room temperature indicating stability of created cavities in the network of polymer preparation. A multipurpose device for protein concentration, dialysis and buffer exchange was designed and the patent filed for the same.

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CHAPTER - 1

Introduction

1. INTRODUCTION

The incessant need for new fast and efficient methods within the chemical, biological and pharmaceutical sectors fuel research into better and more selective and sensitive analytical procedures. The increasing number of analyses requires fast methods amenable to automation. Trace analytical methods for complex matrices rely on efficient sample enrichment and selective assays. In the research into new analytical techniques molecular imprinted polymers have gained interest as a novel type of sorbent with attractive properties.

The concept of molecular recognition and related chemistry can be a powerful tool for the understanding of physiological and pharmacological phenomena because the generation and maintenance of the life is governed by combining many simple but specific chemical reactions based upon molecular recognition. Since molecular recognition is the origin of biological functions, the preparation and combination of synthetic molecules capable of molecular recognition may enable us to regenerate bio-functionalized artificial molecules. As one biomimetic strategy involving the formation of these type molecules, 'molecular imprinting' is recognized as a tailor made way of preparing functionalized synthetic polymers capable of molecular recognition of given molecules. Molecular imprinted polymers represent a new class of materials possessing high selectivity and affinity for the target molecule. The target molecule can be small molecules like amino acids as well as large molecules like proteins. Imprinting of molecules occurs by the polymerization of functional and cross-linking monomers in the presence of a template. The template monomer system is chosen in such a way that in solution the imprint molecule complexes one or more functional monomers, which then become spatially fixed in a solid polymer by the polymerization reaction. The resultant imprints possess a steric (size and shape) and chemical (spatial arrangement of complementary

functionality) memory for the template. Following the removal of the imprint molecules these imprints enable the polymer selectively to rebind the imprint molecule from a mixture.

These high selective recognition sites enable imprinted polymers to be used as the mimics of enzymes, receptors and antibodies for screening various kinds of compounds from a mixture of abundant interferences. Moreover, the functional molecular imprinting has provided the polymers with specified selectivity that can be utilized for chromatographic separations, chiral separations and more recent biosensors.

The outstanding advantages of molecular imprinted polymers include their physical robustness, high strength, resistance to elevated temperatures and pressures and inertness to acids, bases, metal ions and organic solvents as well as the low cost and ease of preparation, which have enabled them to be used in a large number of systems and also make them as a fast analytical tool for the detection of contaminants, like, pesticides, herbicides, hormones and antibiotics in food systems. Application of molecular imprinted polymers in separation of small as well as large molecules will be enormous and it can replace some of the classical separation methods. Preparation methods of these imprinted polymers are not well defined and the work in different labs for their preparation is actively pursued. Milk contains diversified molecules differing in size and chemical make-up. These molecules are present in different concentrations. Even environmental contaminants are present in low concentrations. Many of existing analytical methods lack specificity and sensitivity. Molecularly imprinted polymers in such cases can be an enormous help in developing methods specific to analytes existing in low concentrations. In view of this, the present project is being proposed with the following objectives :

1. To prepare molecular imprinted polymers against model molecules present in milk.
2. To evaluate the performance of the prepared imprinted polymers as separation materials.

CHAPTER - 2

Review of Literature

2. REVIEW OF LITERATURE

2.1 HISTORY OF MOLECULAR IMPRINTING

Nature is adept at producing molecules that can recognize and bind other molecules such as antibody molecules, which search out and bind a single foreign molecule called antigen from among other natural substances. This type of exquisite molecular recognition has long inspired chemists who for decades have tried to make molecules that are capable of performing such tasks (Kloeppel, 2002). In 1940, Linus Pauling first suggested the idea of artificial antibodies. But these initial imprints used were either poor in specific recognition or the reported results were irreproducible (Mosbach and Ramstrom, 1996). Nevertheless, these attempts laid the foundations for the area of molecular imprinting.

The history of molecular imprinting is usually traced back to the experiments of Dickey in the 1950s (Dickey, 1955) that was inspired to create affinity for dye molecules in silica gel by a theory of Linus Pauling as to how antibodies are formed (Pauling, 1940). Dickey's silica can be considered to be the first imprinted materials. Imprinting in organic polymers first appeared in the 1970s when covalent imprinting in vinyl polymers was reported (Wulff and Sarhan, 1972). Non-covalent imprinting was introduced about a decade later (Norllow *et al.*, 1984).

One attractive biomimetic approach to study or mimic nature is molecular imprinting technology, which can be described as a way of making artificial locks to molecular keys. The molecular key may be any type of molecule ranging from small molecules such as antibiotics, amino acids, or steroid hormones to larger molecules such as nucleic acids or proteins. But the difficulty of making the imprinted materials increases with the size of the selected molecular key.

2.2 GENERAL PRINCIPLE OF MOLECULAR IMPRINTING

The technology of molecular imprinting allows for the preparation of synthetic polymers with specific binding sites for a target molecule. This can be achieved if the target is present during the polymerization process, thus acting as a molecular template. There are two basic approaches for molecular imprinting : (1) The pre-organized approach where covalent forces maintain aggregates in solution prior to polymerization, and (2) The self-assembly approach, where pre-arrangement between print molecule and functional monomers is formed by non-covalent or metal coordination interactions (Kriz *et al.*, 1997). Both imprinting procedures make use of high percentage of cross-linker resulting the polymers of substantial rigidity. Following polymerization with a high degree of cross-linking, the functional groups of monomers is held in position by polymer network. Subsequent removal of the template by solvent extraction or chemical cleavage leaves cavities that are complementary to the template in terms of size, shape and arrangement of functional groups. The highly specific receptor sites are capable of rebinding the target molecule with a high specificity that is comparable to that of antibodies (Alexander *et al.*, 2003).

2.2.1 Covalent Imprinting

Covalent imprinting refers to molecular imprinting strategies whereby the template and one or more polymerizable units are attached by covalent bonds to form a "template-monomer" by a chemical step independent of polymer formation. Co-polymerization of this template-monomer with additional polymer-forming components, including a high proportion of cross-linker, in a porogenic solvent, results in a polymer which has template covalently bound within the polymer structure. Removal of this template and subsequent rebinding steps involve chemical reactions. Semi-covalent methods differ in that the rebinding step is non-covalent in nature.

Early work carried out by Damen and Neckers (1980) and Shea *et al.* (1980) employed template strategies in which strong covalent bonds, notably ester bonds, were used to assemble the polymerizable template monomer

species, followed by incorporation into divinylbenzene-based matrices. The templates were removed by treatment with a suitable reagent or by hydrolysis. The figure 2.1 depicts a molecular imprinting reaction based on covalent approach. Rebinding to the polymers involved reaction of an acyl chloride with the alcohol component or displacement of bromide by a salt of the carboxylic acid. In each case, the aim of the study was to discover how templated sites were formed by an imprinting process. These experiments had significance that, rebinding using carboxylic ester formation is not practical for the vast majority of applications for which molecular imprinted polymers (MIPs) are currently used. Due to the complexity of covalent imprinting, this method is not widely used.

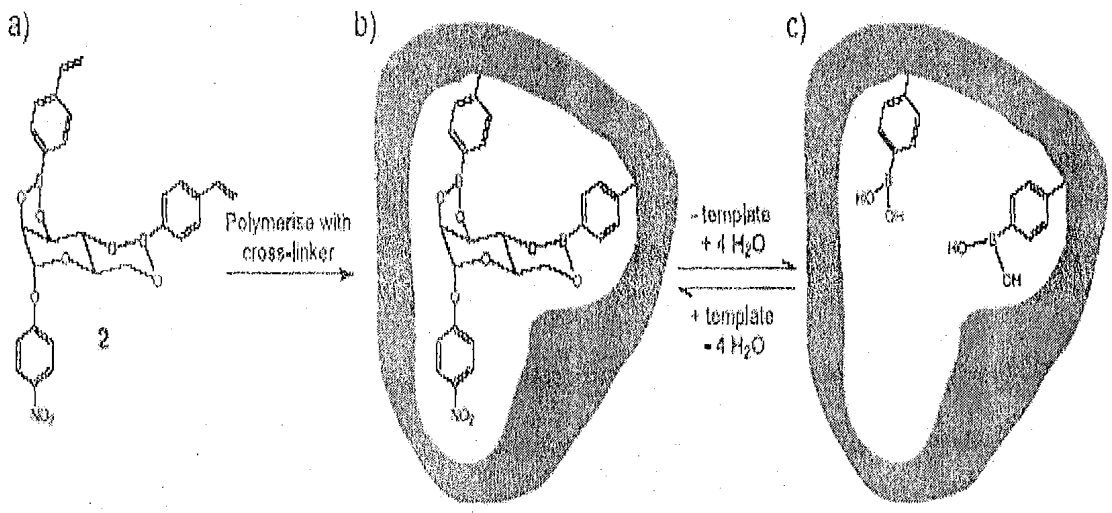


Fig. 2.1 Imprinting of 4-nitrophenyl-α-D-mannopyranoside-2,3:4,6-di-O-(4-vinylphenylboronate) (2): the covalent template monomer, prepared by condensation of 4-vinylbenzeneboronic acid with 4-nitrophenyl-α-D-mannopyranoside (a) is copolymerised with cross-linker (divinylbenzene or ethyleneglycol dimethacrylate) to give the polymer (b). Template removal involves hydrolysis of the boronate ester groups and the addition of 4 equivalents of water to reveal the imprinted site (c). Template uptake and release are reversible and rapid enough for the polymers to be used as HPLC stationary phases (Wulff and Sarhan, 1972).

2.2.1.1 Pros and cons of the covalent methods

There are some advantages of covalent imprinting. This helps to lower non-specific interactions. A further advantage of the semi-covalent methods is compatibility with a wider range of polymerization conditions, which has allowed them to be used in the synthesis of imprinted emulsions. The distinct disadvantages of all covalent strategies are the need for some level of synthetic chemistry to be carried out on the template before polymerization and a chemical treatment on the polymer to release template. A further consequence can be low to moderate template recovery but, at least with the semi-covalent methods, unremoved template remains covalently bound to the polymer, and are not subject to leaching under normal conditions of use (Mayes and Whitcombe, 2005).

2.2.2 Non-covalent Imprinting

Non-covalent imprinting uses the typical forces of attraction between molecules such as hydrogen bonds, ion-pairs, dipole-dipole interactions and Van der Waals forces to generate adducts of template and functional monomers in solution. Unlike those used in covalent methods of imprinting, these adducts are unstable and dynamically rearrange on a time scale relevant to the imprinting process. This has major implications for the design of polymers using non-covalent imprinting methods. One of the key developments in this area of imprinting is the emergence of methods aimed at generating much more stable adducts that can lead to a greater yield of more uniform receptor sites (Mayes and Whitcombe, 2005).

The use of non-covalent interactions can be traced back to the earliest reports of imprinting, and through the era of imprinting in silica matrices, but it has been popularized by the work of Mosbach's group in the 1980s, who showed that this approach has a viable method for producing imprinted receptors in synthetic polymers (Arshady and Mosbach, 1981). Today, it is the predominant method used, because it offers much more flexibility in terms of the functionalities on a template that can be targeted. It also requires much less chemistry than the pre-synthesis of covalent adducts.

It is generally assumed that the non-covalent imprinting a pre-polymerization complex is formed between the template and functional monomers, which is then incorporated into the growing polymer network. This provides a good static model from which predictions and optimization strategies can be developed, even though the real mechanism during polymerization is uncertain. During polymerization, small sections of polymer structure will develop carrying multiple functional groups that will have their local environment and configuration influenced by a template. This will produce a cooperative effect since the multiple interactions will display a much higher avidity than single monomer-template interactions. As polymerization proceeds, these structures will further develop by changing shape and by addition of more functional monomer, leading to an ever-higher avidity than traps the template properly in the receptor site. When considering factors pertinent to the design of non-covalently imprinted polymers, the actual mechanism of receptor formation is not crucial, since any factor that strengthens the interaction between a single functional monomer and a template, will also enhance the avidity effect, so either way it will be beneficial to receptor a yield and affinity (Yu *et al.*, 1997).

2.2.2.1 Non-covalent imprinting with a single functional monomer

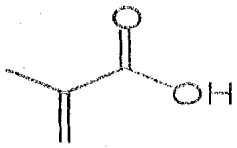
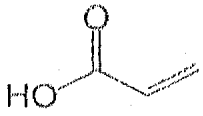
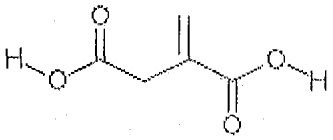
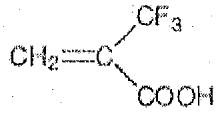
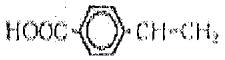
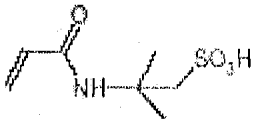
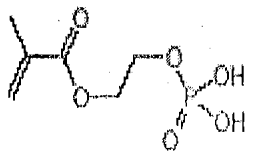
This is the simplest approach to non-covalent imprinting. It is also the first method that was demonstrated to work historically and is by far the most widespread in the literature. But the nature of the pre-polymerization complexes present is far from simple and a number of interactions need to be taken into account. These include desirable template-functional monomer interactions, and interactions of either the template or the functional monomer with the cross-linker. The former may be important in defining or refining functional receptor sites, whereas the latter is probably undesirable. Additional complications can arise from self-association of functional monomer. This is a particular problem with carboxylic acids, which have a strong tendency to dimerise. Finally, possible interactions with the initiator should also be considered because it is often present at a concentration similar to that of the template (Takeuchi *et al.*, 1999).

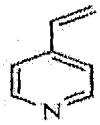
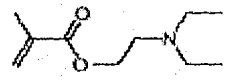
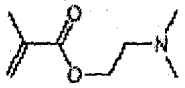
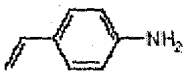
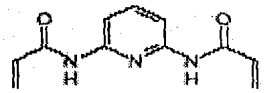
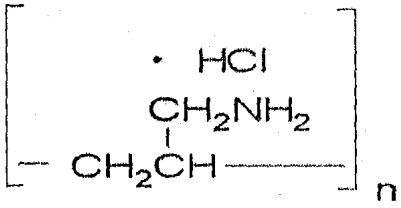
During the last 20 years or so, many different functional monomers have been tested in non-covalent imprinting. Some have found wide utility, while others have been used only in one or a few specialized situations. The examples of these monomers are given in Table 2.1, where they are classified according to whether they are acidic, basic or neutral in nature.

Despite the apparent drawback of strong dimerisation under the conditions generally employed in non-covalent imprinting (high concentration, low solvent polarity), carboxylic acid-based monomers, principally methacrylic acid (MAA) have been by far the most successful. This is probably due to (i) they have relatively few bonds with rotational degrees of freedom and (ii) their ability to interact in various ways with template as H-bond donors, H-bond acceptors and through formal ion-pair formation, as well as weaker dipole-dipole interactions, etc. Methacrylic acid also benefits from the bulk of the methyl group, which probably restricts rotation and conformational flexibility (compared with acrylic acid) and also provides additional Van der Waals interactions, which help to define the general shape-selective elements of the receptor sites (Ramstrom et al., 1993). In a few cases trifluoromethyl acrylic acid has proved superior, probably due to its enhanced acidity (Matsui *et al.*, 1997). Vinylbenzoic acid can be considered an attractive option, due to the bulk and π-electron system of the aromatic ring; but in practice, it has generally provided disappointing results (Andersson *et al.*, 1984). This may be largely due to its relatively poor reactivity ratios with typical (meth)acrylate cross-linkers and hence its non-random incorporation into the final polymer network, rather than unfavourable interactions with template molecules.

There is by now a considerable literature using much more acidic monomers, such as sulphonates, phosphonates, phosphates, etc (Wulff and Shonfeld, 1998). These are particularly used to form formal ion pairs when carrying out imprinting under aqueous conditions. In principle, they should be highly effective, but the large number of bonds probably compromises their performances with relatively unrestricted rotational degrees of freedom in the structures of the commercially available members of this group. Phosphonates and phosphates have also found utility in imprinting based on metal ion complexation (Kim *et al.*, 2001).

Table 2.1 Structures of commonly used monomers for different templates

Monomers	Application (References)
Monomers bearing acidic residues	
Methacrylic acid 	Amino acid derivatives (Sellergren *et al.*, 1988) Peptides (Ramstrom *et al.*, 1994) Propranolol (Andersson, 1996) Morphine (Andersson , 1996) Monosaccharide derivatives (Mayes *et al.*, 1994)
Acrylic acid 	Amino acid derivatives (Sellergren *et al.*, 1985)
Itaconic acid 	β blockers (Timolol) (Fischer *et al.*, 1991) norephedrine (Suedee *et al.*, 1999)
Trifluoromethylacrylic acid (TfMAA) 	Atrazine Nicotine (Matsui *et al.*, 1997) Cinchona alkaloids (Matsui *et al.*, 1998)
p-Vinylbenzoic acid 	Amino acid derivatives (Andersson *et al.*, 1984)
2-Acrylamido-2-methyl-1-propane sulphonic acid (AMPSA) 	2,6-Diaminoanthroquinone (Dunkin *et al.*, 1993) Desmetryn (herbicide) (Piletsky *et al.*, 2000)
2-(Methacryloyloxy) ethyl phosphate 	Sterols (Kugimiya *et al.*, 2001)

Monomers	Application (References)
Monomers bearing basic residues	
4-Vinylpyridine 	Naproxen (Kempe and Mosbach, 1994) Amino acid derivatives (Kempe *et al.*, 1993)
Diethylaminoethyl methacrylate 	Atrazine (Piletsky *et al.*, 1995)
Dimethylaminoethyl methacrylate 	Acidic templates (Simon and Spivak, 2004)
p-Aminostyrene 	Food colourants (Ju *et al.*, 1999)
1-Vinylimidazole 	Amino acid derivatives (Kempe *et al.*, 1993)
4(5)-Vinylimidazole 	ATP (Mathew and Buchardt, 1995)
2,6-Bis-acrylamidopyridine 	Barbiturates (Tanabe *et al.*, 1995) Alloxan (Yano *et al.*, 1998)
Hydrogels	
Poly(allyl) amimine hydrochloride 	D-Glucose 6-phosphate (Parmpi and Kofinas, 2004) Baculoviruses (Linden *et al.*, 2004)

From the basic monomers, the vinyl pyridines have been the most widely applied, though in reality these monomers are quite acidic in nature (pKa = 5.62 for 4-vinylpyridine and pKa = 4.92 for 2-vinylpyridine; Pietrzyk *et al.*, 1957). In their basic form, these represent electron rich π-electron ring systems, which allow them to interact strongly with electron deficient aromatic rings, as well as through acid-base interactions and H-bond acceptance (or donation if in the conjugate acid form). These monomers often interact strongly with templates, which suggest that they should be highly successful in imprinting. They have proved useful in many cases, but their use can also have significant drawbacks. A particular problem is the very strong π-π interactions that occur during template rebinding in aqueous conditions. It generally leads to extremely high levels of non-specific binding of analytes to the polymer, so much so that imprinted and reference polymers often show identical abilities in that they both effectively bind near 100 percent of the template (Kempe and Mosbach., 1994).

4(5)-vinyl imidazole is an interesting monomer, but has been little used, except in metal complexation systems due to its lack of commercial availability and the relative difficulty of preparing it in the laboratory. Other basic monomers include the family of tertiary amino monomers. These have been effective in a few cases, but in general yield less satisfactory results than acidic monomers, possibly due to their much greater chain flexibility and the fact that the amine functionality is more remote from the polymer backbone (Kempe *et al.*, 1993).

A variety of neutral monomers have also been shown to produce effective imprints. Perhaps the most successful has been acrylamide, or its N-alkyl derivatives. Acrylamide shows superior H-bonding ability compared with MAA under conditions of low polarity, as demonstrated by comparisons of MAA and acrylamide MIPs in HPLC mode (Yu and Mosbach, 1997). Under aqueous rebinding conditions, however, H-bonding is less important than electrostatic and hydrophobic interactions, and MAA polymers often show

superior recognition in this case. Although better known for its hydrogel properties, hydroxy-ethyl methacrylate has also been shown to yield effective imprints under some conditions.

Biomolecule sensitive hydrogels have attracted considerable attention as intelligent materials for imprinting purposes in recent years. Hydrogels have been used primarily in the pharmaceutical field as carriers for delivery of various drugs, peptides and proteins. There is the possibility of utilizing molecular imprinting strategies to impart analyte specificity and responsiveness within these systems (Miyata *et al.*, 2002). A molecularly imprinted polymer hydrogels having an affinity to glucose over fructose have been developed (Parmpi and Kofinas, 2004). Non covalent imprinting of poly(allylamine hydrochloride) with D-glucose 6-phospate monobarium salt has been used to produce the molecularly imprinted hydrogels. The ionic association of an imprint molecule with the polymer helps to form the imprinted hydrogels. The chosen polymer matrix, poly(allylamine hydrochloride), has good water solubility and presents a high density of amine groups (Kofinas and Cohen, 1997). A molecularly imprinted hydrogels capable of quantitative, isomerically specific binding of glucose have also been developed using poly (allylamine hydrochloride) and glucose phosphate mono-sodium salt (Wizeman and Kofinas, 2001).

2.2.2.2 Imprinting with combinations of monomers

It seems highly attractive to combine the specific interaction potential of a variety of different monomers even though it is much more complex mechanism. In practice almost all imprinting methods use a mixture of monomers since the cross linkers can interact with both template and monomers and are also present at significant concentrations. Recent work by Spivak has shown that if the cross-linker is appropriately functionalised, no further functional monomers are required to achieve effective imprinting (Sibrian and Spivak, 2004a).

In order to be successful, the adducts formed between the template and the functional monomers need to be stronger than any interactions between the functional monomers. Early work by Ramstrom *et al.* (1993) used mixtures of MAA and 2-Vinyl Pyrridine (2-VP) in the imprinting of amino acid derivatives. They showed that when the carboxyl terminus was free, better imprinting occurred with the mixture of MAA and 2-VP than with either monomer alone. This was assumed to be due to a strong interaction between the 2-VP and the carboxyl group enhancing the affinity of the receptor. Mixtures of acrylamide and 2-VP have been used in a series of studies by Meng *et al.* (1999). This method has been shown to give good results with a variety of templates. Overall, there is a vast array of polymerizations that could be carried out with different mixtures of monomers.

2.2.2.3 Inclusion complexes

To date, most imprinting work in this area has used polymerisable cyclodextrin derivatives. Piletsky *et al.* (1998) used an acrylate derivative of cyclodextrin together with 2-acrylamido-2-methyl-1-propane sulphonic acid (AMPSA) to imprint phenylalanine under aqueous conditions. The cyclodextrin cavity provided a strong interaction with an aromatic ring of the template, while the AMPSA formed an ion pair to add selectivity to the site around the cavity. The opportunities for imprinting presented by modified supramolecular hosts (to utilize a performed cavity that creates part of the binding site and provides strong interactions with the template) is attractive, but as yet has been little explored (Piletsky *et al.*, 1999).

2.2.2.4 Stoichiometric imprinting

In principle, stoichiometric imprinting is non-covalent molecular imprinting using carefully selected high affinity monomers, but it offers features that make it much more like covalent imprinting in many respects. In stoichiometric imprinting, the interaction between template and functional monomer is arranged to be strong enough to ensure that the vast majority of monomer is associated with template under the conditions (concentration,

temperature, solvent polarity, etc.) of imprinting. The classic example of the stoichiometric interaction in non-covalent imprinting is the interaction of polymerizable amidines with carboxylic acids, phosphates and phosphonates, introduced by Wulff and Schonfeld (1998), the latter group of templates proving particularly effective in the preparation of hydrolytic catalysts.

2.2.2.5 Advantages and disadvantages of non-covalent imprinting

The most obvious strength of the approach is the relative simplicity of the process itself. Little or so synthetic chemistry is needed. Components are simply mixed together and left to interact; the species formed as a result being entirely dictated by the thermodynamics of the system, resulting in a mixture of different solution adducts which are in dynamic equilibrium. Exactly how this translates into functional imprinted receptor sites is still not clear but it is generally accepted that stronger interactions between functional monomer and template at this stage lead to better receptor sites in the final polymer. The other great strength of the approach is the range of chemical functionalities that can be targeted. Using covalent imprinting, only a few different types of functional groups can be easily targeted that can be readily and dynamically reversed to allow free exchange of analytes in and out of the receptor sites. With non-covalent approaches, almost any functionality can be addressed while retaining this essential reversibility. Some, such as ion-pairs or hydrogen-bonding groups, are easy to target while others, such as aromatic rings, may need more subtle approaches. Since such a wide range of monomers is available, suitable interactions can be arranged with any functional group unlike covalent imprinting (Tanabe et al., 1995).

Three significant drawbacks are also associated with the non-covalent approach. The first of these is the heterogeneity of the receptor sites produced. Since a variety of solution adducts of functional monomer and templates are present in the pre-polymerization mixture, a heterogeneous population of receptor sites is formed. This range from the very high affinity (Kd in the nM range or better) to very low affinity (Kd is mM or worse) and this system is often compared with that of polyclonal antibodies. For certain

applications, this range of receptor affinity might be beneficial since it can increase the dynamic range of some assay systems, but in general it is a disadvantage, which makes the materials more difficult to work with (Shea *et al.*, 1993).

The second problem is the fact that a lot of functional monomer is spread around the polymer network, outside receptor cavities. This leads to very non-specific single point interactions between analyte molecules and the polymer, which is undesirable for most practical applications (e.g., it increases background signals in sensors and reduces the purity of extracts in solid phase extraction). But, it is also possible that this non-specifically anchored functional monomer increases the hydrophilicity of the polymer surface, so while it may increase non-specific interactions through, e.g., hydrogen bonding, it may at the same time reduce non-specific binding due to Van der Waals interactions. The balance of these effects depends entirely on the specific nature of the polymer and the matrix in which it is being applied (Righetti and Gelfi, 1997).

The third key problem in non-covalent imprinting is the very low yield of functional high-affinity receptor sites relative to the amount of template present in the pre-polymerization mixture. It could be that template-monomer adducts present prior to polymerization are destroyed during the violent and dynamic process of radical based network polymerization due to conformational or thermal effects. It could be due to the lack of true chain cross-linking, giving inadequate rigidity to maintain receptor conformational integrity (the swellability of MIPS suggests that they are much less cross-linked than their stoichiometric monomer ratios would suggest). It may be that most receptor sites are located in inaccessible regions of the polymer nano-nodules formed during phase separation, even though the extraction of most of the template, suggests that these sites should be accessible for rebinding. The large amount of "wasted template" in non-covalent imprinting remains a problem, and contributes to issues such a template leaching (Mayes and Whitcombe, 2005).

2.3 PREPARATION OF MOLECULAR IMPRINTED POLYMER

The general step of imprints preparation includes mixing monomer with template followed by pre-arrangement of functional groups and then addition of cross-linker and initiator, which leads to polymerization. The extraction of template using appropriate solvent yields cavities with specific recognition properties. Figure 2.2 explains the general polymerization process.

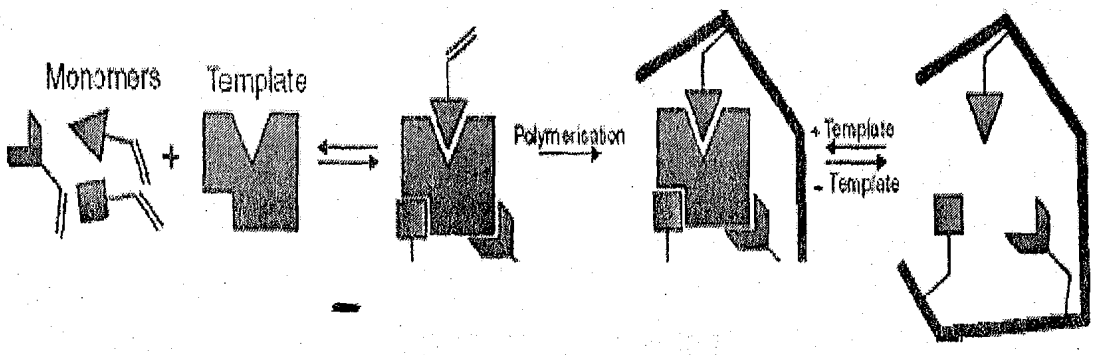


Fig. 2.2 Schematic generalization of molecular imprinting process

2.3.1 Target Molecule

A wide variety of print molecules have been used in various imprinting protocols. Compounds such as drugs (Chen *et al.*, 2001; Kempe and Mosbach, 1994)), amino acids (Reddy *et al.*, 2002; Kempe *et al.*, 1993)), carbohydrates (Oral and Peppas, 2004), proteins (Rachkov and Minoura, 2001), nucleotide bases (Spivak and Shea, 1998), antioxidants (Bruggemann *et al.*, 2004), hormones (Kugimiya *et al.*, 2001), pesticides and coenzymes (Piletsky *et al.*, 2001) have been used for the preparation of selective recognition matrices.

Because biological interactions are mainly based on non-covalent interactions, many workers have tried artificial recognition systems with such interactions and it is suggested that the use of non-covalent interactions between the print molecule and the functional monomers is the more versatile.

2.3.2 Functional Monomers

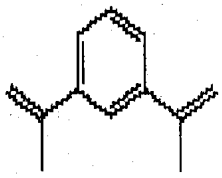
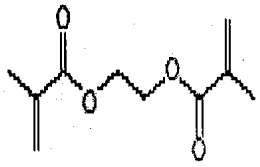
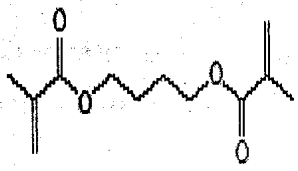
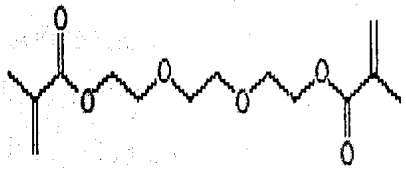
The careful choice of functional monomer is of the utmost importance to provide complementary interactions with the template and substrates. Commonly used monomers are listed in Table 2.1. Conventional polymer synthesis and processing is very time consuming, so to optimize even one of the many possible compositional or operational variables is a substantial synthetic undertaking. Various approaches have been developed to make a more systematic and wide ranging evaluation of possible polymer compositions and several polymer systems have been developed for use in molecular imprinting technology. By far the most readily used are either polyacrylate-based or polyacrylamide-based systems. The functional monomers are responsible for the binding interactions in the imprinted binding sites and are normally used in excess relative to the number of moles of template to favour the formation of template functional monomer assemblies. It is important that the functionality of the template should match with the functionality of monomer in a complementary fashion in order to maximize complex formation and thus the imprinting effect (Lu *et al.*, 2002).

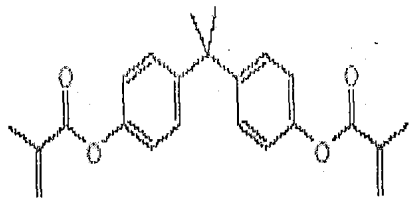
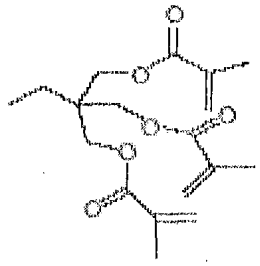
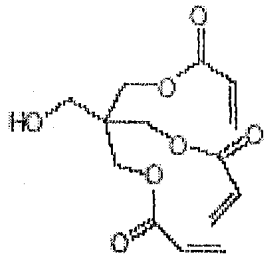
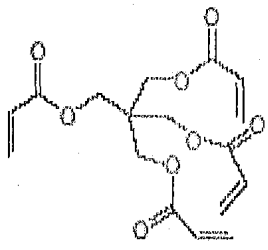
Typical functional monomers used are carboxylic acids (acrylic acid, methactrylic acid, vinyl benzoic acid) sulphonic acids (acrylamido-methyl propane sulphonic acid) and hetero aromatic weak bases (vinyl pyridine, vinyl imidazole). For metal chelating interactions imido acetic acid derivative is commonly used. Another system that has been used for polymer preparation is polysiloxane bases strategy. In this, various silanes are used as monomers.

2.3.3 Cross-linkers

Many different cross linkers have been used in preparation of MIP (Table 2.2). Cross-linker typically can make up anything between about 70 and 98 percent of the final MIP. In an imprinted polymer, the cross-linker fulfills three major functions. First of all, the cross-linker is important in controlling the morphology of polymer matrix, whether it is a gel type, macro porous, or a microgel powder. Secondly, it serves to stabilize the imprinted binding site. Finally, it imparts mechanical stability to the polymer matrix.

Table 2.2 Structures of commonly used cross-linking monomers

Cross-linker	References
Styrenic cross-linkers	
Divinylbenzene (DVB) 	Shea and Sasaki (1989)
1,3-Diisopropenylbenzene 	Shea and Sasaki (1989)
Acrylate and metacrylate ester-based linkers	
Ethyleneglycol dimethacrylate (EDMA) 	Wulff *et al.* (1987)
1,4-Butanediol dimethacrylate 	Zhan *et al.* (1999)
Tri(ethylene glycol) dimethacrylate 	Sergeyeva *et al.* (1999)

Cross-linker	References
Bisphenol A dimethacrylate 	Wulff *et al.* (1987)
Trimethylolpropane trimethacrylate (TRIM) 	Glad *et al.* (1995)
Pentacrythritol triacrylate (PETRA) 	Dong *et al.* (2002)
Pentacrythritol tetraacrylate (PETEA) 	Kempe (1996)

A few systematic investigations of the effect of cross-linkers on the recognition properties of MIPs have been carried out, by the groups of Wulff (Wulff *et al.*, 1987) and Sibrian and spivak (2004). Although some systems require the more chemically resistant divinylbenzene (DVB), ethyleneglycol dimethacrylate (EDMA) is by far the most commonly used cross-linker.

Since a very high degree of cross-linking (70-80%) is necessary for achieving specificity, a limited number of cross-linkers have been used. The solubility of cross-linker itself in the pre-polymeric solution and the solubility of monomerized template reduce the number of possible alternatives. But several cross-linkers have been tried with different degrees of success. It was found that acrylic or methacrylic acid based systems could be prepared with much higher specificity using EDMA or trimethylopropane trimethacrylate as cross-linkers (Ramstrom *et al.*, 1996).

2.3.4 Solvents

Solvent plays an important role in the formation of the porous structure of MIPs, which are a subset of a larger class known as macro porous polymers. The morphological properties of porosity and surface area are determined by the type of solvent, referred as "porogen", used in the polymerization. Porosity arises from phase separation of the porogen and the growing polymer during polymerization. Porogens with low solubility phase separate early and tend to form larger pores and materials with lower surface areas. Conversely, porogens with higher solubility phase separate later in the polymerization providing materials with smaller pore size distributions and greater surface area. It is not necessary that binding and selectivity in MIPs is dependent on a particular porosity. MIPs made without any porogen do not exhibit any selectivity because substrate cannot access the polymer. Polarity index of different solvents is given in Table 2.3.

Another important role for solvent in the formation of MIPs is the effect it has on the complexation of functional monomers with the template before, during, and after polymerization. Before (and during) polymerization, the extent of the non-covalent pre-polymer complex is affected by the polarity of

Table 2.3 Polarity index of different solvents

Solvent	Polarity Index
Heptane	0.0
Hexanol	0.0
Carbon tetrachloride	1.6
Toluene	2.4
Methyl t-butyl ether	2.5
Diethyl ether	2.8
Dichloromethane	3.1
Isopropanol	3.9
Tetrahydrofuran	4.0
n-propanol	4.0
Chloroform	4.1
Ethyl acetate	4.4
1,4-Dioxane	4.8
Acetone	5.1
Methanol	5.1
Ethanol	5.2
Acetonitrile	5.8
Acetic acid	6.2
Dimethyl sulfoxide	7.2
Water	9.0

the porogen solvent. As porogen in the polymerization, the solvent governs the strength of non-covalent interactions in addition to its influence on the polymer morphology. Generally, the more polar the porogen, the weaker resulting recognition effect becomes, as a consequence of the influence of the solvent polarity in non-covalent interactions. Since all non-covalent forces are influenced by the properties of solvent, non-polar solvents normally lead to the best recognition. Less polar solvents such as chloroform or benzene will increase complex formation by Le Chatelier's principle, facilitating polar non-covalent interactions such as hydrogen bonding or bridging of ionic salts. On the other hand, more polar solvents tend dissociate the non-covalent interactions in the pre-polymer complex, especially protic solvents that afford a high degree of disruption to hydrogen bonds (Yoshizako *et al.*, 1998).

An important discovery in MIPs is that after polymerization, the rebinding performance is optimized when carried out in the same solvent used for imprinting (Yu *et al.*, 1997). The underlying cause for this effect has been postulated to arise from differences in solvation of the polymer structure in the binding site microenvironment. Different solvation properties of different solvents, such as chloroform and acetonitrile, may play a role in determining shape and distance parameters that are built into the forming polymer. In order to recreate and maintain this shape and distance parameters, it is possible that optimum rebinding conditions require the same, or very similar, solvation conditions used for polymerization.

The solvent serves to bring the entire components in polymerization, i.e., template, functional monomer, cross-linker and initiator into one phase. When macro porous polymers are being made, the nature and level of porogen can be used to control the morphology and the total pore volume. The use of thermodynamically good solvent tends to lead polymers with sell-developed pore structures and high specific surface areas. The solvent should be chosen in such a way that it simultaneously maximize the template functional monomer complex formation (Cormack and Elorza, 2004).

2.3.5 Initiator

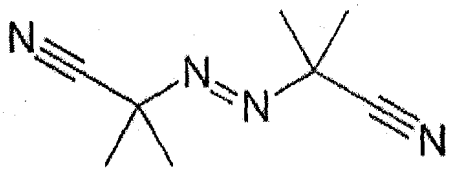
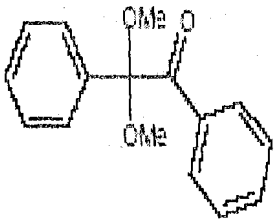
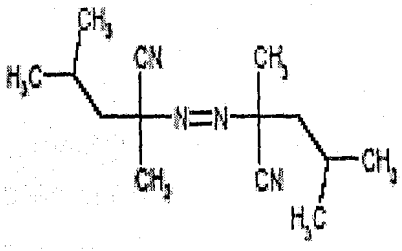
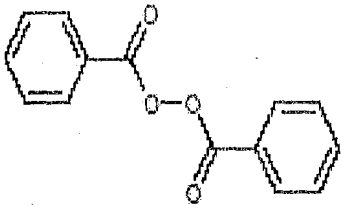
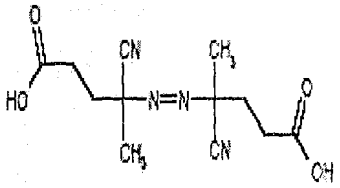
The proper selection of initiator is also necessary for an efficient imprint formation. The decomposition of the initiators to form free radicals can be induced by heat energy, light energy or catalysts. The free radicals formed by photodecomposition of an initiator are the same as those formed by its thermal decomposition. Here the rate of decomposition mainly depends on intensity and wavelength of radiation not so much on temperature. But the polymerization reaction initiated by UV light fall under the class of photo initiated polymerization. If the template is photochemically or thermally unstable, then initiators that can be triggered photochemically and thermally will not be suitable. Where complexation is driven by hydrogen bonding, lower polymerization temperatures are preferred and in that case photochemically active initiators are well suited as these can operate efficiently at low temperatures. Molecular imprints of L-phenylalanine anilide were prepared using azo bisnitriles as either thermal initiators or photo initiators at temperature ranging from 0 to 60°C (O'Shannessy *et al.*, 1989). The chemical structures of important polymerization initiators are shown in Table 2.4 (Cormack and Elorza, 2004).

2.4 MOLECULAR MODELLING APPROACHES

In an attempt to avoid the time-consuming process of making and testing large numbers of MIPs, the group of Subrahmanyam (Subrahmanyam *et al.*, 2001) have taken a different approach using a computationally based "virtual imprinting" approach. In this, they have assembled a liberty of functional monomers, which is then screened for the strength of the interactions each monomer is capable of making with a template molecule of interest using molecular modeling software. Monomers showing strong interactions are then used for a simulated annealing (molecular dynamics) process with the template under conditions that reflect those used during imprinting (solvent polarity, etc.), leading to a model of likely template-monomer interactions and stoichiometry. The results of this process are then

used to define the monomers and ratios used in the real imprinted polymer. Several successes for this process have been reported. The best example to date is probably the use of this technique to generate MIPs for Microcystin-LR, an algal toxin (Chianella *et al.*, 2002). This would have been difficult to achieve using conventional imprinting approaches due to the expense and toxicity of the template, but was attempted with some success following virtual imprinting to give likely recipes for successful polymers.

Table 2.4 Chemical structures of selected initiators

Initiator	Initiator
Azobisisobutyronitrile (AIBN) 	Dimethylacetal of benzyl 
Azobisdimethylvaleronitrile (ABDV) 	Benzoylperoxide (BPO) 
4,4'-azo(4-cyanovaleric acid) 	

A real strength of the approach is its ability to deal with mixtures of functional monomers and predicts likely complexes and stoichiometries. While computational imprinting has many attractions in terms of cost and time saved, it does have some drawbacks. Firstly, it is a purely thermodynamic model of imprinting based on association in the prepolymer mixture and takes no account of dynamic processes occurring during polymerization.

2.5 MOLECULAR IMPRINTED POLYMERS AGAINST TARGET MOLECULES

Molecular imprinted polymers represent a new class of materials possessing high selectivity and affinity for the target molecule. Since their discovery in 70's these molecular imprinted polymers have attracted considerable interest from bio and chemical laboratories to pharmaceutical institutes. They have been utilized as sensors, catalysts, sorbents for solid phase extraction, stationary phase for liquid chromatography, mimics of enzymes, receptor and antibodies (Piletsky *et al.*, 2001). Several imprinted polymers have been made against target molecules that are small as well as large. These target molecules include amino acids, peptides, proteins, carbohydrates, enzymes, pesticides, drugs, etc. (Hilt and Byrne, 2004).

2.5.1 Amino Acids

Stationary phases to be used in high performance liquid chromatography for the separation of amino acids, peptides, and proteins were prepared using molecular imprinting technology by Kempe and Mosbach (1995). In their approach, low molecular mass compounds were imprinted in bulk polymers by co-polymerization of functional monomers and cross-linkers in the presence of the compound of interest. These polymers after extraction of template were successfully applied as chiral stationary phases showing high resolution and load capacity. A methacrylic acid-ethylene glycol dimethacrylate co-polymer imprinted with amino acid derivatives like Boc-L-Phe-OH (Boc = ter-butyloxycarbonyl) was investigated by frontal chromatography. The dissociation constant for Boc-L-Phe-OH was determined to be lower than that of Boc-D-Phe-OH. This shows the higher

affinity for L-enantiomer compared to the D-enantiomer since the L-enantiomer was used as the print molecule. They choose a ratio of 1:4:20 between template, monomer and cross-linker, respectively. The other print molecules used were Z-L-Phe-OH (Z = benzyloxycarbonyl), Z-L-Asp-OH, Z-L-Glu-OH and Z-L-Ala-OH.

An optically active polymeric absorbent was developed for the use in chromatographic resolution of non-derivatized amino acids (Lee and Hong, 2000). The chiral selectivity of the absorbent was based on ligand exchange of coordinated copper (II) complexes of D or L amino acids and molecular imprinting technique by modifying the resin surface with polypyrrole coating. Applying a potential difference of 1.5 V to the chiral and conductive column racemic amino acids like DL-Lysine, DL-aspartic acids were separated according to their charge characteristics and simultaneously resolved with respect to their optical properties.

2.5.2 Proteins

Surface imprinting of RNase A was done where a metal binding monomer, N-(4-Vinyl)-benzyl iminodiacetic acid and the imidazole groups of RNase A were allowed to coordinate metal ions (Kempe *et al.*, 1995). Silica particle derivatized with methacrylic groups were added and the polymerization was initiated. After the extraction of template, the resulting silica particles were used as stationary phase in HPLC. Alternatively polysaccharide like surfaces with tailored protein binding nano cavities were prepared by a novel templating approach based on radio frequency plasma deposition of thin layer films (Shi and Ratner, 2000). The template imprinted proteins included albumin, immunoglobulin, fibrinogen, lysozyme, ribonuclease A, α-lactalbumin and glutamine synthetase. Here, the proteins coated with disaccharide were adsorbed to a mica sheet and further plasma deposition of hexaflouropropylene was done. Transmission Electron Microscopy (TEM) showed that nanometerized pits in the shape of imprinted proteins were formed on the surfaces of template imprinted polymer films (Shi *et al.*, 1999). Specific protein recognition was clearly demonstrated by the

competitive adsorption experiments. Even though there was non-specific binding also, molecular imprinting of ricin and it's A and B chains to organic silanes were done and specificity was detected by scanning flourometer (Lulka *et al.*, 2000).

2.5.3 Peptides

Hart and Shea (2002) developed a molecular imprinted polymer for recognition of peptide using peptide metal interactions. Here, both polymerization and recognition were carried out in aqueous systems. This approach utilized a strong Ni (II) –His binding to attract the N-terminus histidine of the dipeptide to the polymer surface and secondary interactions between peptide and polymers to discriminate between the peptide sequence. These developments were enabled by utilizing N,N-ethylene bis (acrylamide) as a water soluble cross linking monomer and a polymerizable NTA (nitriloacetic acid) ligand, which could be used to incorporate nickel and other metal into these polyacrylamides. The Ni-NTA complex provided a strong histidine binding site that drew the dipeptide to the polymer surface. Variation of monomer composition revealed an optimum cross linking for achieving maximum selectivity for these polymers. An epitope approach based on using a short peptide that represents only part of a larger peptide or protein as template, which in turn can be recognized by the synthesized polymer was used for the recognition of larger peptide like oxytocin. Here methacrylic acid was selected as monomer, EGDMA as cross linker and a tetrapeptide, YPLG (Tyr-Pro-Leu-Gly-NH_2) as template. Following polymerization and processing, the properties of synthesized polymers were evaluated by HPLC. The results of chromatographic evaluation showed successful imprinting with YPLG, since the molecular imprinted polymers retained YPLG much strongly than non imprinted polymers and also it recognized oxytocin, a larger molecule bearing the same 3-amino acid C-terminus as that of template used (Rachkov and Minoura, 2001). Molecular imprinted polymer selective for [Sar' (sarcosine), Ala] angiotensin-II were prepared using sodium acrylate as monomer and polyethylene glycol diacrylate as cross-linker in aqueous solutions (Rachkov *et al.*, 2004).

2.5.4 Bio-imprinting of Proteins

The lyophilization of proteins in the presence of template molecules was suggested as a means of creating binding and catalytic sites. The bio-imprinting of bovine serum albumin was investigated using the binding of p-hydroxybenzoic acid and the β-elimination of 4-fluoro-4(-p-nitrophenyl) butan-2-one as model systems (Slade, 2000). It was found that both binding and catalytic activity could be enhanced by a factor of three over that of native protein but no increased specificity towards template was introduced.

2.5.5 Carbohydrates

The non-covalent imprinting procedure was used for the preparation of polymers selective for various carbohydrate derivatives like peracetylated phenyl α- and β- D-glycosides of galactose. The selectivities of resulting polymers were tested in a HPLC procedure. The selectivity was influenced by anomeric configuration of the glycoside. Thus, the polymer prepared with *p*-aminophenyl *β*-galactoside as a print molecule, gave a relatively high selectivity (α = 1.27) for the β-galactoside over the α-galactoside (Nilsson *et al.*, 1995). Striegler (2003) prepared sugar templated polymers using poly(acrylates). Here alkaline pH was used to enable strong chelation of underivatized carbohydrates to the mononuclear copper (II) complex during the preparation of sugar templated polymers. Positive ion ESI (electrospray ionization) mass spectrometry showed only weak copper (II)-saccharide interactions under sugar rebinding conditions. The formation of multiple hydrogen bonding between the polymer backbone and the sugar template were demonstrated to be advantageous to overall material binding capacity. Biomimetic hydrogels have used for the preparation of glucose imprinted polymer material. Poly(allylamine hydrochloride) was imprinted with glucose phosphate mono-sodium salt in order to produce a molecular imprinted polymer which could specifically bind the glucose (Wizeman and Kofinas, 2001). Further, Parmpi and Kofinas (2004) prepared an imprinted polymer of D-glucose-6-phosphate using a preformed polymer poly (allyl) amine hydrochloride and cross-linker epichlorohydrin.

2.5.6 Pesticides, Drugs and Steroids

New types of molecular membranes containing molecular recognition sites for a pesticide named atrazine has been prepared by molecular imprinting approach (Piletsky *et al.*, 1995). The membrane synthesis includes radical polymerization of ethyl amino, ethyl methacrylate and ethylene glycol dimethacrylate in the presence of atrazine as a template.

Different drugs like ketoprofen (Suedee *et al.*, 2002), naproxen (Haginaka *et al.*, 1999), paracetamol (Tan *et al.*, 2001), sulfonamides (Zheng *et al.*, 2002), tetracycline (Cai and Gupta, 2004), theophyline, diazepam (Vlatakis *et al.*, 1993), caffeine (Carter and Rimmer, 2002), ephedrine (Piletsky *et al.*, 2002), cephalexin (Lei and Wu, 2003), ampicillin (Lubke *et al.*, 2000), chloraphemine (Chen *et al.*, 2001), etc. have been used as templates for the preparation of molecular imprinted polymers. Besides these imprinted polymers have been developed against steroids like cholesterol (Hwang and Lee, 2002), estradiol (Kugimiya *et al.*, 2001), cortisone (Ramstrom *et al.*, 1996) and also nucleotide bases like adenine, adenosine 5'-tri-phosphate (Kempe, 1996), nucleoside derivatives like tri-O-acetyl adenosine, 9-ethyladenine (Spivak and Shea, 1998).

2.6 IMPROVED FORMATS FOR POLYMERIZATION

Over the last decade, significant progress has been made in developing polymerization methods that deliver MIPs with good binding properties in the formats required. A few representative examples of polymer formats that have been produced include :

- Micron-sized spherical beads by suspension polymerization in a liquid fluorocarbon (Mayes and Mosbach, 1996).
- Mono-disperse micron-sized beads by aqueous two step swelling methods (Hosoya *et al.*, 1996).
- Sub-micron particles by precipitation polymerization (Ye *et al.*, 1999).
- Imprinted core-shell nano particles (Pierez *et al.*, 2001).

- Nano particles prepared by mini-emulsion polymerization (Vaihinger *et al.*, 2002).
- MIP films grafted onto surfaces and particles (Wang *et al.*, 1997).
- Micro-moulded and micro-fabricated MIP structures (Yan and Kapua, 2001).

A significant limitation is that most of the above techniques use particular conditions that may not be compatible with all imprinting recipes. For instance, the fluorocarbon suspension method does not work very reliably when acetonitrile is used as a porogenic solvent. Methods using aqueous phases (core-shell nano-particles and two-step swelling) work poorly with more polar templates. Since no truly universal approaches have yet been developed, it is important that researches recognize the limitations of each technique and select carefully.

2.7 CHARACTERISTICS OF MOLECULAR IMPRINTED POLYMERS

Molecular imprinted polymers are highly resistant to physical and chemical factors. They are remarkably stable against mechanical stress, high temperatures and pressures as well as resistant to treatment with acid, base, metal ions and a wide range of solvents (Kriz *et al.*, 1995). The polymers can be reused more than 100 times and stored for over 8 months at ambient temperature without reduction of memory (Fischer *et al.*, 1991).

A small drawback in molecular imprinting is the need of substantial amount of print molecule, which it may be expensive or difficult to obtain. Normally 50 to 500 µmol imprint molecule/g dry polymer is used. Up to 99 percent of imprint molecule can be subsequently recovered from the polymer by extraction (Andersson *et al.*, 1990).

Molecular imprinted polymers have been prepared using various techniques like the preparation of bulk polymers that thereafter dried and grounded into small particles. Other techniques have been evolved such as *in situ* polymerization in chromatographic columns and also in capillary electrographic columns. Since the flow properties are dependent on particle

size and shape, attempts have been made to develop molecular imprinted particle of uniform size. This can be accomplished by grafting / coating of the imprinted polymer to preformed particles such as silica or preparation of beads through suspension or emulsion polymerization. For analytical or sensor device applications, thin layer or polymer membranes have been developed (Ulbricht, 2004).

2.8 EVALUATION OF IMPRINTED POLYMERS

The polymer obtained after polymerization is checked for its binding capacity. Different workers have selected different terms to express the binding capacity. However, the molecularly imprinted polymers initially incubated with a solution mixture of template. After incubation, supernatant was collected and checked for remaining template (unbound) concentration. The amount of template bound to polymer was calculated by subtracting the unbound concentration of template from initial amount loading. Partition coefficient K = Cp/Cs was used to characterize the binding extent, where Cp is the concentration of template inside the polymer, and Cs is the concentration of template in the solution (Cai and Gupta, 2004). Alternatively Imprinted polymers are slurry packed into columns and samples are eluted at a suitable flow rate (Rachkov *et al.*, 2004). The capacity factor (k') was calculated as $(t-t_0)/t_0$, where't' is the retention time of the analyte and 't_0' is the retention time of the void marker (Synder and Kirkland, 1979). The imprinting factor was defined as $I = k'_{imp} / k'_n$, where k'_{imp} and k'_n are the capacity factors of the same analyte on the imprinted and non-imprinted polymers, respectively (Cheong *et al.*, 1997).

2.9 APPLICATIONS

A large number of molecules have been imprinted for various practical applications. Four main applications include: (1) Separation materials, (2) Antibody and receptor binding mimics in recognition and assay systems, (3) Catalytic applications as enzyme mimics, and (4) Recognition elements in biosensors.

2.9.1 Molecular Imprinted Separation and Screening

2.9.1.1 Solid phase extraction

In the last few years, solid phase extraction has become the most used sample preparation technique for trace analysis. Molecular imprinting solid phase extraction (MISPE) has been intensively developed in the last few years. The experimental set-up has varied from different modes of on line SPE (Sellergen, 1994), conventional SPE where the MIP is packed into columns or cartridges (Muldon and Stanker, 1997) to batch mode SPE where the MIP is incubated with the sample (Andersson *et al.*, 1997). The first application of MIP was its use as stationary phases in affinity chromatography, specifically for enantioseparation of racemic mixtures of chiral compounds. The advantages of MISPE-PE include easy operation, rapid analysis and low solvent consumption (Haupt, 2001). The other benefit of this technique is that the selectivity of the MIP can be pre-determined by the choice of template employed for its preparation, which combined with the high selectivity of the sorbent lead to efficient sample clean up (Andersson, 2000). MISPE has been widely used for the pre-concentration of template itself, where extraction of other related compounds is still under the development of its application (Xu *et al.*, 2004).

The various analytes extracted by MISPE include pentamidine, propanolol, tamoxifen, atrazine, dariifenancin, atenolol, bentazone, etc. (Andersson, 2000). The quantification of the herbicide atrazine in beef liver is a good demonstrative example of the utility of imprinted polymers in SPE. Initially, atrazine was extracted from liver tissue with chloroform. The imprinted polymer was then used to clean up the chloroform extract and to concentrate the analyte further prior to quantification. In this experiment, the binding capacity of the polymer for atrazine in chloroform was found to be 19μmol g^{-1}. The analyte was eluted from the polymer with a suitable solvent and quantified after drying and reconstitution in acetonitrile by RP-HPLC. The SPE step with the imprinted polymer considerably improved the accuracy and precision of the HPLC method and lowered the detection limit from 20 to 5

ppb (Muldon and Stanker, 1997). The molecularly imprinted polymer enabled the selective extraction of four organophosphorus pesticides successfully from water and soil samples. The recoveries of organophosphorus pesticides extracted from a 5-g soil sample at the 100µg/Kg spike level were in the range of 79.3-93.5%. The use of MISPE improved the accuracy and precision of the GC method and lowered the limit of detection (Zhu *et al.*, 2005).

2.9.1.2 Liquid chromatography

The use of molecular imprinted polymer, as stationary phase for chromatographic separations is by far the most extensively studied application of these polymers. In chromatographic methods, the selectivity is dependent on differences in interaction (specific and non-specific) between analytes and imprint. The selectivity is generally defined as 'α', where 'α' is the ratio of the capacity factors of two compounds. These are proportional to the interaction of the analytes with the stationary phase. Chromatographic systems using molecular imprints described by various authors show that extreme α-values can be obtained, but that hardly adequate resolutions were reported (Andersson and Mosbach, 1990). The particularity of the molecular imprinted polymers compared with conventional chiral stationary phases is that they are tailor made for a specific target molecule, hence their selectivity is pre determined. A very pronounced stereo selectivity has been observed with an molecular imprinted polymer specific for the cinchona alkaloids cinchonidine and cinchonine, resulting in chromatographic α values of upto 31 (Matsui et al., 1998). The objects analyzed in the liquid chromatography using molecular imprinted polymer include amino acids and peptides, nucleotide bases, drugs, sugars, steroids, etc. The asymmetric shape of the chromatographic peaks on molecular imprinted column due to the imprinted site heterogeneity is always one of the disadvantages of imprinted polymers and limits its application in chromatographic systems. The preparation of uniform sized particles for the polymers as the stationary phase will improve column efficiency remarkably. For separation process, it can be concluded that molecular imprints can add different types of selectivity. It should be noted that both the production

process of polymers and the selection of mobile phase or run buffers should be focused on improvement of mass transfer processes. Low affinity binding sites are essential. The selection of monomers and cross-linkers and the extent of cross-linking are important parameters. The selection of mobile phases or run buffers should be directed to a reduction of strength of interaction between functional groups of the analytes and of the polymer and not to the elimination of a particular type of interaction, that may lead to the situation where "recognition" by an imprinted polymer is comparable to the recognition by non-imprinted polymers.

2.9.1.3 Membrane separation

The relevance of membranes as a special format for molecularly imprinted polymer (MIPs) has received a lot of attention in recent years (Yoshikawa, 2002). Three main approaches can be used for the preparation of molecular imprinted membrane (MIM): (1) sequential approach—preparation of membranes from previously synthesized "conventional" MIPs, i.e. particles, (2) simultaneous formation of MIP structure and membrane morphology, (3) sequential approach—preparation of MIPs *on* or *in* support membranes with suited morphology.

A molecular imprinted membrane (MIM) is a membrane either composed of a MIP or containing a MIP (Barboiu *et al.*, 2000). A high membrane performance depends on well-defined membrane morphology with respect to barrier pore size and layer topology, especially the thickness of the barrier layer. Yoshikawa *et al.* (1995) had used polystyrene resins with peptide recognition groups, in a blend with a matrix polymer, for the MIM formation. In this process, the polymer solidification was achieved by solvent evaporation. Remarkably, the permeability was much higher for the MIM as compared with the blank membranes. Alternatively, Kobayashi *et al.* (1995) had used functional acrylate co-polymers for yielding asymmetric porous MIM. In that case, the polymer solidification was achieved by a precipitation induced via contact with a non-solvent.

The unique feature of MIM is the interplay of selective binding and trans-membrane transport of molecules, making them potentially superior to synthetic separation membranes already applied in various industries (Sergeyeva *et al.*, 1999). Receptor and transport properties of microporous MIM can be based on template-specific binding sites in trans-membrane pores, which serve as fixed carriers for "facilitated" transport. Furthermore, template binding in microporous MIM can lead to a "gate effect" which either increases or decreases membrane permeability. Alternatively, MIM can also function as adsorbers, leading to a retardation of template transport, followed by breakthrough once the binding capacity has been saturated. Finally, the development of nano-MIPs will facilitate other synergistic combinations with separation membranes for effective separations based on MIPs. The literature indicates that much further work will be necessary to really explore the potential of MIM (Ulbricht, 2004).

2.9.2 Antibody and Receptor Binding Mimics

Artificial antibodies and receptors prepared by molecular imprinting are attractive complements to their counterparts. Over the last few years, several studies have demonstrated that molecular imprinted polymers can serve as artificial versions of natural antibodies and can be used as recognition elements in immunoassays. Imprints against bronchodilator drug theophylline and the tranquilizer diazepam have shown specific recognition (Vlatakis *et al.*, 1993). The anti-theophylline imprints were able to completely distinguish between the imprinted species and the closely related substance caffeine, which only differs in single methyl group. The binding results showed excellent correlation between molecular sorbent assays and immuno-sorbent assays. For ligand-binding assays, it can be concluded that molecular imprints do have potential advantages and also the imprints can be made in a reproducible fashion. Compatibility with biological, aqueous samples is highly commendable. It is unclear whether or not ligand-binding assays based on imprints can compete with the sensitivity and selectivity of immuno- and receptor assays. More complex monomer and cross-linking mixtures and step-by-step polymerization procedures might attribute to obtain imprinted polymers with the desired binding characteristics (Turkewitsch *et al.*, 1998).

2.9.3 Catalysis and Artificial Enzymes

One of the most intriguing challenges for the use of molecular imprinted polymers is their use as enzyme mimics. But the studies initiated by various scientists have been shown that the molecular imprinted polymers can be made to achieve catalytic activity. To date, four different approaches have been pursued: (1) The use of co-enzyme analogues for providing a useful pre-determined catalytic mechanism, (2) The use of coordination compounds for mediation of catalytic reaction, (3) The use of transition state analogues in the imprinting protocol, (4) The use of designed 'bait and switch' strategies for the correct organization of catalytic groups in the sites. The transition state of carboxylic ester hydrolysis can be mimicked by phosphonate derivatives and this has been used for the preparation of molecular imprinted enzyme mimics (Robinson and Mosbach, 1989). Molecular imprints have been made against different enzymes like α-amylase (Silvestri *et al.*, 2004), glucose oxidase (Burow and Minoura, 1996), horse radish peroxidase (Piletsky *et al.*, 1995), lysozyme (Hirayama *et al.*, 2001) and trypsin (Vaidya *et al.*, 2001) . A polymer layer with a lysozyme recognition site on the silica-bead surface was prepared by Hirayama et al (2001). In this example, they polymerized acrylamide and acrylic acid/ acrylamide and N, N-dimethylaminopropylacrylamide with $(NH_4)_2S_2O_8$ in a phosphate buffer containing the lysozyme, surface modified silica beads and cross linkers. The binding results showed that modified silica beads in contrast to unmodified silica beads can selectively adsorb lysozymes. High selectivity was also demonstrated by tests with other protein solutions.

2.9.4 Biosensors

One of the most appealing applications developed in the area of molecular imprinting is the use of these imprinted polymers as recognition elements in biosensor like devices (Kriz *et al.*, 1997). A recognition element such as enzyme antibody or receptor is immobilized at an interface between the sensor and the analyte. A chemical signal results from the binding of analyte to the recognition element and is subsequently transmitted into electric signal that is amplified and monitored. Substitution of natural

recognition elements with molecular imprinted polymers has a number of potential advantages over those found with antibodies (Sellergen, 1997). Imprints are attached to a surface, forming a thin layer. Binding of analytes to the imprint will change a number of physicochemical properties of that layer, which can be monitored (Hedborg *et al.*, 1993). Only for the measurement of separate samples, indirect detection protocols are possible. Essentially it can be regarded as a competitive ligand binding assay in which a unique property, e.g., fluorescence of a labeled ligand, which also binds to the imprint, is monitored. This implies that a fixed concentration of a labeled ligand is added to a sample, which needs incubation with the sensor until equilibrium is reached. The binding molecule, the imprinted polymer, is re-used for many assays. These imprinted polymers are far more stable with the potential of performing in harsh environments and they have the potential to become highly resistant sensing element alternatives. Molecular imprinted polymer against an amino acid derivative L-phenylalanine anilide have been applied in a flow through column electrode to selectively distinguish between two enantiomers of the imprint molecule (Andersson *et al.*, 1990). In conclusion, due to their stability, molecular imprints can be readily used as the sensing surface of a chemical sensor. A thin layer with a high density of imprints is a pre-requisite for a sensitive sensor. The sensitivity is strongly dependent on the interaction of the analyte, and in some cases, the interaction of a labeled ligand with the imprint. In cases, where the detection principle is based on properties of the analyte itself, interaction of the analyte and other matrix components with non-imprinted parts of the polymer affects the sensor signal. Particularly, in cases, where the matrix composition is constant and the analyte is present in variable concentrations, sensors can have an application.

2.10 FOOD ANALYSIS

The detection of contaminants in foodstuffs needs fast analytical tools. Molecular imprinted polymer due to their high selectivity and durability can be employed for either extraction of the eligible group of analyte or for the actual separation of imprinted species from the rest of the compounds (Bruggemann

et al., 2000). It has been demonstrated that these imprinted polymers are applicable for their determination of food additives such as carbohydrates, peptides (Kempe and Mosbach, 1995) or flavour additives, galactose, fucose and mannose as well as glucose (Parmpi and Kofinas, 2004). Several molecules like amino acids and proteins (Shi *et al.*, 1999), ribonuclease A (Kempe *et al.*, 1995), transferrin (Glad *et al.*, 1985), etc. have been used as templates. Furthermore, vitamins and nucleotides can specifically be analyzed by utilizing the imprinted polymers. Another important application was imprinting of pesticides and herbicides and their detection in food and water with molecular imprinted polymers. The imprinted polymers have been applied for the determination of atrazine with sensors (Piletsky *et al.*, 1995) and assays or for investigating 2,4-dichlorophenoxyacetic acid contaminations. Furthermore, drugs (Ansell and Mosbach, 1998) like local anesthetics and in particular antibiotics have been employed as template molecules such as chloramphenicol and clenbuterol (Masci *et al.*, 2002). A molecular imprinted polymer was prepared and was evaluated as a selective solid phase extraction sorbent, coupled to Volta metric detection for the efficient pre-concentration and determination of sulfamethazine in milk (Guzman *et al.*, 2005).

Materials and Methods

3. MATERIALS AND METHODS

3.1 PREPARATION OF IMPRINTED POLYMER AGAINST TETRACYCLINE

3.1.1 Materials

Tetracycline hydrochloride, ethylene glycol dimethacrylate (EGDMA), methacrylic acid and benzoyl peroxide were purchased from M/s Sigma Aldrich, USA. Methanol and acetonitrile were of HPLC grade and purchased from SRL, Mumbai.

3.1.2 Method

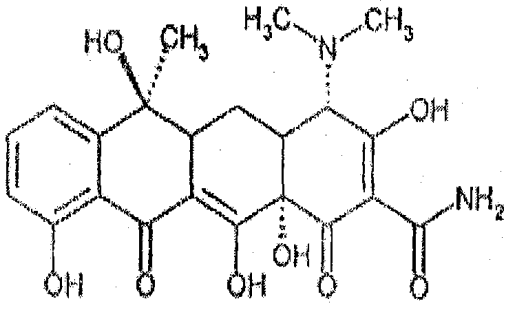
3.1.2.1 Molar ratio of different components

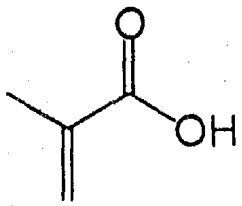
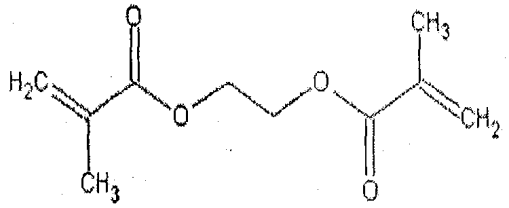
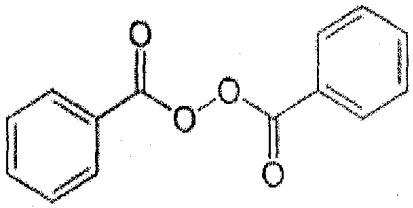
The molar ratio of imprint, monomer, cross-linker and initiator was essentially same as described by Cai and Gupta (2004). The molar ratio of these components used for preparation of polymer is given below :

Imprint : Monomer : Cross-linker : Benzoyl peroxide = 0.25 : 3 : 25 : 0.16

The structure and molecular weight of these components is given in Table 3.1.

Table 3.1 Different components used for preparation of imprinted polymer of tetracycline

Component	Specific Name of Component	Structure	Mol. Weight	Ratio
Imprint	Tetracycline		444	0.25

Monomer	Methacrylic acid		86	3.0
Cross-linker	EGDMA		198	25.0
Initiator	Benzoyl peroxide		242	0.16

3.1.2.2 Preparation of imprinted polymer

3.1.2.2.1 Polymerization reaction

The amount of tetracycline (imprint), methacrylic acid (monomer), ethylene glycol dimethacrylate (cross-linker) and benzoyl peroxide (initiator) was calculated according to molar ratio. Then 114 mg of tetracycline, 262 µl methacrylic acid and 4.76 ml ethylene glycol dimethacrylate were added to glass ampoule (20 ml). Then 7.5 ml methanol or acetonitrile was added. The contents were mixed and nitrogen was bubbled for 10 min. Then 40 mg benzoyl peroxide was added and glass ampoule was properly sealed. The glass ampoule containing the mixture was placed in a water bath maintained at 65°C for 24 h. Then the glass ampoule was broken and the resulted bulk rigid mass was grounded into fine powder.

3.1.2.2.2 Removal of tetracycline and unreacted components

The tetracycline (bound and free), unreacted methacrylic acid and ethylene glycol dimethacrylate were removed using a Soxhlet extraction apparatus. The fine powder was transferred to a thimble, which was then

placed in the Soxhlet extraction apparatus. The extraction was done using a solvent mixture of acetonitrile and methanol (50 : 50 v/v) for 48 h. After extraction, the polymer was transferred to a clean beaker and was again washed with a mixture of methanol and distilled water (50/50 v/v) until the washing solution became free from tetracycline. The removal of tetracycline in the washing solution was monitored by measuring tetracycline content at 280 nm. A standard curve for tetracycline is shown in Figure 3.1. The polymer was further washed with distilled water to remove methanol. The resulted powder was then air-dried. The quantity of polymer obtained was approximately 2.5 g.

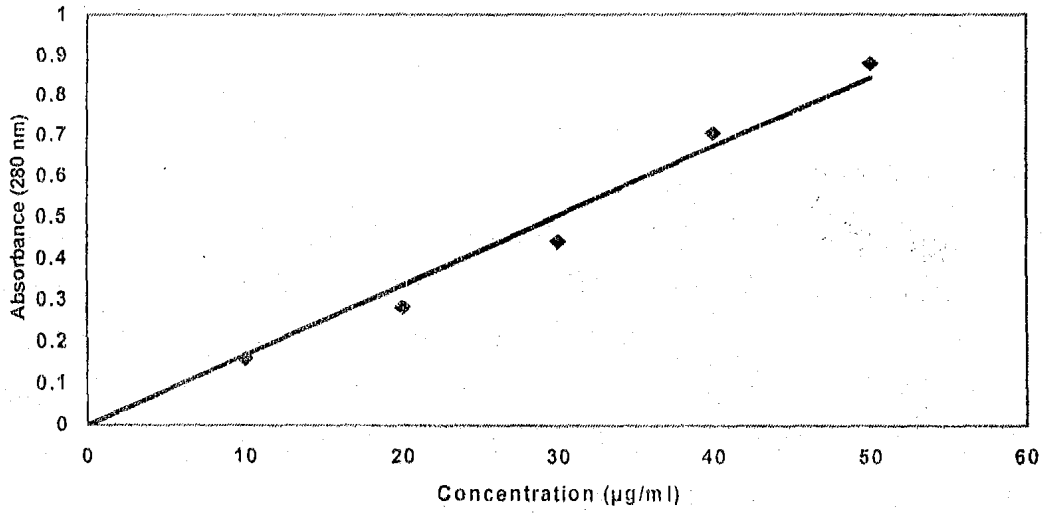


Fig. 3.1 Standard curve of Tetracycline

3.1.2.3 Preparation of non-imprinted polymer

Non-imprinted polymer was prepared similar to imprinted polymer except tetracycline (imprint) was omitted. Prepared polymer was similarly treated for removing unreacted components. The resulted powder was then air-dried.

3.1.3 Evaluation of Binding Characteristics

3.1.3.1 Partition coefficient

The 20 mg imprinted or non-imprinted polymer was taken in 6 ml glass tubes. The 4 ml of tetracycline solution (20 µg/ml distilled water) was added. The tubes were properly sealed and then placed in a vertical rotatory shaker.

The contents of tubes were end-to-end mixed gently in rotatory shaker at 30°C for 24 h. After incubation, supernatant was collected from the tubes and absorbance was recorded at 280 nm. The amount of tetracycline bound to polymer was calculated by subtracting the amount in the supernatant from the initially added amount. The partition coefficient was calculated (Cai and Gupta, 2004) as detailed below :

$$\text{Partition coefficient, } K = C_p / C_s$$

Where,

C_p = Concentration of tetracycline inside polymer, and

C_s = Concentration of tetracycline in supernatant.

3.1.3.2 Calculation of selectivity factor

The selectivity factor was calculated from partition coefficient values of tetracycline for polymer prepared in presence and absence of tetracycline.

$$\text{Selectivity factor, } S = K_{imp} / K_{non\text{-}imp}$$

Where,

K_{imp} = Partition coefficient of tetracycline imprinted polymer, and

$K_{non\text{-}imp}$ = Partition coefficient of non-imprinted polymer.

3.1.3.3 Calculation of binding efficiency

Binding efficiency in percent was calculated as detailed below :

$$\text{Binding efficiency, \%B} = \frac{\text{Bound tetracycline}}{\text{Total tetracycline}} \times 100$$

3.1.3.4 Effect of solvents on binding

The binding of tetracycline to imprinted or non-imprinted polymer was studied in different solvents, viz., water, 1 M NaCl, methanol and acetonitrile. The experiment was similarly conducted as explained in Section 3.1.3.1,

except different solvents used for dissolving tetracycline and for further incubation. Partition coefficient and selection factors were also similarly calculated.

3.1.3.5 Effect of pH on binding

Effect of pH on partition coefficient and selectivity factor was studied and for this purpose 0.02 M acetate buffer of pH values 4.0 or 5.0 and 0.02 M phosphate buffer of pH values 6.0 and 7.0 were used for solubilizing the tetracycline. Incubation of polymer with tetracycline was done in these different buffers. Incubation time, temperature and binding conditions were same as explained elsewhere. Partition coefficient and selectivity factor were then calculated.

3.1.3.6 Binding experiment at different temperatures

The binding experiment was done at two different temperatures, viz., 30 and 40°C as explained in Section 3.1.3.1. The partition coefficient and binding activity of molecular imprinted and non-imprinted polymers were calculated. The selectivity factor was calculated from partition coefficient.

3.1.3.7 Effect of tetracycline concentration

The effect of tetracycline concentration (20, 40, 60, 80 and 100 µg/ml distilled water) on binding was studied by incubating varied concentrations of tetracycline to a fixed quantity of matrix. The 20 mg of both imprinted and non-imprinted polymers were taken in 6 ml clean glass tubes. The 3 ml of tetracycline solution was added to the polymer. The tubes were further kept for incubation at 30°C for 24 h in a rotary shaker. After incubation, the supernatant was collected and tetracycline content was estimated. The tetracycline bound to the polymer was calculated by subtracting the amount in supernatant from the initial amount of added tetracycline. The partition coefficient and selectivity factor were then calculated.

3.1.3.8 Effect of amount of polymer matrix

Binding experiment was done by using different weights (10, 20, 30 and 40 mg) of both imprinted and non-imprinted polymer. The concentration of tetracycline was kept constant. The 4 ml of tetracycline solution (20 µg/ml distilled water) was added to tube containing polymer. The mixture was kept for incubation at 30°C for 24 h. After incubation, the supernatant was collected from each tubes and tetracycline content was estimated. The partition coefficient and selectivity factor were then calculated.

3.1.3.9 Binding on chromatographic column

3.1.3.9.1 Method for evaluating binding and elution of pure tetracycline solution

Polymer (imprinted or non-imprinted) was washed with distilled water and packed into chromatographic glass column (size 1 x 2 cm). The column was equilibrated with distilled water. One ml of tetracycline (0.1 mg/ml) was loaded to the column. The column was washed with 15 ml water. Subsequently, a linear gradient of water and acetonitrile mixture (water 30 ml; acetonitrile 30 ml) was applied. Fractions of 3 ml each at a flow rate of 12 ml/h were collected. Absorbance of the fractions was determined spectrophotometrically (Specord 200) at 280 nm. The experiment was repeated with non-imprinted polymer under same conditions.

3.1.3.9.2 Method for evaluating binding and elution of tetracycline present in milk

4.7 ml cow milk was spiked with 2 ml tetracycline solution (1 mg/ ml). 13.3 ml of trichloro acetic acid (15%) was then added. The contents were mixed and the solution was allowed to stand for ten minutes at room temperature (18°C). The contents were filtered through Whatman No.1 filter paper. 1 ml of the filtrate was loaded to chromatographic column (1 x 2 cm) packed with imprinted polymer. Column was then washed with distilled water. The bound material was then eluted by application of pure acetonitrile

fractions of 3 ml each were collected at a flow rate of 12 ml/h. The absorbance was recorded at 280nm. The milk without added tetracycline was similarly treated and evaluated.

3.1.3.10 Determination of cross reactivity of imprinted polymer

Tetracycline imprinted polymer was checked for its reactivity with ciprofloxacin and amoxicillin. For this purpose, the prepared polymer was packed in column (1 x 2 cm). The binding and elution of these antibiotics were similarly studied as explained in Section 3.1.3.9. The concentrations of ciprofloxacin and amoxicillin in fractions were monitored by measuring absorbance at 280 nm and 220 nm, respectively.

3.1.3.11 Storage Stability of Tetracycline Imprinted Polymer

Tetracycline imprinted polymer was stored at room temperature (22-35°C) up to 9 months. A fixed quantity of polymer was withdrawn at different time intervals (3, 5, 7 and 9 months). The binding capacity of polymer was determined under similar conditions as explained in Section 3.1.3.1. For comparative purpose, storage stability of non-imprinted polymer was also checked. The percentage binding, partition coefficient and selectivity factor were calculated.

3.2 PREPARATION OF IMPRINTED POLYMER AGAINST LACTIC ACID

3.2.1 Materials

Lithium lactate, polyallylamine hydrochloride (PAA.HCl) and epichlorohydrin (EPI) were purchased from M/s Sigma Aldrich, USA.

3.2.2 Method

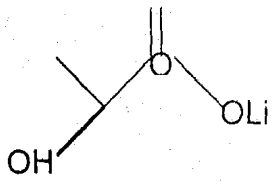
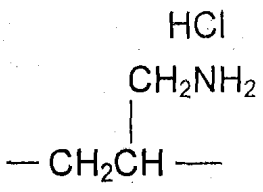
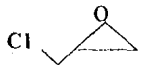
3.2.2.1 Molar ratio of different components

The molar ratios of imprint, polymer, cross-linker and neutralizer were essentially same as described by Parmpi and Kofinas (2004). The molar ratio of these components used for preparation of polymer is given below :

Imprint : Monomer : Cross-linker : Neutralizer = 0.143 : 10 : 1 : 5

Structure and molecular weight of components are given in Table 3.2.

Table 3.2 Different components used for preparation of imprinted polymer of lithium lactate

Component	Specific Name of Component	Structure	Molecular Weight	Ratio
Imprint	Lithium lactate		96	0.143
Polymer	Poly (allylamine hydrochloride)		~ 15,000	10
Cross-linker	Epichlorohydrin		92	1
Neutralizer	Sodium hydroxide	NaOH	42	5

3.2.2.2 Preparation

The amounts of lithium lactate (imprint), polyallylamine hydrochloride (polymer), epichlorohydrin (cross-linker) and NaOH (neutralizer) were calculated according to molar ratio. The 2.5 g PAA.HCl was dissolved in 8 ml distilled water and then 70 mg lithium lactate was added. The whole mixture was stirred for 2 h. Then, 2.2 ml of 10 M NaOH solution was added with constant stirring. The stirring was continued for next 10 min and then 460 µl of epichlorohydrin was added. Thereafter, the contents were vortexed and monitored for start of gelation. At the start of gelation, the entire mixture was poured down to a petri dish. The polymer was kept for incubation at room temperature for 24 h. The polymer was then cut into nearly 4 mm squares and

washed with 4 M aqueous NaOH solution for 48 h to remove the lactate and any other unreacted reagents. The washing solution was checked for lactate content by the method approved by BIS (1984). Then, the polymer was washed with distilled water until the traces of NaOH were removed. This was ensured by checking the pH of the washing solution. Gels were finally dried under air. Preparation without template (lithium lactate) was also prepared. Figure 3.2 explains the synthesis of poly (allylamine) polymer by cross-linking with epichlorohydrin.

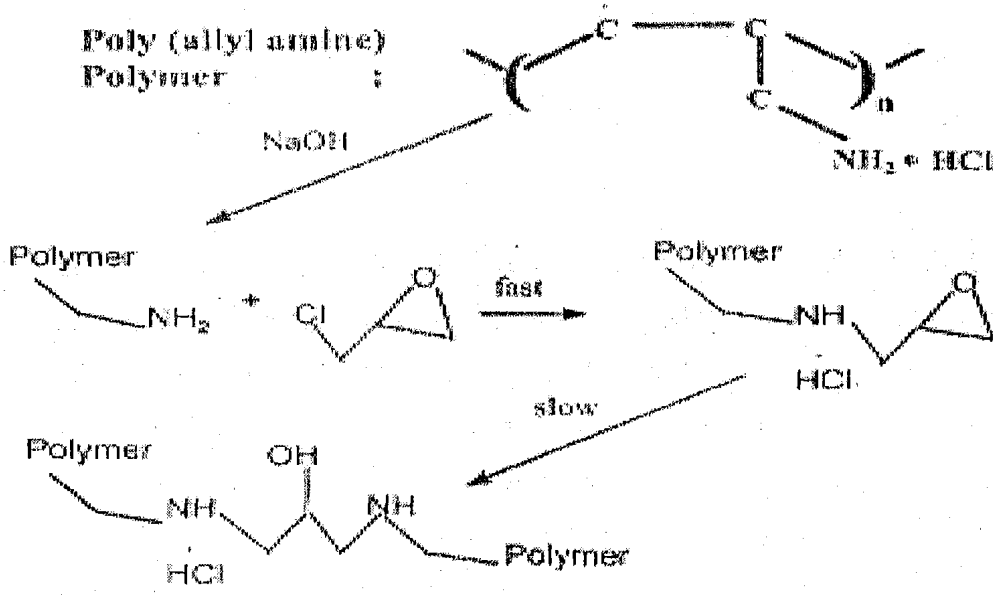


Fig. 3.2 Hydrogel synthesis by cross-linking with EPI

3.2.3 Determination of Lactate Content by BIS Method

Reagents :

(i) Copper (II) sulphate solution

The 250 g of copper (II) sulphate ($CuSO_4.5H_2O$) was dissolved in distilled water and diluted to 1000 ml in a volumetric flask.

(ii) Calcium hydroxide suspension

The 300 g of calcium hydroxide ($Ca(OH)_2$) was mixed properly with a total of 900 ml distilled water. The suspension was kept in a tightly stoppered bottle.

(iii) Copper (II) sulphate – sulphuric solution

The 0.5 ml of copper (II) sulphate solution was added to 300 ml of concentrated sulphuric acid.

(iv) p-Hydroxydiphenyl ($C_6H_5C_6H_4OH$) solution

The 0.75 g of p-hydroxydiphenyl was dissolved in 5 ml of an aqueous 5 percent solution of sodium hydroxide with slight shaking and heating, and diluted to 50 ml with water in a volumetric flask. The solution was stored in a brown glass bottle in a dark and cool place.

(v) Lithium lactate ($CH_3CHOHCOOLi$) standard solution (0.1 mg of lactic acid per millilitre)

The 10.67 g of lithium lactate was dissolved in distilled water and diluted to 100 ml in a volumetric flask.

Method :

One ml lithium lactate solution containing 2 to 10 µg lithium lactate was pipetted into a 50 ml calibrated glass tube and was diluted to about 35 ml with water. Then 5 ml of the copper (II) sulphate solution was added and was allowed to stand for 10 min. Then 5 ml of calcium hydroxide solution was added. After 10 min, volume was made up to 50 ml with distilled water. After 10 min, the contents were filtered using Whatman No.1 filter paper. One ml of the filtrate and 6.0 ml of sulphuric copper (II) sulphate solution were mixed. The resultant solution was boiled for 5 min and cooled to ambient temperature. Cooling of solution (containing boiled water) was achieved by placing test tube in running tap water. The 40 µl p-hydroxydiphenyl was added to the test tube and vigorous shaking was done for the uniform mixing. The test tubes were placed in a water bath at 30°C for 15 min. Then the test tubes were placed in a boiling water bath for 90 sec. Further, the tubes were cooled to ambient temperature in running water and the absorbance was measured at 570 nm. The standard curve for lithium lactate is given in Figure 3.3.

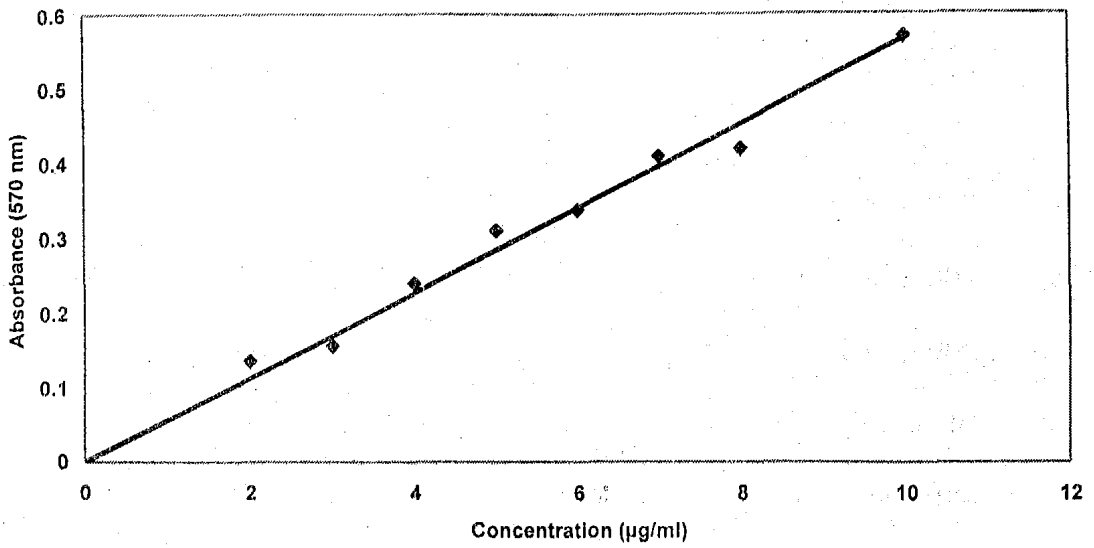


Fig. 3.3 Standard curve of lithium lactate

3.2.4 Evaluation of Binding Characteristics

3.2.4.1 Percent binding efficiency

Imprinted and non-imprinted polymers were evaluated for their binding to lactate. The binding experiments were done in distilled water and buffered media. The buffered media used for binding was 10 mM BES (N, N-bis [2-Hydroxyethyl]-2-aminoethanesulfonic acid) containing 160 mM NaCl (pH 7.0). The 1.0 g polymer was added to a clean 50 ml beaker. Then, 8.0 ml of buffered or aqueous lactate solution (1 mg/ml) was added and beaker was covered with aluminium foil. The incubation was done at room temperature (22-35°C) for 18 h. After incubation, 1 ml supernatant was collected and lactate content in the supernatant was estimated by BIS (1984) method. Percent binding efficiency was calculated by using the following formula :

$$\%BE = \frac{(L_i - L_s)}{L_i} \times 100$$

Where,

L_i = Amount of lactate initially added, and

L_s = Amount of lactate in supernatant.

Then, partition coefficient and selectivity factor were calculated.

3.2.4.2 Effect of pH on binding

Effect of pH on binding of lithium lactate to polymer was studied by conducting the binding experiment in buffers of different pH as given below :

(i) 200 mM acetate buffer of pH 4.0

(ii) 200 mM acetate buffer of pH 5.0

(iii) 200 mM acetate buffer of pH 5.6

(iii) BES buffer of pH 7.0

Measurement of bound lactate after incubation of polymer with lactate was done similarly as explained in Section 3.2.4.1.

3.2.4.3 Binding of L- and D- forms of lactic acid

The prepared polymer was evaluated for its binding of L(+) lactic acid and D(-) lactic acid. The binding of these two stereo isomers of lactic acid was done in distilled water and was explained in Section 3.2.4.1.

3.2.4.4 Binding on chromatographic column

Polymer (imprinted or non-imprinted) was washed with distilled water and packed into chromatographic glass column (size, 1 x 2 cm). The column was equilibrated with distilled water. One ml of aqueous lithium lactate solution (0.5 mg/ml) loaded to the column. The column was washed with 7-8 ml water. Subsequently, 6 ml of 1 M NaCl was applied. Fractions of 1 ml each at a flow rate 12 ml/h were collected. Lactate in each fractions was measured and method of measurement was similar as explained in Section 3.2.3.

3.2.4.5 Binding experiment in milk

Cow milk was collected from Cattle Yard of National Dairy Research Institute, Karnal. Dilution of milk was carried out by distilled water in the ratio of 1 : 5 and 1 : 10 (milk : water). Lithium lactate was added to the diluted samples to get a final concentration of 500 µg/ml. The 0.7 g of imprinted or

non-imprinted polymer was taken in a 25 ml glass tubes. Four ml of lactate solution (500 µg/ml) was added. The tubes were properly sealed and then placed in a rotatory shaker. The contents of tubes were end-to-end mixed gently in rotatory shaker at room temperature (26°C) for 4 h. After incubation, one ml of supernatant was collected and the lactate content was measured. Percent binding efficiency was calculated.

3.2.4.6 Storage stability of lactate imprinted polymer

Lithium lactate imprinted polymer was stored at room temperature (22-35°C) up to 9 months. A fixed quantity of polymer was withdrawn at different time intervals (3, 5, 7 and 9 months) and binding of lactate was measured. The binding experiment was done in distilled water under similar conditions as explained in Section 3.2.4.1.

3.3 PREPARATION OF IMPRINTED POLYMER AGAINST VITAMIN A

3.3.1 Materials

Retinyl acetate, ethylene glycol dimethacrylate, methacrylic acid and benzoyl peroxide were purchased from M/s Sigma Aldrich, USA. Methanol used was of HPLC grade and was purchased from SRL Mumbai.

3.3.2 Method

3.3.2.1 Molar ratio of different components

The molar ratio of imprint, monomer, cross-linker and initiator was essentially same as described by Cai and Gupta (2004) as detailed below :

Imprint : Monomer : Cross-linker : Initiator = 0.25 : 3 : 25 : 0.16

The imprint molecule was vitamin A (Retinyl acetate, molecular weight = 328) whose structure is given below (Fig. 3.4). The monomer, cross-linker and initiator were methacrylic acid, ethylene glycol dimethacrylate, benzoyl peroxide, respectively.

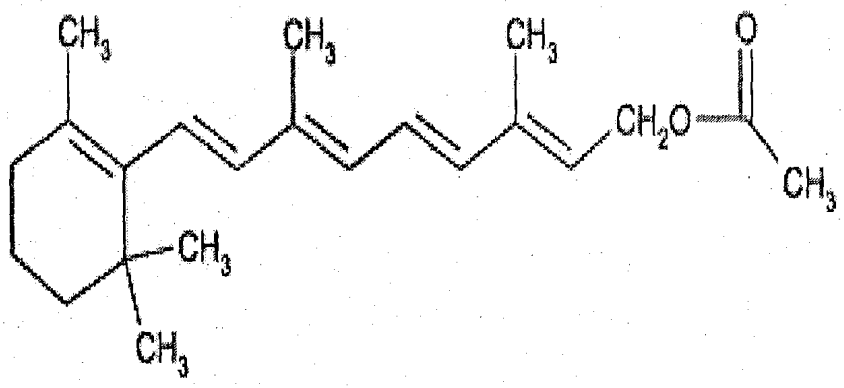


Fig. 3.4 Structure of Retinyl acetate

3.3.2.2 Preparation of imprinted polymer

3.3.2.2.1 Polymerization reaction

The amount of retinyl acetate, methacrylic acid, ethylene glycol dimethacrylate and benzoyl peroxide was calculated according to molar ratio. The 114 mg of retinyl acetate, 262 µl methacrylic acid and 4.76 ml ethylene glycol dimethacrylate were added to glass ampoule (20 ml). Then, 7.5 ml acetonitrile was added. The contents were mixed and nitrogen was bubbled for 10 min. Then 40 mg benzoyl peroxide was added and glass ampoule was properly sealed. The glass ampoule containing the mixture was placed in a water bath maintained at 65°C for 24 h. Then the glass ampoule was broken and the resulted bulk rigid mass was grounded into fine powder.

3.3.2.2.2 Removal of retinyl acetate and unreacted components

The retinyl acetate (bound and free), unreacted methacrylic acid and ethylene glycol dimethacrylate were extracted using a Soxhlet extraction apparatus. The extraction procedure was same as that described for tetracycline. The extraction was done using a solvent mixture of acetonitrile and methanol (50:50 v/v) for 48 h. After extraction, the polymer was transferred to a clean beaker and was again washed with a mixture of acetonitrile and distilled water (50/50) until the washing solution became free from retinyl acetate. The removal of retinyl acetate in the washing solution was monitored by measuring retinyl acetate content at 325 nm. Thus obtained polymer was further washed with distilled water in order to remove the traces

of acetonitrile. The resulted polymer particles were then air-dried. The standard curve of retinyl acetate A is given in Figure 3.5.

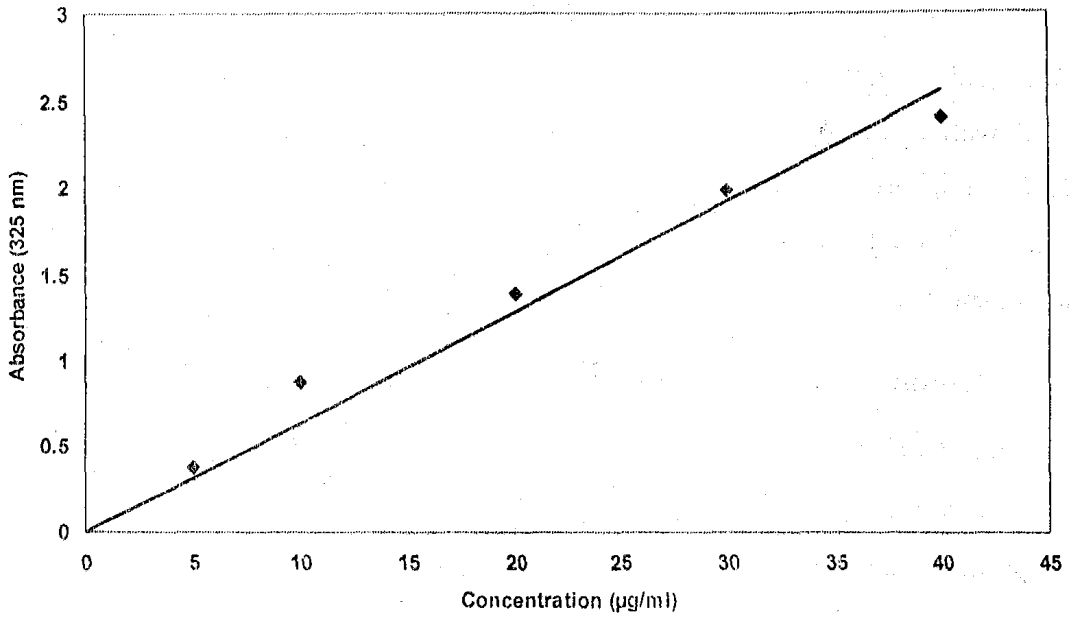


Fig. 3.5 Standard curve of retinyl acetate

3.3.2.3 Preparation of non-imprinted polymer

Non-imprinted polymer was prepared similar to imprinted polymer except retinyl acetate (imprint) was omitted. Prepared polymer was similarly treated for removing unreacted components. The resulted powder was then air-dried.

3.3.3 Evaluation of Binding Characteristics

3.3.3.1 Calculation of partition coefficient

Twenty mg of polymer (imprinted or non-imprinted) was taken in a 6 ml clean glass test tubes. Four ml retinyl acetate (20 µg/ml, 1:1 mixture of acetonitrile and water) was added. The glass test tubes were then placed in a rotatory shaker and contents were end-to-end mixed at 30°C for 24 h. After incubation, 3 ml of supernatant was collected from the tubes and absorbance was measured at 325 nm. The amount of retinyl acetate bound to polymer was calculated by subtracting the amount in the supernatant from the initial amount. Partition coefficient, percent binding and selectivity factor were then calculated and the method used for these calculations were essentially same as described for tetracycline imprinted polymer.

3.3.3.2 Effect of solvents on binding

The binding of retinyl acetate to imprinted and non-imprinted polymer was studied in methanol, acetonitrile and water. The mixtures of methanol and water, and acetonitrile and water were also used. For this purpose, water was mixed with methanol or acetonitrile in different ratios. Retinyl acetate was dissolved in these solvents. The binding was measured as explained in Section 3.3.3.1. Partition coefficient and selectivity factor were then calculated.

3.3.3.3 Binding on chromatographic column

3.3.3.3.1 Method

Molecular imprinted or non-imprinted polymer was washed with distilled water and packed into chromatographic columns (1 x 2 cm). The column was equilibrated with 2:1 mixture of water and acetonitrile. One ml of the retinyl acetate (0.1 mg/ml) dissolved in 2:1 mixture of water and acetonitrile was loaded to the column. The column was washed with about 21 ml of 2:1 mixture of water and acetonitrile. Subsequently, linear gradient of water-acetonitrile mixture [water and acetonitrile (2:1), 30 ml; water and acetonitrile (1:2), 30 ml] was applied. Fractions of 3 ml each at a flow rate 12 ml/h were collected. The concentration of retinyl acetate in each fraction was measured by recording absorbance at 325 nm.

3.3.3.4 Cross reactivity of imprinted polymer

The binding of two antibiotics: i) Tetracycline and ii) Ciprofloxacin was checked against imprinted polymer of vitamin A in chromatographic columns (1 x 2 cm). The binding conditions applied in this case were similar to that used for tetracycline binding in glass column. The absorbance of the fractions was monitored at 280 nm.

3.3.3.5 Storage stability of vitamin A imprinted polymer

Retinyl acetate imprinted polymer was stored at room temperature (22-35°C) up to 7 months. A fixed quantity of polymer was withdrawn at different time intervals (3, 5 and 7 months) and binding of retinyl acetate was

measured. The binding conditions were similar as that explained in Section 3.3.3.1. The percentage binding, partition coefficient and selectivity factor were calculated.

3.4 PREPARATION OF IMPRINTED POLYMER AGAINST CIPROFLOXACIN

3.4.1 Materials

Ciprofloxacin hydrochloride was purchased from CDH, New Delhi. Ethylene glycol dimethacrylate (EGDMA), methacrylic acid, methyl methacrylate, 4-vinyl pyridine, poly-allylamine hydrochloride, epichlorohydrin and benzoyl peroxide were purchased from M/s Sigma Aldrich, USA. Methanol, dimethyl sulfoxide and acetonitrile were of HPLC grade and purchased from SRL, Mumbai.

3.4.2 Acrylate Based Polymer Preparation

3.4.2.1 Molar ratio of different components

The molar ratio of imprint, monomer, cross-linker and initiator was essentially same as described by Cai and Gupta (2004). The molar ratio of these components used for preparation of polymer is given below :

Imprint : Monomer : Cross-linker : Benzoyl peroxide = 0.25 : 3 : 25 : 0.16

The imprint molecule was ciprofloxacin hydrochloride (molecular weight = 385), whose structure is given below (Fig. 3.6). The monomers selected were 4-vinyl pyridine, methyl methacrylate and methacrylic acid. Cross linker and initiator were EGDMA and benzoyl peroxide, respectively.

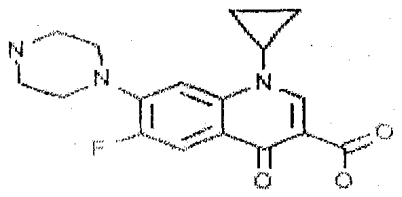


Fig. 3.6 Structure of ciprofloxacin

3.4.2.2 Preparation of imprinted polymer

The method of preparation of ciprofloxacin imprinted polymer using methacrylic acid, 4-vinyl pyridine and methyl methacrylate was similar as that described for imprinted polymer of tetracycline. The solvents used were methanol / acetonitrile / dimethyl sulfoxide. A non-imprinted polymer in absence of ciprofloxacin was also prepared in all cases.

3.4.2.3 Standard curve of ciprofloxacin

Standard curve of ciprofloxacin was prepared by measuring absorbance of ciprofloxacin solution at 280 nm at different concentrations of ciprofloxacin (Fig. 3.7).

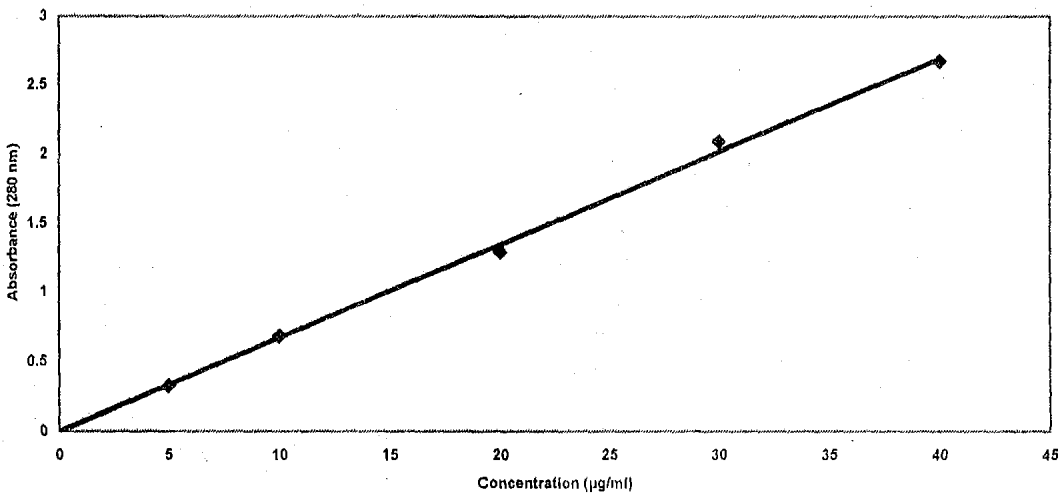


Fig. 3.7 Standard curve of ciplofloxacin

3.4.3 Poly-allylamine Based Polymer Preparation

3.4.3.1 Molar ratio of different components

The molar ratios of imprint, polymer, cross-linker and neutralizer were essentially same as described by Parmpi and Kofinas (2004). The molar ratio of these components used for preparation of polymer is given below :

Imprint : Monomer : Cross-linker : Neutralizer = 0.143 : 10 : 1 : 5

3.4.3.2 Preparation of imprinted polymer

The preparation of imprinted polymer using poly-allylamine hydrochloride was as described for the lithium lactate imprinted polymer.

3.4.4 Evaluation of Binding Characteristics

The binding studies were carried out in different solvents, viz., distilled water, methanol, 1 M NaCl, acetonitrile and dimethyl sulfoxide and also different combinations of methanol and acetonitrile with water. For acrylate based polymers, the binding procedure and conditions applied were similar to that explained in Section 3.1.3.1 and the binding was studied in water for poly-allylamine based polymer as that explained in section 3.2.4.1.

3.5 PREPARATION OF IMPRINTED POLYMER AGAINST ASCORBIC ACID

3.5.1 Materials

Ascorbic acid, ethylene glycol dimethacrylate, methacrylic acid and benzoyl peroxide were purchased from M/s Sigma Aldrich, USA. Methanol used was of HPLC grade and was purchased from SRL Mumbai.

3.5.2 Method

3.5.2.1. Molar ratio of different components

The molar ratio of imprint, monomer, cross-linker and initiator was essentially same as described by Cai and Gupta (2004) as detailed below :

Imprint : Monomer : Cross-linker : Initiator = 0.25 : 3 : 25 : 0.16

The imprint molecule was ascorbic acid (molecular weight = 176) whose structure is given below (Fig. 3.8). The monomer, cross-linker and initiator were methacrylic acid, ethylene glycol dimethacrylate and benzoyl peroxide respectively.

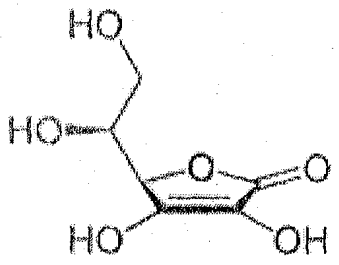


Fig. 3.8 Structure of ascorbic acid

3.5.2.2. Preparation of imprinted polymer

The method of preparation of ascorbic acid imprinted polymer was similar as that described for imprinted polymer of tetracycline. Methanol was used as the solvent.

3.6 PREPARATION OF IMPRINTED POLYMER AGAINST LYSOZYME

3.6.1 Materials

Lysozyme, polyallylamine hydrochloride (PAA.HCl), *Micrococcus lysodeikticus* and epichlorohydrin (EPI) were purchased from M/s Sigma Aldrich, USA.

3.6.2 Method

1.5 g of polyallylamine hydrochloride was dissolved in 8 ml distilled water and was mixed with 300 mg of lysozyme. Then 2.0 ml 10M NaOH was gradually added with continuous stirring. After 10 minutes of stirring, 460 µl epichlorohydrin was added. Removal of template and further steps in preparation were same as described for lactate-imprinted polymer preparation.

3.6.3 Evaluation of Binding Characteristics

3.6.3.1 Method

One g of imprinted or non-imprinted polymer was taken in a clean 50 ml beaker. The 4 ml of lysozyme solution (0.2 mg/ml distilled water or other solvent) was then added. The contents were incubated at room temperature (22°C) for 2 h. After incubation, lysozyme activity from 50 µl supernatant was determined by measuring change in absorbance at 450 nm (Selested and Martinez, 1980).

3.6.3.1.1 Lysozyme assay

Reagents :

(i) Potassium phosphate buffer (0.05 M, pH 7.4)

(ii) Substrate : A suspension of killed *Micrococcus lysodeikticus* (35 mg/100 ml) prepared in 0.05 M potassium phosphate buffer (pH 7.4)

(iii) 1% BSA in 50 mM phosphate buffer (pH 7.4)

(v) Stock egg white lysozyme (1 mg/ml water)

Procedure :

Reaction mixture contained 2.1 ml substrate, 0.3 ml BSA, 0.55 ml phosphate buffer (pH 7.4) and 50 µl lysozyme. Before addition of enzyme, absorbance at 450 nm was recorded. Just after addition of lysozyme, absorbance at 450 nm at regular intervals was measured.

3.7 MULTIPURPOSE DEVICE FOR PROTEIN CONCENTRATION, DIALYSIS AND BUFFER EXCHANGE

A multipurpose device for protein concentration, dialysis and buffer exchange was developed. This device was designed with the purpose of removing salts from the lysozyme solution. A patent for the multipurpose device has been filed. The said device can be used for protein concentration and buffer exchange as well.

3.7.1 Construction of Multipurpose Device

A multipurpose device was designed and constructed from commercially available 1.5 ml microcentrifuge tube. By utilizing the recess inside the lid and a dialysis membrane, a chamber for protein concentration, dialysis and buffer exchange was constructed. The details of construction of this device and its utilization are presented in results (section 4.7).

3.7.2 Efficiency

The efficiency of the multipurpose device for protein concentration was checked by placing 20% or 40% or 60% or 80% or 100% glycerol in holed tube. After different period of rotation (15, 30 and 60 min) sample was recovered and its volume and amount of protein was measured (Bradford, 1976). The efficiency of device was also checked with other water binding material, Sephadex G-100. The efficiency of device for dialysis was checked by placing 200 µl of potassium dichromate solution in recessed face of lid and 1.5 ml distilled water to holed tube. Absorbance of solution was measured after 20 times dilution with water at different time.

Results and Discussion

4. RESULTS AND DISCUSSION

The recognition of antigen by antibody molecule is through non-covalent interactions. These interactions are largely ionic interactions, hydrogen bonds and Van der Walls interactions. Although these interactions became known few decades ago, exploitations of these interactions for making an affinity molecule have come to notice recently. The molecular imprinted polymers are capable of selectively interacting with the template. With the development in polymer chemistry, it has now become possible to prepare molecular imprinted polymers from commercially available monomers. These monomer molecules have groups through which non-covalent interactions with the template are possible. The monomers can be selected on the basis of structure of the template. Acrylate based monomers are preferred, because these can undergo free radical reactions and result in quick formation of polymer. Among the acrylates, methacrylic acid is widely used. Similarly, cross-linker, ethylene glycol dimethacrylate is preferred in the preparation of imprinted polymers. In all these preparations, relatively large ratio of cross-linker over monomers is used in order to provide a close interaction between template and the polymer. The preparation of imprinted polymer against different templates is of interest in different laboratories for different applications. In general, standard protocol is used for preparation of imprinted polymers. The prepared polymers are then evaluated extensively for ascertaining affinity for the template.

In present work, efforts were made to synthesize polymers having imprints against tetracycline, ciprofloxacin, lactic acid, vitamin C, vitamin A and lysozyme. These molecules have been selected as either as model molecules or with possible implications in quality related aspects of milk and milk products. For example, tetracycline and ciprofloxacin are antibiotics and could be present in milk and thus can affect milk quality. Lactic acid is also equally important molecule connected to milk quality since its level in milk is

related to hygienic status of milk. Acrylate based imprinted polymers were prepared against tetracycline, ciprofloxacin, vitamin C and vitamin A. There are few reports where imprinted polymers are prepared using materials other than acrylates. One such example is hydrogels, which are prepared by cross-linking a pre-formed polymer in presence of template. Such polymers are prepared against lactic acid and lysozyme. The work reported in this thesis pertains to preparation of acrylate based polymers and hydrogels having affinity against templates. Prepared polymers were further characterized for their specificity.

4.1 RESULTS

4.1.1 Acrylate Based Imprinted Polymers

4.1.1.1 Preparation of imprinted polymer against tetracycline

The imprinted polymer against tetracycline was prepared using methacrylic acid (monomer), ethylene glycol dimethacrylate (cross-linker), benzoyl peroxide (initiator) and in presence of tetracycline (template). The ratio of different components was essentially same as described by Cai and Gupta (2004), where large ratio of cross-linker over monomer was used. The polymerisation reaction was carried out in acetonitrile. The polymer was also prepared in absence of tetracycline. The polymerising mix, which was yellow in colour (due to the presence of tetracycline) turned light brown colour as the polymerisation reaction proceeded. This colour change was observed when tetracycline was present. When the preparation was treated with 50:50 mixtures of methanol and acetonitrile (v/v) using Soxhlet apparatus, the solvent turned yellow. This indicated that tetracycline was extracted from the polymer matrix. For effective extraction, fresh solvent (mixture of methanol and acetonitrile) was used in Soxhlet apparatus for every 12 h of 48 h long extraction. The intensity of the colour decreased with subsequent solvent change, indicating that the removal of tetracycline was better during the initial stage of extraction. At the end of 48 h extraction, the solvent was nearly colourless. The final preparation was hard and grainish in texture and was light brown in colour (Plate 4.1). It was noted that from 262 µl methacrylic acid and 4.76 ml of ethylene glycol dimethacrylate, approximately 2.5 g of polymer

matrix was obtained. The polymer prepared in absence of tetracycline (Plate 4.1) was white in colour and was of similar in appearance and hardness as that of polymer prepared in presence of tetracycline.

The selection of methacrylic acid as monomer and ethylene glycol dimethacrylate as cross-linker for preparation of molecular imprinted polymer against tetracycline is justified since methacrylic acid can interact with tetracycline through hydrogen bonds (Fig. 4.1) as well as through hydrophobic and Van der Walls interactions.

4.1.1.2 Effect of solvents on binding of tetracycline to imprinted polymer

Binding of tetracycline to prepared polymers was studied in presence of different solvents, viz., distilled water, 1 M NaCl, methanol and acetonitrile and results are presented in Figure 4.2 and Table 4.1. The binding efficiency of imprinted polymer was highest in water (86%), followed by 1 M NaCl (58%), methanol (45%), acetonitrile (40%). The binding efficiency of non-imprinted polymer in water, 1 M NaCl, methanol and acetonitrile was 26, 38, 22 and 15 percent, respectively. In all the four solvents, the binding of template to imprinted polymers was considerably high than in the non-imprinted polymers. In water, 3.45 mg of tetracycline could bind to 1 g of imprinted polymer. The binding in this range is considered high binding of template to the polymer (Cai and Gupta, 2004). The binding of tetracycline in methanol or acetonitrile to imprinted polymer was between 45 and 53 percent to that of binding in distilled water indicating relatively less binding in less polar solvents. The binding in 1 M NaCl to imprinted polymer was around 67 percent to that of binding in water. Tetracycline contains both hydrophobic and hydrophilic moieties. Thus, the repulsion forces between tetracycline and less polar solvent such as methanol and acetonitrile will be weak as compared between tetracycline and water. Therefore, tetracycline will prefer to remain with matrix in water as compared to less polar solvents. These results suggest that hydrophobic interactions are playing a major role in binding of tetracycline to the prepared polymers.

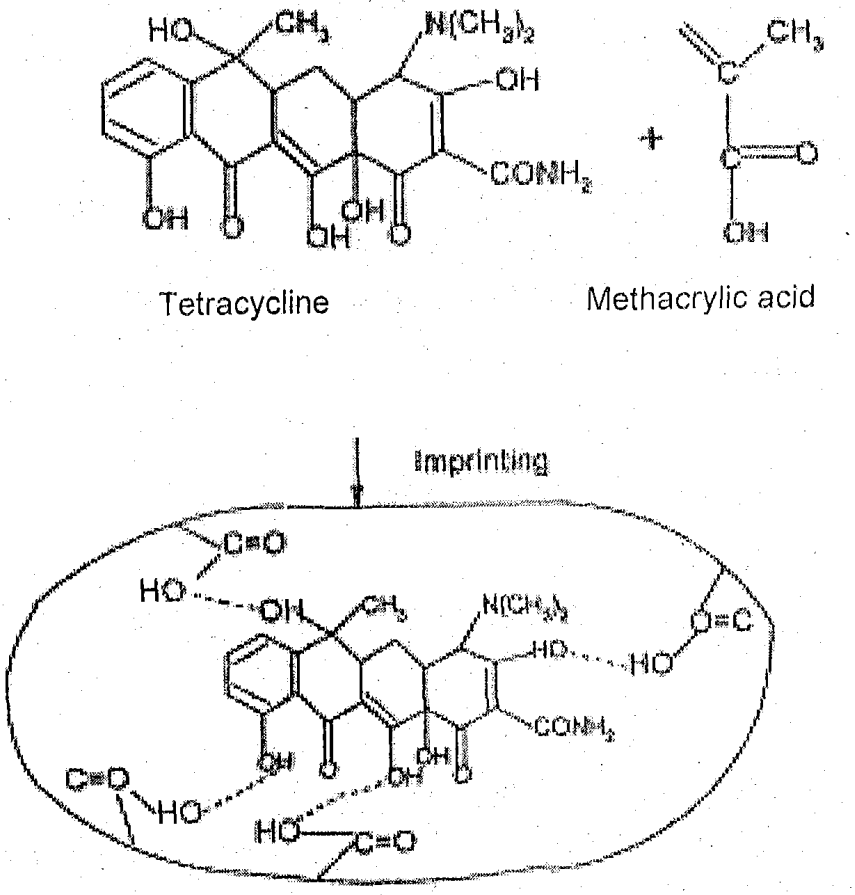


Fig. 4.1 Proposed model of hydrogen bonding between tetracycline and methacrylic acid

Table 4.1 Effect of solvents on percentage binding efficiency and capacity of imprinted and non-imprinted polymer of tetracycline

Solvent	Polymer	Percent Binding Efficiency (Mean ± SD*)	Binding Capacity (mg tetracycline/ g polymer)
Water	Imprinted	86 ± 3.0	3.45
	Non-imprinted	26 ± 2.6	1.04
1 M NaCl	Imprinted	58 ± 4.0	2.32
	Non-imprinted	38 ± 3.0	1.52
Methanol	Imprinted	45 ± 4.0	1.80
	Non-imprinted	22 ± 2.2	0.88
Acetonitrile	Imprinted	40 ± 2.0	1.60
	Non-imprinted	15 ± 3.0	0.60

* Mean of seven samples.

Table 4.2 Effect of solvents on partition coefficient of imprinted and non-imprinted polymer

Solvent	Polymer	Partition Coefficient	Selectivity
Water	Imprinted	94	3.9
	Non-imprinted	24	
1 M NaCl	Imprinted	63	1.8
	Non-imprinted	35	
Methanol	Imprinted	43	2.0
	Non-imprinted	22	
Acetonitrile	Imprinted	36	2.6
	Non-imprinted	14	

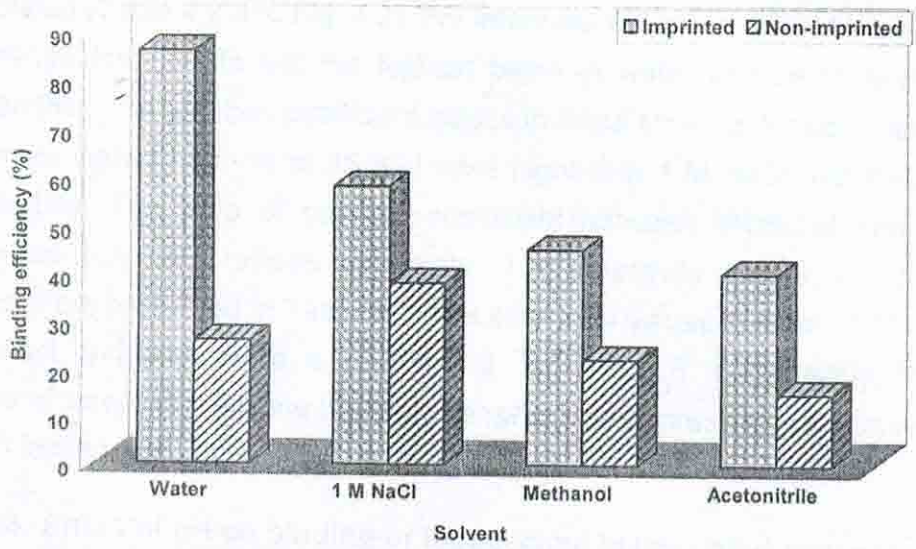


Fig. 4.2 Effect of solvent on binding efficiency of imprinted and non-imprinted polymer of tetracycline

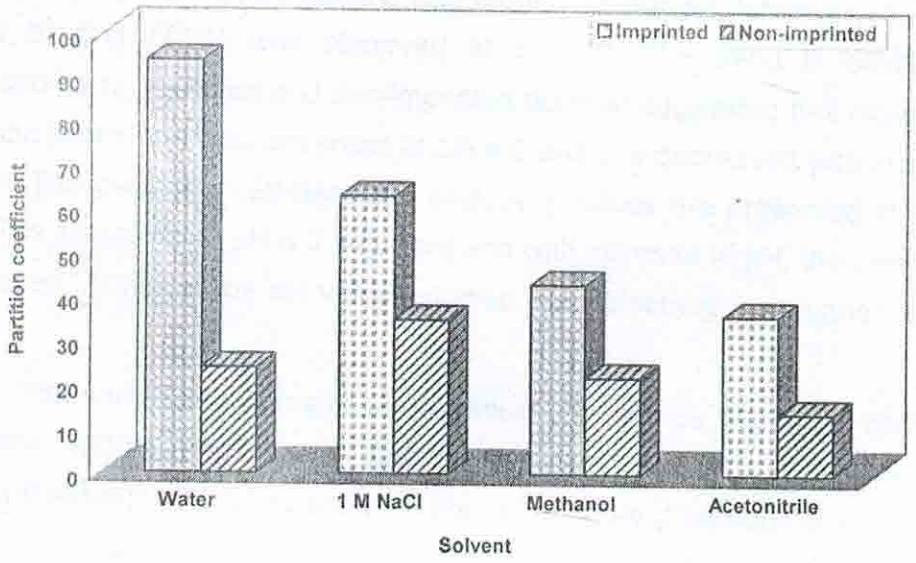


Fig. 4.3 Effect of solvent on partition coefficient of tetracycline imprinted and non-imprinted polymer

The partition coefficient values in different solvents have also been calculated (Table 4.2 and Fig. 4.3). For imprinted polymers, the values were in the range from 36 to 94; the highest being in water and being lowest in acetonitrile. The partition coefficient values in these solvents for non-imprinted polymers varied from 14 to 35 and were highest in 1 M NaCl and lowest in acetonitrile. The ratio of partition coefficient between imprinted and non-imprinted polymers reflects selectivity. The selectivity values in different solvents are presented in Table 4.2. The selectivity values in water, 1 M NaCl, methanol and acetonitrile were 3.9, 1.8, 2.0 and 2.6, respectively. These values of selectivity indicate that polymer prepared in presence of tetracycline has in fact imprints of tetracycline.

4.1.1.3 Effect of pH on binding of tetracycline to imprinted polymer

The binding efficiency of imprinted and non-imprinted polymers was evaluated at pH 4.0, 5.0, 6.0 and 7.0. The binding of tetracycline at different pH and selectivity are shown in Figure 4.4 and Table 4.3. The binding of tetracycline to the non-imprinted polymers decreased from 71 to 37 percent, when pH was raised from 4.0 to 7.0. This trend was not however noticed with imprinted polymer. At pH 4.0, 5.0 and 6.0, the binding of tetracycline to the imprinted polymer was similar and was around 70 percent. However, relatively more binding (77%) was observed at pH 7.0. The trend in binding of tetracycline to imprinted and non-imprinted polymer suggested that more non-specific interaction was observed at pH 4.0 and this decreased with increase in pH. The partition coefficient and selectivity values are presented in Table 4.3. The selectivity at pH 4.0 was zero and with increase in pH, the selectivity increased. Amongst the pH values studied, the selectivity was highest at pH 7.0.

The tetracycline contains three distinct acid groups, like the tri carbonyl methane system (ring A), the ammonium cation (ring B) and the phenolic diketone system (ring C) as shown in the below figure (Stephens *et al.*, 1956).

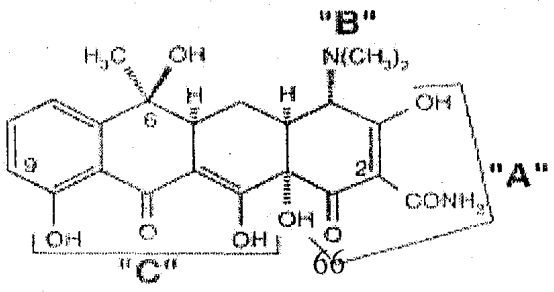


Table 4.3 Effect of pH on tetracycline binding to imprinted and non-imprinted polymer

pH	Polymer	Percent Binding Efficiency	Partition Coefficient	Selectivity
4.0*	Imprinted	71	70	0
	Non-imprinted	71	70	
5.0*	Imprinted	69	66	1.5
	Non-imprinted	57	42	
6.0**	Imprinted	67	57	1.9
	Non-imprinted	43	30	
7.0**	Imprinted	77	85	3.7
	Non-imprinted	37	23	

*, 20 mM Acetate buffer ; **, 20 mM Phosphate buffer.

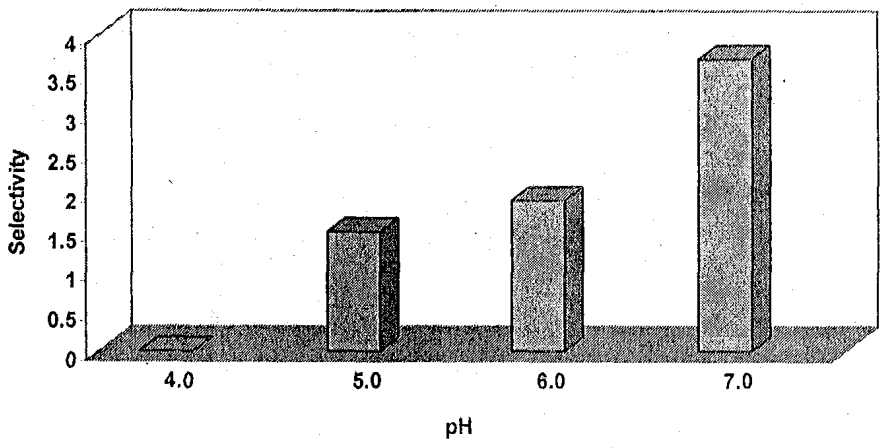


Fig. 4.4 Effect of pH on selectivity

Because of this, the tetracycline has three pKa values such as 3.3, 7.68 and 9.69 (Stephens *et al.*, 1956). At pH 7.0, the proton from tricarbonyl methane system will not be available for H-bond formation with negatively charged carboxyl groups from either methacrylic acid or EGDMA and as well as with oxygen present in ether linkage in EGDMA. In this situation, a decrease in contribution of H-bond is expected as the pH is raised from 4.0 to 7.0.

4.1.1.4 Effect of temperature on tetracycline binding to imprinted polymer

Since the non-ionic interactions can change with temperature, polymers were evaluated for their binding at two different temperatures, viz., 30 and 40°C in acetonitrile. The results of percentage binding efficiency, partition coefficient, capacity of the polymer and selectivity are presented in Table 4.4. Although there was not much difference in selectivity, but nevertheless, slightly better selectivity was observed at 30°C. It is known that the hydrogen bonds are more preferably formed at lower temperature and perhaps this interaction might be involved in recognition by the imprinted polymers to tetracycline. Since the binding has been done in acetonitrile, hydrogen bonds are expected to be formed.

4.1.1.5 Effect of tetracycline concentration on binding to imprinted polymer

The effect of tetracycline concentration ranging from 20 to 100 µg/ml on the binding efficiency of imprinted and non-imprinted polymer is shown in Figure 4.5 and Table 4.5. The binding efficiency of imprinted polymer remains same at the entire range of tetracycline concentration (20 to 100 µg/ml) and was around 83 percent. However, in case of non-imprinted polymer, the binding efficiency at 20 µg/ml tetracycline concentration was only 25 percent and the binding efficiency, in general, increased with increase in tetracycline concentration. The maximum binding with non-imprinted polymer was 59 percent, which was observed at 80 µg/ml. At all the concentrations of

tetracycline, the binding efficiency was considerably high for imprinted polymer as compared to non-imprinted polymer. These results suggest that the imprinted polymer has additional binding sites of tetracycline. This is also clear from the partition coefficient values of imprinted and non-imprinted polymer and selectivity value as shown in Table 4.5. The maximum selectivity (4.0) was achieved at 20 µg/ml tetracycline concentration. Cai and Gupta (2004) reported that in case of both imprinted and non-imprinted polymers, percentage-binding efficiency increased with increase in tetracycline concentration. The increased concentration of template might have led to increased non-specific interaction between non-imprinted polymer and the template (Sellergen, 1997). This indicated that the polymer matrix had some affinities to tetracycline even without imprinting attributed to the hydrogen bonding interaction between poly-methyacrylic acid (PMAA) matrix and tetracycline molecules (Cai and Gupta, 2004). Since at 20 µg/ml, there was higher selectivity (4.0) and higher percentage binding (83%) by imprinted polymer, this concentration was selected for the binding studies.

4.1.1.6 Effect of quantity of polymer in binding of tetracycline

The effect of quantity of polymer ranging from 20 to 40 mg on the binding efficiency of imprinted and non-imprinted polymer is shown in Table 4.6. The binding efficiency of imprinted polymer varied from 74 percent at 40 mg to 80 percent at 20 mg. In case of non-imprinted polymer, the percentage binding efficiency at 20 mg polymer was 30 percent and increased binding (40 to 43%) was observed at higher quantity of polymer (30 or 40 mg). Therefore, the selectivity was higher when 20 mg polymer matrix was used. At all the quantity of polymer used, the binding efficiency of imprinted polymer was more when compared to non-imprinted polymer. These results indicate that the imprinted polymer had additional binding pockets for tetracycline. The higher partition coefficient values of imprinted polymer also support this conclusion. The non-specific interaction between polymer matrix and

Table 4.4 Effect of temperature on percentage binding efficiency and partition coefficient of imprinted and non-imprinted polymer of tetracycline in acetonitrile

Polymer	Percent Binding Efficiency (Mean ± SD*)		Partition Coefficient (Mean ± SD*)		Binding Capacity (mg tetracycline/ g polymer)		Selectivity	
	30°C	40°C	30°C	40°C	30°C	40°C	30°C	40°C
Imprinted	40 ± 2	49 ± 4	29 ± 3	34 ± 2	1.6	2.0	2.2	2.0
Non-imprinted	16 ± 3.0	21 ± 2.7	13 ± 1.2	17 ± 1.2	0.6	0.6		

* Mean of five samples.

Table 4.5 Effect of tetracycline concentration on binding of tetracycline to imprinted and non-imprinted polymer

Tetracycline Concentration (µg/ml)	Polymer	Percent Binding Efficiency	Partition Coefficient	Selectivity
20	Imprinted	83	90	4.0
	Non-imprinted	25	22	
40	Imprinted	82	89	3.4
	Non-imprinted	32	26	
60	Imprinted	83	90	2.8
	Non-imprinted	47	32	
80	Imprinted	84	91	2.2
	Non-imprinted	59	42	
100	Imprinted	82	89	2.3
	Non-imprinted	53	38	

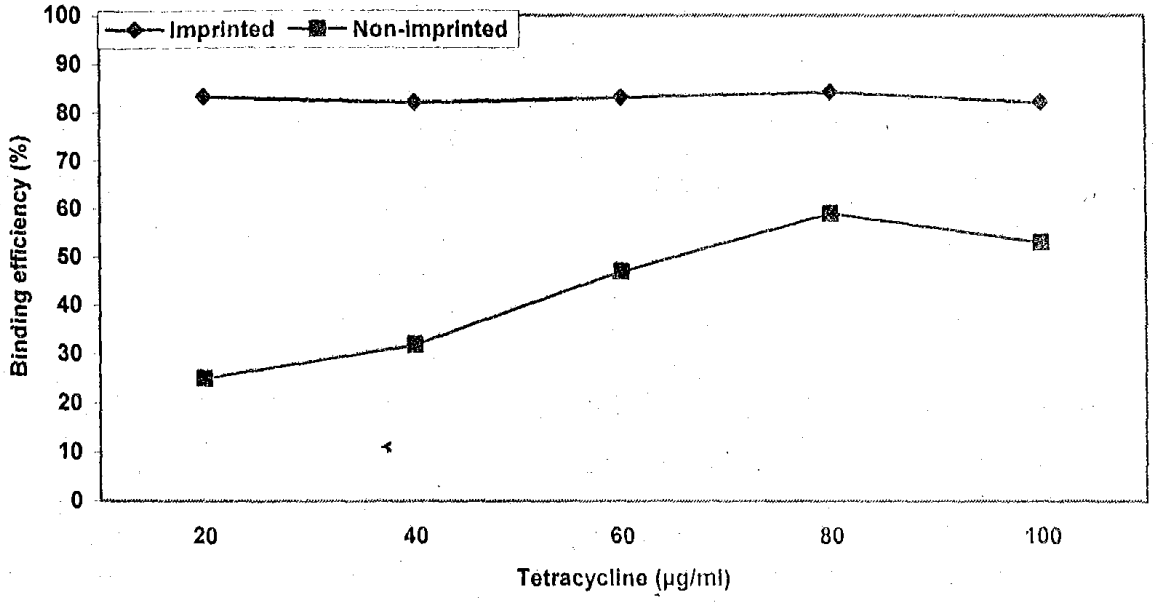


Fig. 4.5 Effect of tetracycline concentration on binding of tetracycline to imprinted and non-imprinted polymer

Table 4.6 Effect of quantity of polymer on binding of tetracycline to imprinted and non-imprinted polymer

Quantity of Polymer (mg)	Polymer	Percent Binding Efficiency	Partition Coefficient	Selectivity
20	Imprinted	80	86	3.2
	Non-imprinted	30	27	
30	Imprinted	75	80	2.2
	Non-imprinted	40	36	
40	Imprinted	74	79	2.0
	Non-imprinted	43	39	

tetracycline is more at 30 or 40 mg as compared to 20 mg polymer. This explains the decreased values of selectivity at increasing quantity of polymer.

4.1.1.7 Evaluation of imprinted polymer as matrix in chromatography

Any matrix is required to be finally evaluated for its efficiency in column chromatography. For this purpose, imprinted polymer was packed into small glass column. Since binding of tetracycline to imprinted polymer was relatively more in water, the column was equilibrated with water. After binding of tetracycline to the column, the increasing concentration of acetonitrile was applied for eluting out the bound tetracycline. The results are shown in Figure 4.6. For comparative purposes, non-imprinted polymer was also separately packed and evaluated similarly (Fig. 4.6). In case of imprinted polymer, 5 percent tetracycline was present in fractions eluted with distilled water. When linear gradient of water-acetonitrile was applied, 84 percent tetracycline got eluted in the fraction. On the other hand, from the column packed with non-imprinted polymer, 49 percent of tetracycline was present in fractions eluted with water and only 28% of tetracycline got eluted with increased concentration of acetonitrile. The pattern of tetracycline elution with water and subsequently with increasing concentration of acetonitrile clearly establishes that imprinted polymer has specific binding sites for the tetracycline. Since tetracycline could be eluted from imprinted polymer by reducing the polarity of eluent (increasing concentration of acetonitrile), the interaction of tetracycline with imprinted polymer is largely hydrophobic in nature (Caro *et al.*, 2005). The peak obtained after the application of linear gradient of water and acetonitrile mixture is not bell-shaped and rather two shoulders were observed. This indicates the heterogeneity in binding sites; some sites have higher affinity over the other sites. This can be again explained by the fact that in this chromatographic experiment, the tetracycline could be in the stationary phase in three different conditions, viz., (i) dissolved in the solvent in the inner pores of the particles, (ii) non-specifically bound to one or two functional groups of the polymer, and (iii) specifically bound in the cavities of imprint. The kinetics of the latter interactions could be the main factor that determined

the peak broadening effect (Ensing and de Boer, 1999). In case of non-imprinted polymer, the interaction between tetracycline and polymer was poor in nature due to the absence of specific sites corresponding to the template. Because of this reason on the loading step itself, the tetracycline got leaked out unlike imprinted polymer. Chromatographic experiments confirmed the presence of imprints in molecular imprinted polymer.

4.1.1.8 Evaluation of binding and elution of tetracycline present in milk

The tetracycline-imprinted polymer was evaluated for its performance in milk. For this purpose, milk samples were spiked with tetracycline. The filtrate obtained after precipitation of proteins was subjected to chromatographic separation and results are shown in Figure 4.7. For comparative purposes, the profile of milk without added tetracycline has also been shown in Figure 4.7. The fractions eluted with water-contained materials, which also show absorbance at 280 nm. Since these were present in milk samples both with and without tetracycline, the material eluted with water appears to be milk origin. When acetonitrile was used as eluent, only a minor peak was observed with milk. However, in case of milk spiked with tetracycline, a major peak was noticed. The difference in the area of peaks eluted with acetonitrile is an indicator of the quantity of tetracycline present in milk. It has been noticed that entire amount of the tetracycline added to the milk was recovered in fractions eluted with acetonitrile and, therefore, the recovery was quantitative. Thus, it can be safely concluded that acrylate based tetracycline imprinted polymer can be applied to milk system and these materials can be used for sample concentration before their analysis.

4.1.1.9 Evaluation of tetracycline imprinted polymer towards other antibiotics

Although molecular imprinted polymers are prepared against the targeted molecules, and in present case polymer has been prepared against tetracycline, it is desirable to check binding of other antibiotics to the prepared-polymer. The binding of amoxicillin and ciprofloxacin was evaluated

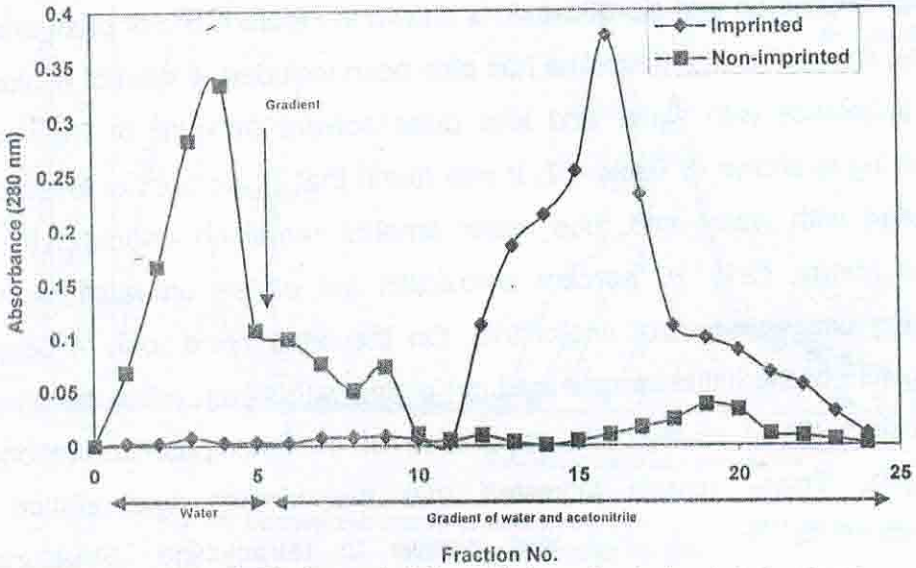


Fig. 4.6 Binding and elution of tetracycline to imprinted and non-imprinted polymer

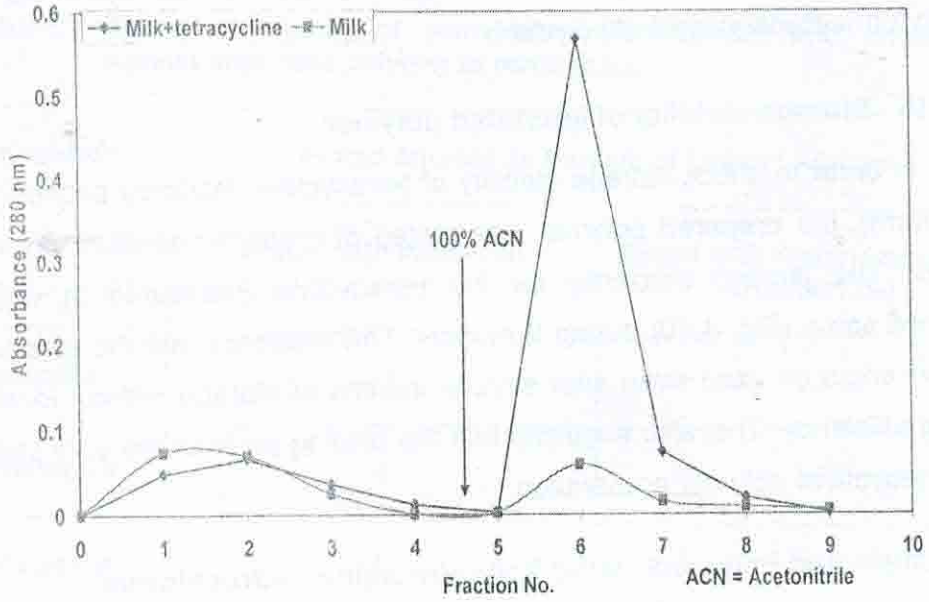


Fig. 4.7 Binding and elution of tetracycline present in milk to imprinted polymer of tetracycline. Chromatographic profile of milk without tetracycline (control) is also shown in figure

in column packed with tetracycline-imprinted polymer. The column was equilibrated with water. After sample loading, the column was first eluted with water and then a gradient of water-acetonitrile mixture was applied. The profile of amoxicillin and ciprofloxacin is shown in Figure 4.8. For comparative purpose, the profile of tetracycline has also been included. Extent of elution of these antibiotics with water and less polar solvent (mixture of water and acetonitrile) is shown in Table 4.7. It was found that 71 percent of amoxicillin got eluted with water and thus major amount remained unbound to the polymer matrix. Only 20 percent amoxicillin got eluted on application of increasing concentration of acetonitrile. On the other hand, only 4 percent ciprofloxacin of the initial sample load got eluted with water, while the bound-ciprofloxacin (85%) eluted with application of increasing concentration of acetonitrile. These results indicated that the binding and elution of ciprofloxacin to the polymer was similar to tetracycline. Structurally, ciprofloxacin and tetracycline (Fig. 4.9) can be considered similar, in view of the fact that most of the structure is present in ring form and structure of ciprofloxacin can be placed in cavities of imprinted polymer. The structure of amoxicillin (Fig. 4.9) is more open as compared to tetracycline or ciprofloxacin and might not be able to fit well in cavities and, therefore, amoxicillin did not bind to tetracycline-imprinted polymer.

4.1.1.10 Storage stability of imprinted polymer

In order to check storage stability of tetracycline-imprinted polymer (in dried form), the prepared polymer was stored at room temperature up to 9 months. The binding efficiency for the tetracycline (measured in water) remained same (Fig. 4.10) during 9 months. This indicates that the prepared polymer could be used even after several months of storage without loosing binding efficiency. This also suggests that the binding pockets are very stable in the network of polymer preparation.

4.1.2 Imprinted Polymers Using Poly-Allylamine Hydrochloride

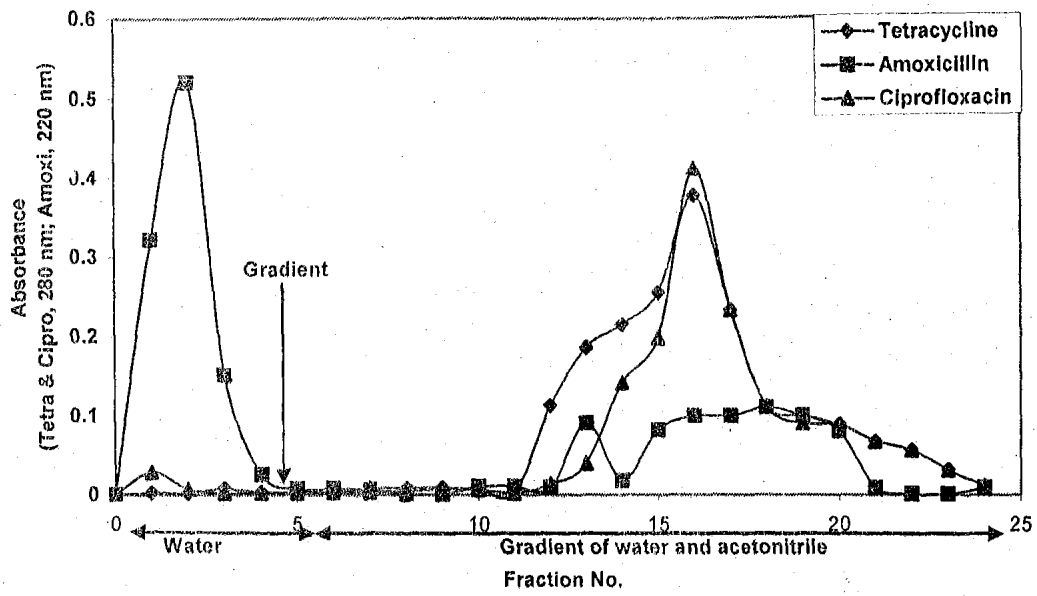
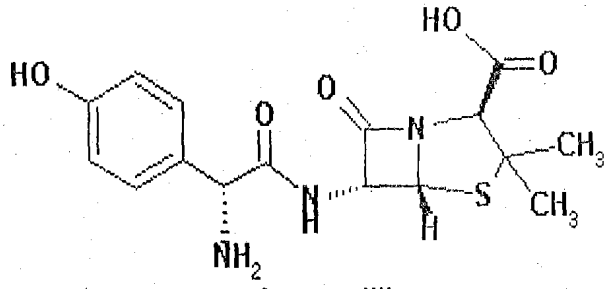


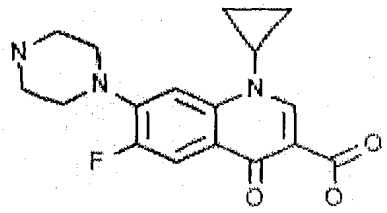
Fig. 4.8 Binding and elution of amoxicillin, ciprofloxacin and tetracycline to tetracycline imprinted polymer

Table 4.7 Extent of elution of amoxicillin, ciprofloxacin and tetracycline against imprinted polymer of tetracycline

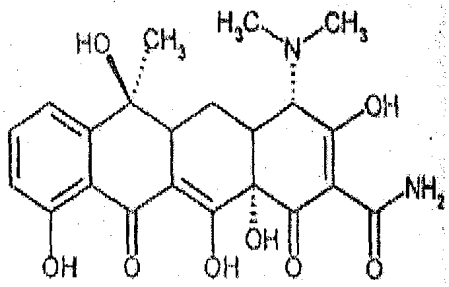
Antibiotic	Eluted Amount as Percent of Loaded Amount	
	Eluted with Water (%)	Eluted with Water-Acetonitrile Gradient (%)
Amoxicillin	71	20
Ciprofloxacin	4	85
Tetracycline	5	84



Amoxycillin



Ciprofloxacin



Tetracycline

Fig. 4.9 Structures of Amoxicillin, Ciprofloxacin and Tetracycline

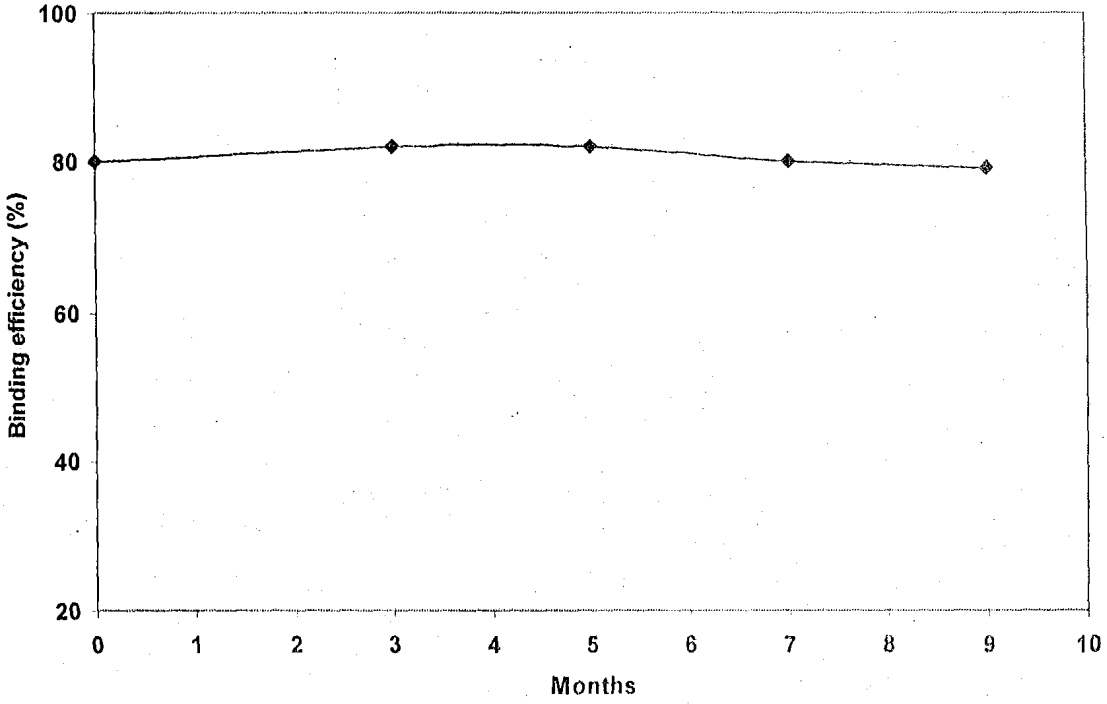


Fig. 4.10 Storage stability of tetracycline imprinted polymer

In majority of the molecular imprinted polymer preparation, organic solvents are used during their synthesis. Non-polar environment is not suited for certain applications. For such situations, hydrogels having imprints of analyte can be proved useful. Not much work has been done in exploiting the potential of hydrogels in creating imprints of target molecule. Poly-allylamine hydrochloride (PAA.HCl) can form hydrogels when it is cross-linked. The amine groups present in poly-allylamine can form ionic interactions with anionic species and also it can participate in hydrogen bonding (Wizeman and Kofinas, 2001). Since epichlorohydrin is a better cross-linker as compared to ethylene glycol diglycidyl ether and glycerol diglycidyl ether (Wizeman and Kofinas, 2001), epichlorohydrin is selected as a cross-linker for preparing imprinted polymers using poly-allylamine hydrochloride in present work. The lactic acid contains carboxylic group and is soluble in aqueous environment and can form ionic interactions with amino groups. These facts encouraged us to prepare imprinted polymer of lithium lactate using poly-allylamine hydrochloride. Large size molecules can permeate to hydrogels and, therefore, lysozyme is also used as template for preparing imprinted polymer.

4.1.2.1 Preparation and characterization of lithium lactate imprinted polymer

While preparing molecular imprinted polymers using PAA.HCl, a portion of the PAA.HCl amine sites was neutralized by adding NaOH under stirring. This is essentially required for cross-linking of amino groups with epichlorohydrin. When epichlorohydrin was added to partly neutralized PAA.HCl in presence of lithium lactate, the contents of the reaction mixture became gelly during the course of reaction. When the reaction was continued overnight, the gelly structure became further hard and at that point, it was possible to cut into required sizes (Plate 4.2). The lithium lactate was removed by treating hydrogels with 4 M NaOH for 48 h. The process of removing NaOH took few days and only then the preparation was used for evaluation.

4.1.2.2 Binding of lithium lactate to imprinted and non-imprinted polymers

The evaluation of imprinted polymers requires comparative studies with non-imprinted polymer. As the binding between lithium lactate and poly-allylamine is likely of ionic in nature, the binding of lithium lactate was evaluated in water and also in 1 M NaCl aqueous. Imprinted and non-imprinted polymers behaved very differently in water and 1 M NaCl. There was large difference in binding efficiency, binding capacity and partition coefficient values for imprinted polymer in comparison to values for non-imprinted polymer when binding was determined in water (Table 4.8 and Fig. 4.11). 2.3 mg of lithium lactate could bind to one gram wet imprinted polymer. Under similar binding condition, only 0.64 mg lactate could bind to one gram wet non-imprinted polymer. Hence, there is a considerable reduced binding of lithium lactate to non-imprinted polymer. The values of binding efficiency and partition coefficient were also lower in non-imprinted polymer. This suggests that imprinted polymer has additional pockets capable of interacting with lactate. The selectivity value of 7.7 in water is an indicator of very high degree of selectivity of imprinted polymer over non-imprinted polymer. The binding of lithium lactate to imprinted and non-imprinted polymer in 1 M NaCl (aqueous) was similar (Table 4.8) and there was no selectivity of imprinted polymer over non-imprinted polymer. The binding to non-imprinted polymer in both water and 1 M NaCl (aqueous) was similar and indicates its non-specific nature. The imprinted polymer was selective over non-imprinted polymer in water and the selectivity was abolished in 1 M NaCl (aqueous). This indicates that lithium lactate interacted with cavities in imprinted polymer through ionic interactions.

4.1.2.3 Effect of pH on binding lactate to imprinted and non-imprinted polymers

Since lactate contains carboxylic group and its dissociation is dependent on pH, it was desirable to study the effect of pH on the performance of imprinted and non-imprinted polymer. The binding was studied at pH 4.0 (acetate buffer), pH 5.0 (acetate buffer), pH 5.6 (acetate buffer) and pH 7.0 (BES buffer, 10 mM) and results are shown in Table 4.9 and Figure 4.12. The imprinted polymers exhibited selectively over non-imprinted polymer at all pH values (4.0, 5.0, 5.6 and 7.0). The selectivity was

Table 4.8 Binding efficiency and capacity of lithium lactate imprinted and non-imprinted polymer in water and 1 M NaCl

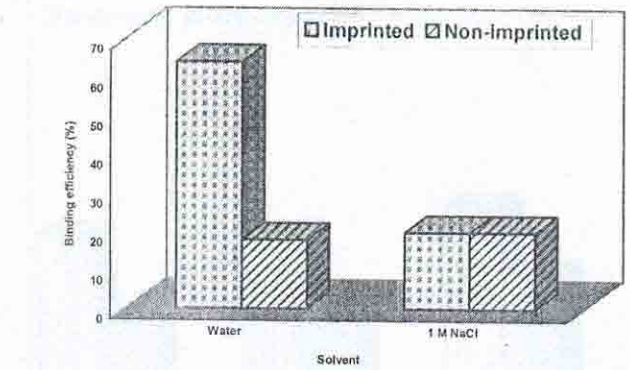
Solvent	Polymer	Percent Binding Efficiency	Binding Capacity (mg lactate/g polymer)	Partition Coefficient	Selectivity
Water	Imprinted	64	2.30	1.70	7.7
	Non-imprinted	18	0.64	0.22	
1 M NaCl	Imprinted	20	0.71	0.24	Nil
	Non-imprinted	20	0.73	0.25	

Table 4.9 Effect of pH on binding of lithium lactate to imprinted and non-imprinted polymer

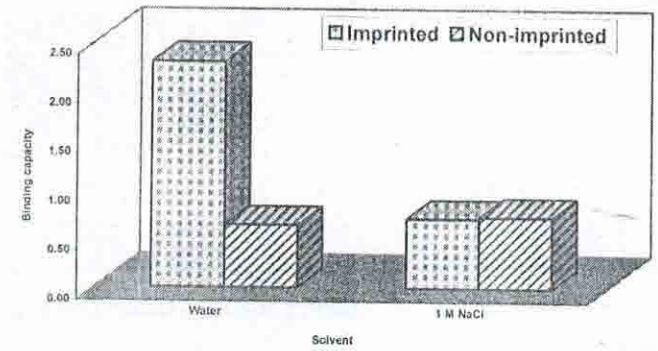
pH	Polymer	Percent Binding Efficiency	Partition Coefficient	Selectivity (Fold)
4.0*	Imprinted	37	0.62	2.0
	Non-imprinted	24	0.31	
5.0*	Imprinted	37	0.62	1.9
	Non-imprinted	27	0.32	
5.6*	Imprinted	41	0.69	1.5
	Non-imprinted	31	0.45	
7.0**	Imprinted	68	2.11	6.8
	Non-imprinted	24	0.31	

*, 20 mM Acetate buffer ; **, 10 mM BES buffer

A



B



C

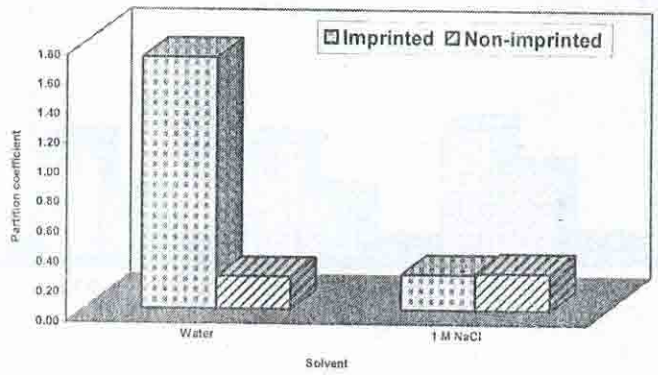


Fig. 4.11 Binding of lithium lactate to imprinted and non-imprinted polymer in water and 1 M NaCl
A) Binding efficiency, B) Binding capacity, and C) Partition coefficient

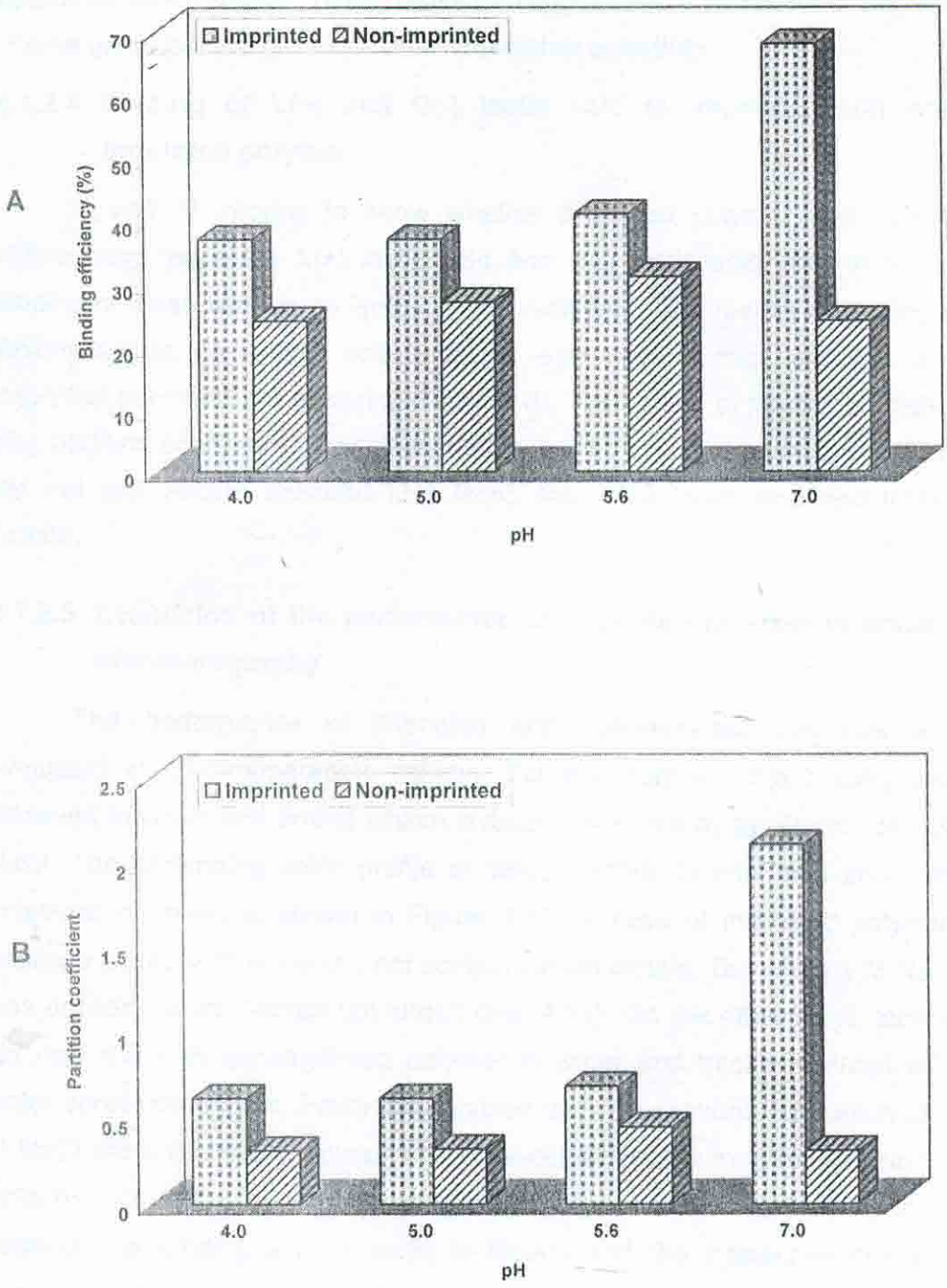


Fig. 4.12 Binding of lithium lactate to imprinted and non-imprinted polymer at different pH
A) Binding efficiency, and B) Partition coefficient

between 1.5 and 2.0 at pH values of 4.0, 5.0 and 5.6. However, at pH 7.0, the selectivity was 6.8 fold. These results do suggest the requirement of negative charge on carboxylic group for obtaining higher selectivity.

4.1.2.4 Binding of L(+) and D(-) lactic acid to imprinted and non-imprinted polymer

It was of interest to know whether imprinted polymer was able to differentially recognize L(+) lactic acid and D(-) lactic acid and, therefore, binding of these two stereo isomers was evaluated. The results of binding of lithium lactate, L(+) lactic acid and D(-) lactic acid to imprinted and non-imprinted polymers are shown in Table 4.10. The values of binding efficiency (%), partition coefficient for all these molecules indicate that imprinted polymer did not differentiate between L(+) lactic acid, D(-) lactic acid and lithium lactate.

4.1.2.5 Evaluation of the performance of imprinted polymer in column chromatography

The performance of imprinted and non-imprinted polymers was evaluated in chromatographic column. For this purpose, the binding was achieved in water and bound lithium lactate was eluted by application of 1 M NaCl. The chromatographic profile of lithium lactate in imprinted and non-imprinted polymers is shown in Figure 4.13. In case of imprinted polymer, fractions eluted with water did not contain lithium lactate. But when 1 M NaCl was applied, bound lactate got eluted (Fig. 4.13). On the other hand, lactate did not bind with non-imprinted polymer in water and fractions eluted with water contained lactate. Fractions obtained with subsequent application of 1 M NaCl did not contain lactate. The clear-cut difference in chromatographic patterns with imprinted and non-imprinted polymer indicated that imprinted polymer has binding sites specific to lactate and the interaction between lactate and the polymer can be broken by the application of NaCl.

4.1.2.6 Binding of lithium lactate present in milk to imprinted and non-imprinted polymer

Table 4.10 Binding of lithium lactate, L(+) lactic acid and D(-) lactic acid to imprinted and non-imprinted polymer

Polymer	Percent Binding Efficiency			Partition Coefficient		
	Lithium Lactate	L(+) Lactic Acid	D(-) Lactic Acid	Lithium Lactate	L(+) Lactic Acid	D(-) Lactic Acid
Imprinted	64	61	63	1.70	1.60	1.67
Non-imprinted	18	24	20	0.22	0.29	0.24

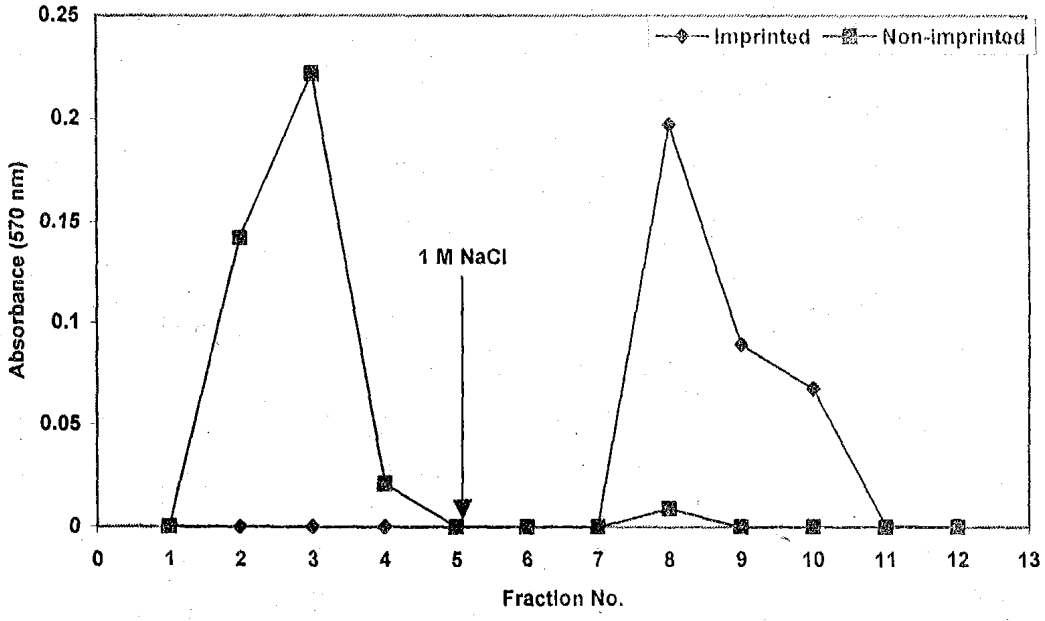


Fig. 4.13 Binding and elution of lithium lactate to imprinted and non-imprinted polymer

Lithium lactate imprinted polymer was evaluated for its performances in milk. Different dilutions of milk were spiked with lithium lactate. The imprinted and non-imprinted polymer was incubated with the lithium lactate containing milk. After incubation, the supernatant was collected and lactate content was estimated. The binding results showed that for 1:5 dilution, the binding efficiency for imprinted polymer was 44% and for non-imprinted polymer, 29%. For 1:10 dilution the binding efficiency for imprinted polymer was 47% while it was 43% for non-imprinted polymer. Even though, for 1:5 dilution, there was selectivity (1.52 fold) for imprinted polymer over non-imprinted polymer compared to 1:10 dilution (no selectivity), the binding by imprinted polymer was slightly influenced due to the presence of different ions at variable concentrations in milk.

4.1.2.7 Storage stability of imprinted polymer of lactate

In order to check storage stability of lithium lactate-imprinted (in wet form), the polymer was stored at room temperature up to 9 months. The binding efficiency for the lactate (measured in water) remained same (Fig. 4.14). This indicates that the prepared polymer could be used over several months without loosing binding efficiency. This also suggests that the binding pockets are very stable in the network of polymer preparation.

4.1.3 Preparation of Imprinted Polymer Against Vitamin A

Retinol, the animal form of vitamin A, is a yellow fat-soluble antioxidant vitamin important in vision and bone growth. In milk, vitamin A is present in ester form mainly as retinyl acetate or retinyl palmitate. Hydrolysis of retinyl esters result in retinal while pro-vitamin A carotenoids can be cleaved to produce retinal. A structurally similar molecule referred as β-carotene (Carotenoids) is an organic pigment, naturally occurring in plants. Because milk contains ester form of vitamin A, retinyl acetate was selected as an imprint molecule. Methacrylic acid can interact with carboxylic acid esters through hydrogen bond and because of many other suitable properties of methacrylic acid, it was selected for the preparation of imprinted polymer of

vitamin A. The preparation protocol was similar to tetracycline-imprinted polymer. For preparation of vitamin A imprinted polymer, the methanol was used as solvent. In methanol, methacrylic acid and EGDMA were miscible and retinyl acetate was soluble. The prepared polymer was hard, grainish texture and white in colour (Plate 4.3). The imprinted polymer was indistinguishable from non-imprinted polymer through naked eye.

4.1.3.1 Effect of solvents on binding

Retinyl acetate is soluble in methanol, acetonitrile, acetonitrile-water mixture and methanol-water mixture and, therefore, binding of retinyl acetate to imprinted and non-imprinted polymer has been studied in these solvents. In 100 percent methanol or 100 percent acetonitrile, retinyl acetate did not bind to either imprinted or non-imprinted polymer. When methanol-water mixture or acetonitrile-water mixture were used as solvent for binding, binding of retinyl acetate to both polymers was noted (Table 4.11 and Fig. 4.15). Binding efficiency, partition coefficient and selectivity values were found to be dependent on ratio of water present in acetonitrile-water or methanol-water mixture. As compared to non-imprinted polymer, the partition coefficient values for imprinted polymer were higher in methanol-water or acetonitrile-water mixtures. Partition coefficient values for imprinted polymer in 1:1 acetonitrile-water mixture was 88, while that for non-imprinted polymer was only 19. Accordingly, the selectivity value (4.6) was highest in solvent containing equal volume of acetonitrile and water amongst different solvents used. The selectivity values were comparatively low in other solvents, viz., 1:3 acetonitrile-water, 1:3 methanol-water and 1:1 methanol-water mixtures. A selectivity of 4.6 is considered very good in view of the fact that most workers using acrylate systems achieved selectivity in the range of 2 to 3 (Wulff, 1995).

4.1.3.2 Performance of imprinted polymers in chromatographic column

Imprinted and non-imprinted polymers were evaluated in chromatographic column (1 x 2 cm) (Fig.4.16). Retinyl acetate was dissolved in 1:2 acetonitrile-water mixtures and loaded to the column. The estimation of

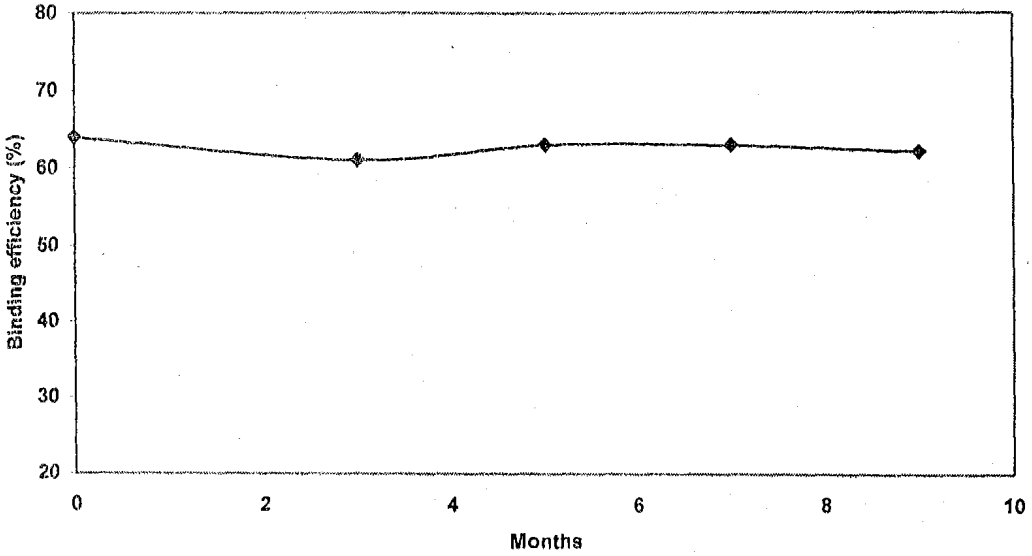


Fig. 4.14 Storage stability of lithium lactate imprinted polymer

Table 4.11 Binding of retinyl acetate to imprinted and non-imprinted polymer in different solvents

Solvent	Percent Binding Efficiency		Partition Coefficient		Selectivity
	Imprinted	Non-imprinted	Imprinted	Non-imprinted	
Methanol	0	0	0	0	Nil
Acetonitrile	0	0	0	0	Nil
1:3 Acetonitrile : Water	78	70	86	67	1.3
1:3 Methanol : Water	52	38	42	28	1.5
1:1 Methanol : Water	74	63	76	49	1.6
1:1 Acetonitrile : Water	79	22	88	19	4.6

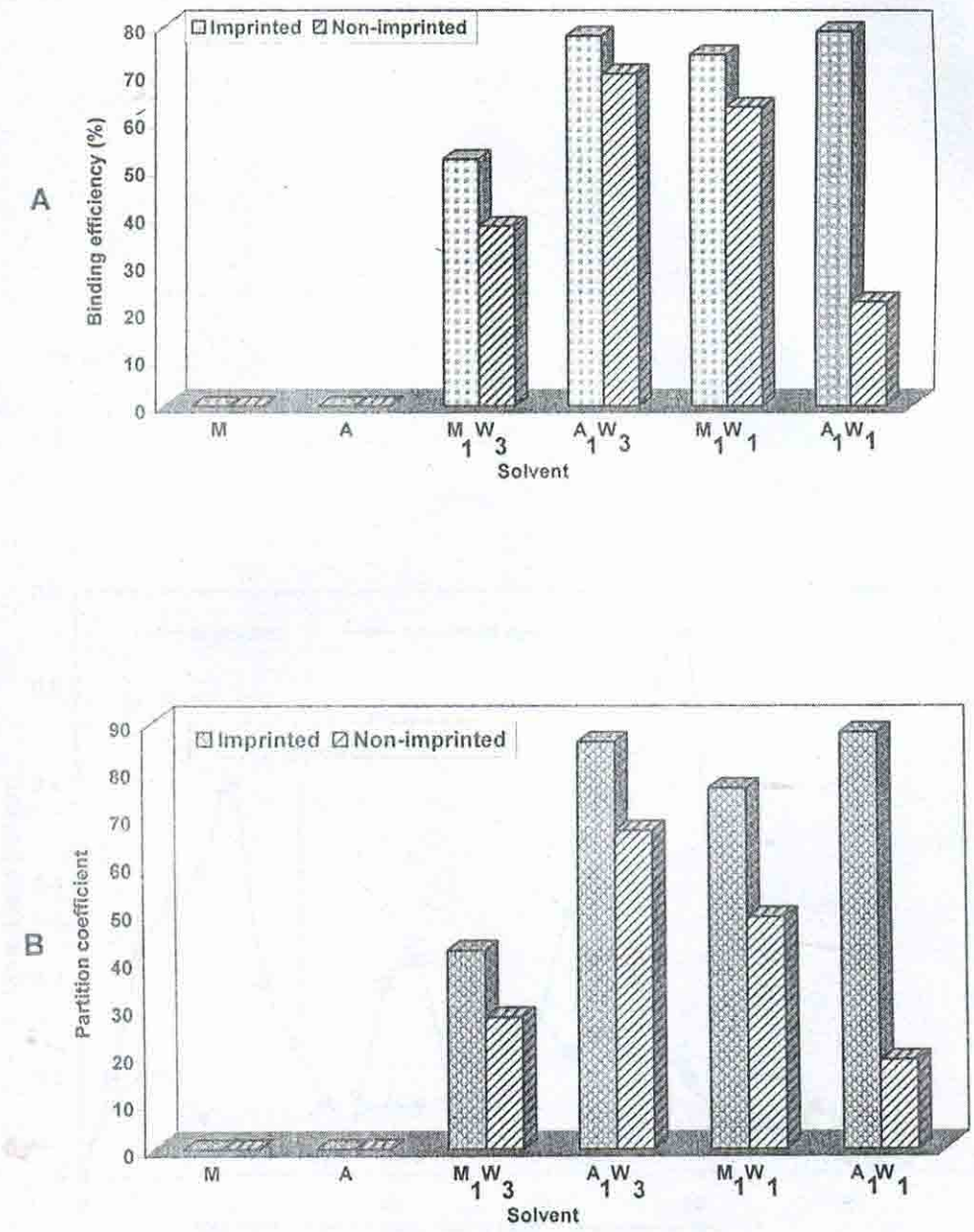


Fig. 4.15 Binding of retinyl acetate to imprinted and non-imprinted polymer in different solvents. A) Binding efficiency, and B) Partition coefficient
M = Methanol; A = Acetonitrile
M_1W_3 = Methanol – Water mixed in ratio of 1:3
A_1W_3 = Acetonitrile – Water mixed in ratio of 1:3
M_1W_1 = Methanol – Water mixed in ratio of 1:1
A_1W_1 = Acetonitrile – Water mixed in ratio of 1:1

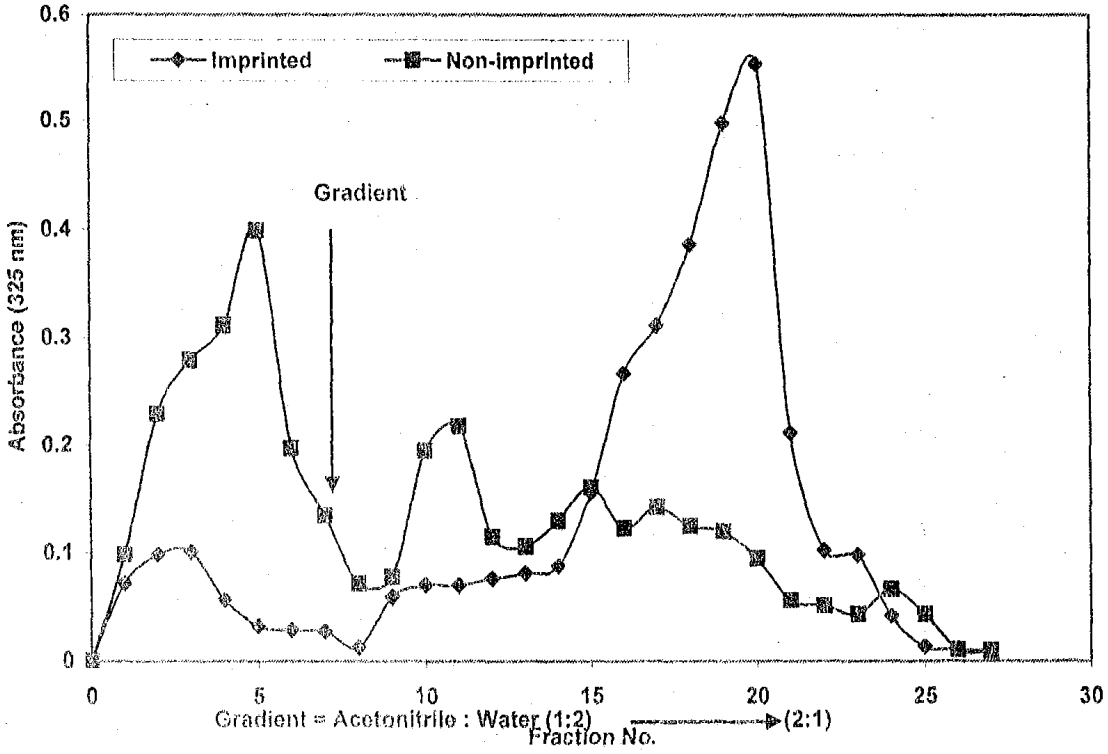


Fig. 4.16 Binding and elution of retinyl acetate to imprinted and non-imprinted polymer

retinyl acetate in fractions collected with 1:2 acetonitrile-water mixtures indicated that 40 percent retinyl acetate did not bind to non-imprinted polymer and 9 percent to imprinted polymer (Fig. 4.16). When elution was carried out with increasing concentration of acetonitrile (linear gradient of 1:2 acetonitrile-water mixture to 2:1 acetonitrile-water mixture) retinyl acetate got eluted in fractions eluted from imprinted polymer. This chromatographic behaviour of retinyl acetate indicated that imprinted polymer has binding pockets for retinyl acetate and disruption of this interaction can be achieved with increasing concentration of acetonitrile. The presence of shoulder in peak obtained with gradient in imprinted polymer indicates the heterogeneous nature of binding pockets. These results also suggest that interaction of retinyl acetate with imprinted polymers is largely hydrophobic in nature.

4.1.3.3 Cross reactivity of vitamin A imprinted polymer

An imprinted polymer is required to be checked for its ability to differentiate the molecules, which are closely related or unrelated to the target molecule. An attempt has been made to see the behaviour of β-carotene, tetracycline and ciprofloxacin on retinyl acetate-imprinted polymer. β-Carotene is not soluble in 1:2 acetonitrile-water mixture and, therefore, β-carotene could not be evaluated for its binding in retinyl acetate imprinted polymer. The chromatographic profile of tetracycline and ciprofloxacin on retinyl acetate-imprinted polymer is shown in Figure 4.17. The fractions eluted with water contained 80 percent of antibiotics loaded to the column and, therefore, major portion remained unbound. The results, therefore, suggested that tetracycline and ciprofloxacin did not interact with retinyl acetate imprinted polymer. The outcome of the result was not unexpected since both of the antibiotics are not structurally similar to retinyl acetate.

4.1.3.4 Storage stability of imprinted polymer

In order to check storage stability of retinyl acetate-imprinted polymer (in dried form), prepared polymer was stored at room temperature up to 7 months. The binding efficiency for the retinyl acetate (measured in 1:1 acetonitrile-water) remained same (Fig. 4.18). This indicates that the prepared

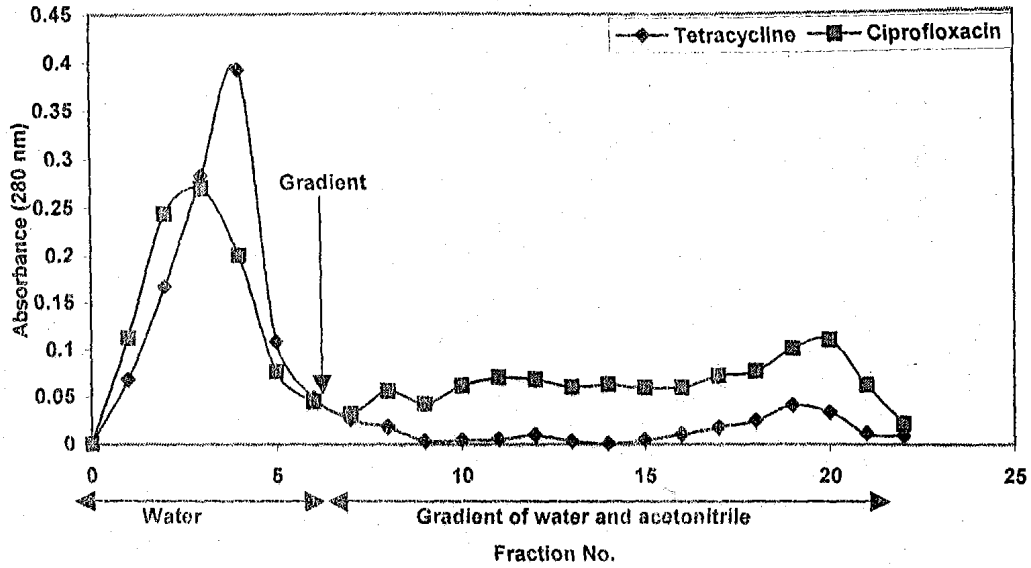


Fig. 4.17 Cross selectivity of imprinted polymer of vitamin A against tetracycline and ciprofloxacin

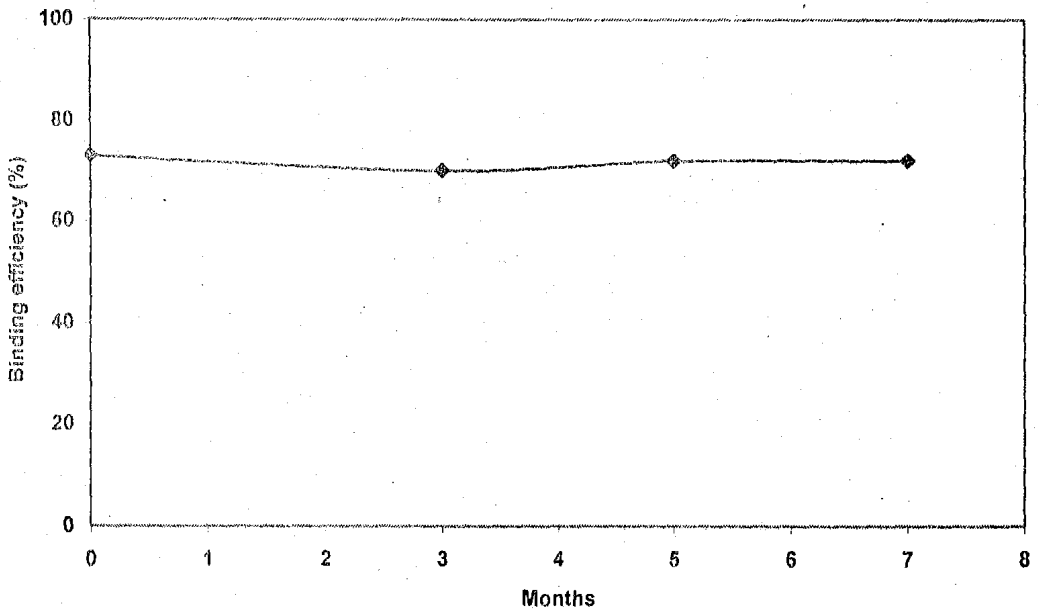


Fig. 4.18 Storage stability of retinyl acetate imprinted polymer

polymer could be used over several months without loosing binding efficiency. This also suggests that the binding pockets are very stable in the network of polymer preparation.

4.1.4 Preparation and Evaluation of Imprinted Polymer Against Ciprofloxacin

4.1.4.1 Evaluation of methacrylate based polymer

Ciprofloxacin comes under the antibiotic group fluoroquinolones. The imprinted polymer against ciprofloxacin was prepared using methacrylic acid and EGDMA (Plate 4.4). The evaluation of prepared polymer was done in distilled water, 1 M NaCl, 50 mM phosphate buffer (pH 3.0) and acetonitrile. The results are shown in Table 4.12. It was found that the binding efficiency of imprinted polymer was low in all cases except water and the selectivity of imprinted polymer over non-imprinted polymer was approximately zero in all solvents including water.

4.1.4.2 Evaluation of 4-vinyl pyridine based polymer

The imprinted polymer prepared with 4-vinyl pyridine was evaluated in different solvents, viz., water, 1 M NaCl and dimethyl sulfoxide. The binding results are shown in Table 4.13. These binding results also showed that there was low binding of ciprofloxacin to the imprinted polymer in water and the selectivity was nearly zero in 1 M NaCl. When the incubation was done in dimethyl sulfoxide, the polymer particles got partially dissolved in the solvent and thus the binding efficiency could not be evaluated.

4.1.4.3 Evaluation of poly-allylamine based polymer

The preparation made by poly (allylamine hydrochloride) also failed to produce specific binding sites for ciprofloxacin and the non-imprinted polymer showed highly non-specific interactions (Table 4.14).

4.1.4.4 Evaluation of methyl methacrylate based polymer

Table 4.12 Binding of ciprofloxacin to imprinted and non-imprinted polymer prepared from methacrylic acid

Solvent	Polymer	Percent Binding Efficiency	Partition Coefficient	Selectivity
Water	Imprinted	70	76	Nil
	Non-imprinted	68	74	
1 M NaCl	Imprinted	40	43	Nil
	Non-imprinted	40	43	
Phosphate buffer	Imprinted	18	13	Nil
	Non-imprinted	17	13	
Acetonitrile	Imprinted	12	19	Nil
	Non-imprinted	12	18	

Table 4.13 Binding of ciprofloxacin to imprinted and non-imprinted polymer prepared from 4-vinyl pyridine

Solvent	Polymer	Percent Binding Efficiency	Partition Coefficient	Selectivity
Water	Imprinted	11	12	1.3
	Non-imprinted	8	9	
1 M NaCl	Imprinted	24	26	Nil
	Non-imprinted	23	25	

When methyl methacrylate was used as a monomer for the preparation of imprinted polymer, complete polymerisation did not take place at the initial stage itself. Hence, thus prepared polymer could not be evaluated for their binding efficiency.

4.1.5 Preparation and Evaluation of Imprinted Polymer Against Ascorbic Acid

Like other imprinted polymers, ascorbic acid imprinted polymer was also evaluated for its binding to ascorbic acid at 268 nm. The results were inconsistent for both imprinted and non-imprinted polymers. As the binding experiments required 24 h incubation of matrix with ascorbic acid solution, there is a scope for its oxidation during incubation. The stability of ascorbic acid in different solvents at different time and temperature combination was evaluated, and results are shown in Table 4.15. It is clear from the table that there was 95% loss in ascorbic acid when it is stored in water at 30°C for 24 h. Addition of meta phosphoric acid, BHA, nitrogen bubbling and ortho phosphoric acid plus glacial acetic acid helped to prevent loss in ascorbic acid but losses were still significant. Therefore, the method used for ascorbic acid estimation could not provide answer to the presence of ascorbic acid imprints in prepared polymer.

4.1.6 Preparation of Imprinted Polymer Against Lysozyme

Lysozyme is an antibacterial agent of molecular weight of 15 kDa. This cleaves β (1→4) glycosidic bonds between N-acetyl muramic acid and N-acetyl D-glucosamine residues in peptide glycan, a constituent of bacterial cell wall. Because of this property, lysozyme plays a major role in food preservation. The molecular imprinted polymer against lysozyme was prepared using poly (allylamine hydrochloride) and epichlorohydrin (Plate 4.5). The polymer preparation was translucent and this is in contrast to non-imprinted polymer; where the preparation was transparent. This might be due to the presence of lysozyme, which has brought out this difference in appearance.

Plate 4.1 (A) Imprinted polymer of tetracycline
(B) Non-imprinted polymer

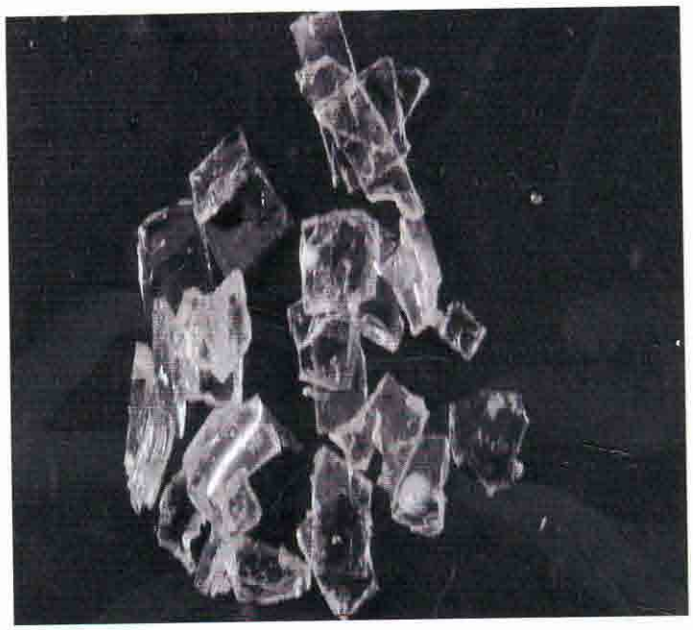


Plate 4.2 Lithium lactate imprinted polymer

Plate 4.3 (A) Imprinted polymer of retinyl acetate
(B) Non-imprinted polymer

Plate 4.4 (A) Polymer prepared in presence of ciprofloxacin
(B) Non-imprinted polymer

Plate 4.5 Polymer prepared in presence of lysozyme

Table 4.14 Binding of ciprofloxacin to imprinted and non-imprinted polymer prepared by poly(allylamine)

Polymer	Percent Binding Efficiency	Partition Coefficient	Selectivity
Imprinted	7.0	7.5	Nil
Non-imprinted	40	43	

Table 4.15 Stability of pure ascorbic acid in different solvent systems

Solvent System	Incubation Time and Temperature	Loss in Ascorbic Acid in Percent
Water	24 h, 30°C	95
Orthophosphoric acid + glacial acetic acid*	24 h, 10°C	84
Water + N_2 bubbling$^{\Delta}$	12 h, 5°C	88
Water + 0.03% BHA	12 h, 5°C	72
Metaphosphoric acid (5-6%)	20 h, 30°C	50

* 900 µl orthophosphoric acid + 4 ml glacial acetic acid + 45 ml distilled water (pH 2.0).

Δ N_2 bubbling is done for 5 min.

4.1.6.1 Evaluation of binding of lysozyme to imprinted polymer

The prepared polymers (imprinted and non-imprinted) were evaluated by measuring binding of lysozyme to these polymers. The binding was studied in water. The unbound activity was measured and is shown in Figure 4.19. The change in absorbance with time for unbound fractions from imprinted polymer and non-imprinted polymer indicate that the activities were nearly similar (Fig. 4.19). The change in absorbance corresponding to lysozyme used in binding (initially added) is also shown in Figure 4.19. The change in absorbance of lysozyme loaded, unbounded fractions from imprinted polymer and non-imprinted polymer further suggest that there was virtually no binding to either imprinted or non-imprinted polymers. Thus, there was no preferential binding of lysozyme to imprinted polymer in water. Then, the selectivity was evaluated in 1:1 mixture of methanol-water and 1:1 mixture of acetonitrile-water. Before binding experiment was to be performed, it was essential to know whether lysozyme will exhibit enzyme activity in these solvents. Accordingly, enzyme activity was evaluated in these solvents and results are shown in Figure 4.20. It is clear from the figure that lysozyme remain active in methanol-water mixture as well as acetonitrile-water mixture. Therefore, binding was evaluated in these solvents, which are less polar compared to water (Figs. 4.21 and 4.22). In these two solvents also, there was no difference in lysozyme activities in unbound fractions collected from imprinted and non-imprinted polymers. Thus, preferential binding of lysozyme to imprinted polymer could not be established in water and less polar solvents.

4.1.7 Multipurpose Device for Protein Concentration, Dialysis and Buffer Exchange

A multipurpose device for protein concentration, dialysis and buffer exchange was designed and constructed. A patent for this device has been filed. This device was developed with purpose of removing salts from lysozyme solution.

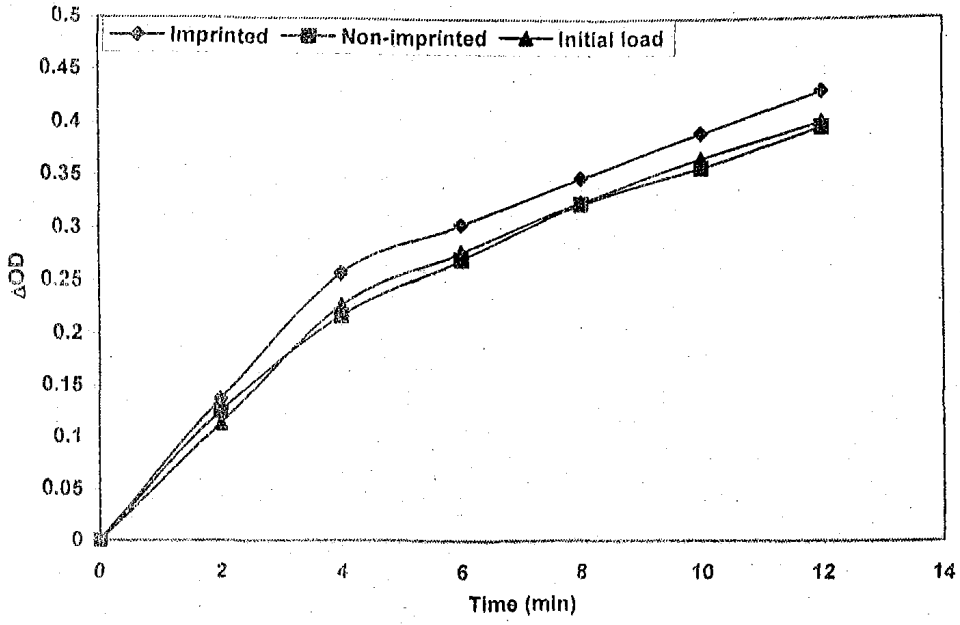


Fig. 4.19 Unbound lysozyme activity to imprinted and non-imprinted polymer in water. The change in absorbance (ΔOD) with time is recorded (at 450 nm)

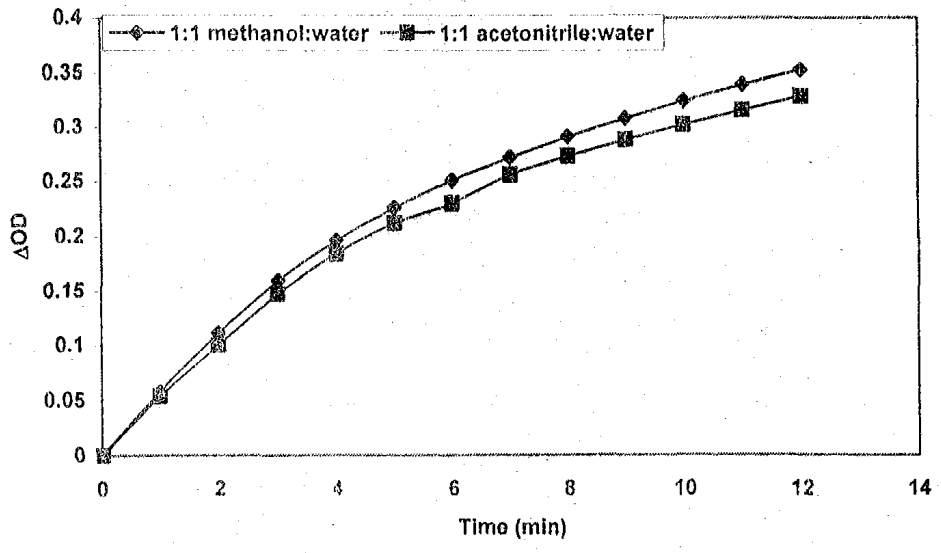


Fig. 4.20 Lysozyme activity in different solvents at 450 nm. Change in absorbance (ΔOD) with time is recorded

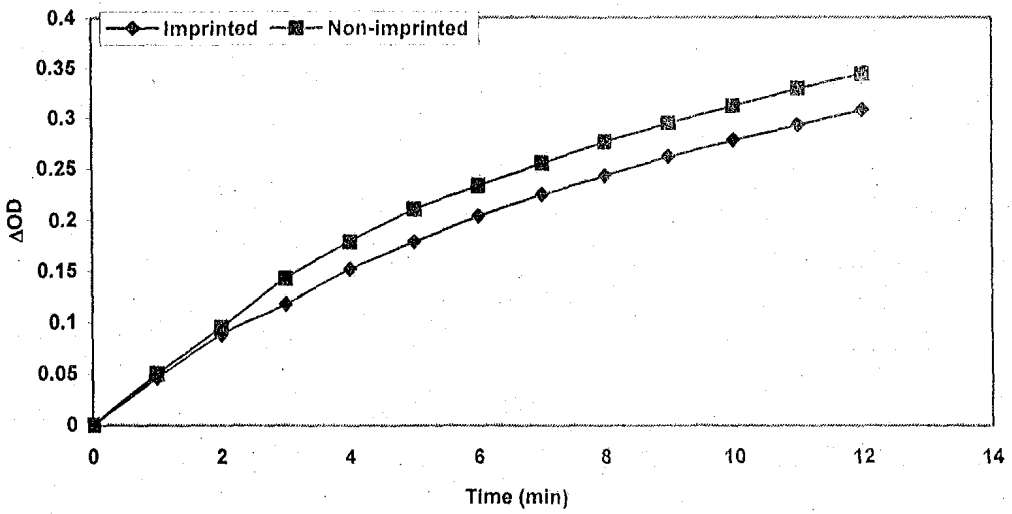


Fig. 4.21 Unbound lysozyme activity to imprinted and non-imprinted polymer in 1:1 methanol-water. Change in absorbance (ΔOD) with time is recorded

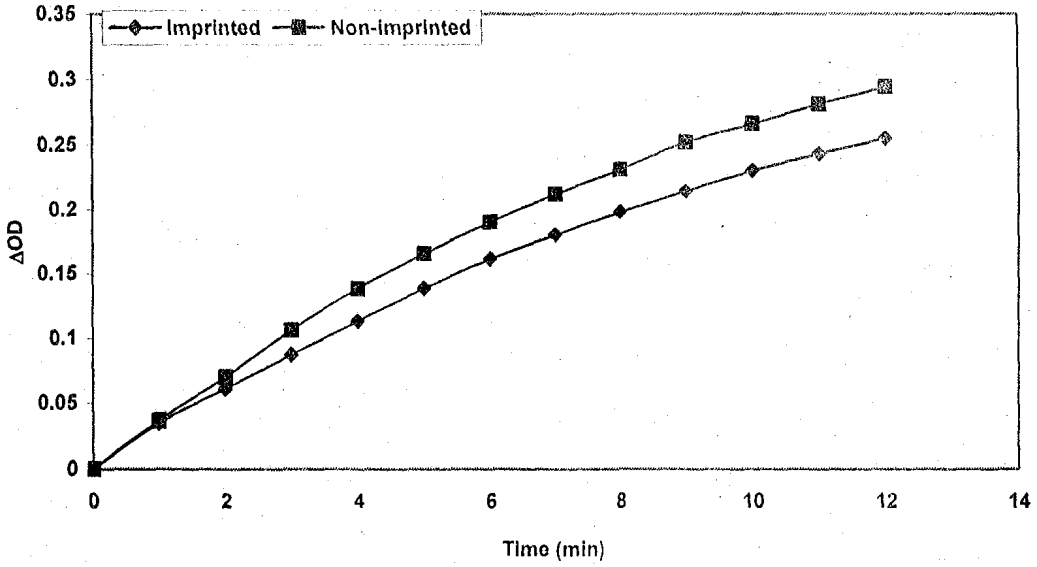


Fig. 4.22 Unbound lysozyme activity to imprinted and non-imprinted polymer in 1:1 acetonitrile-water. Change in absorbance (ΔOD) with time is recorded

4.1.7.1 Construction of multipurpose device

Multipurpose assembly is constructed from a commercially available 1.5 ml microcentrifuge tube (Fig. 4.23a). The tube has a recess inside the lid that forms chamber for placing the protein sample. The lid (Fig. 4.23b) and tube (Fig. 4.23c) are obtained from microcentrifuge tube by cutting attachment between lid and tube. This is followed by cutting of tube from lower end to make holed-tube (Fig. 4.23d). The hole in holed-tube is used for addition of water binding solvent / materials or exchange buffer or water into tube and their subsequent removal after sample concentration / dialysis / buffer exchange. The lid is placed on horizontal surface with recess surface upward (Fig. 4.23b). The sample is added to recessed portion of lid (Fig. 4.23e) and over this dialysis membrane is placed (Fig. 4.23f). The holed-tube in inverted position is then placed over the lid, so as to make perfect seal (Fig. 4.23g). Depending upon purpose for which device is to be used, water or exchange buffer or water binding solvent / material is added through hole of tube leaving some empty space (Fig. 4.23h). The hole in tube is then sealed with parafilm (Fig. 4.23i). The assembly is then fixed in rotary shaker (Fig. 4.23j), which is vertically rotated (Fig. 4.23k). This results in movement of protein solution in lid and water binding solvent / material or exchange buffer or water in holed-tube. During rotation, molecules permeable to membrane permeate across the membrane. Whereas for protein concentration, water binding materials or solvent such as glycerol is added to the tube, water and buffer are added for dialysis and buffer exchange, respectively. After rotation, device is removed from rotary shaker (Fig. 4.23l), followed by peeling off parafilm (Fig. 4.23m). Water binding solvent / materials or exchange buffer or water is then removed (Fig. 4.23n). Subsequently, holed-tube is detached (Fig. 4.23o). The sample is then recovered from recessed face of lid after removal of dialysis membrane (Fig. 4.23p).

4.1.7.2 Efficiency of multipurpose device

The efficiency of device for concentration of protein sample was checked by placing 20 or 40 or 60 or 80 or 100 percent glycerol in holed-tube.

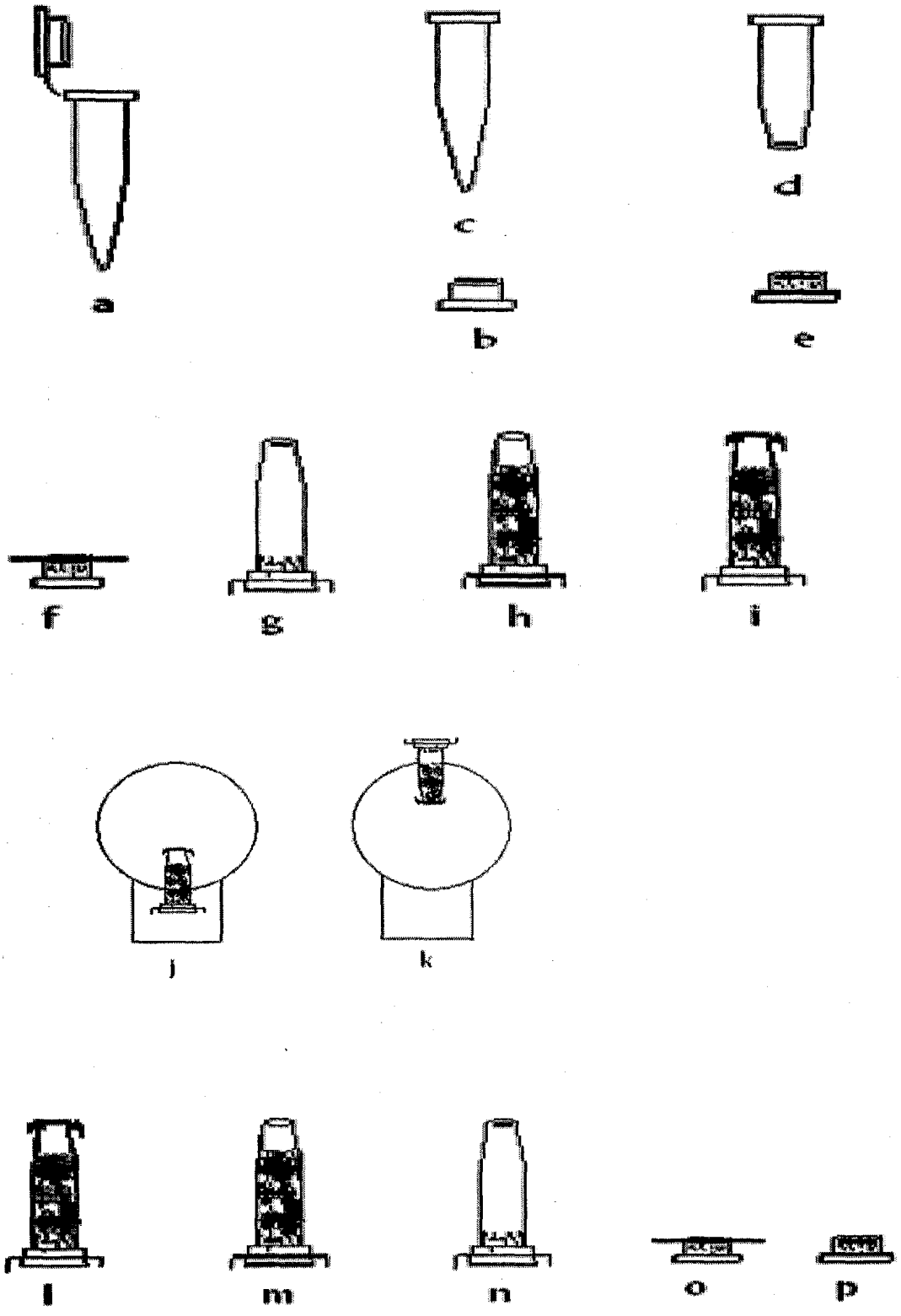


Fig. 4.23 Sequential steps in preparation of multipurpose device and its use

After different period of rotation (15, 30 and 60 min), sample was recovered; its volume and amount of protein measured (Bradford, 1976). The 100 percent glycerol was more effective in concentrating protein sample in comparison to glycerol at lower concentrations. The 150 µl protein samples could be concentrated to 10 µl in 60 min duration (Fig. 4.24). When 20, 40, 60 or 80 percent glycerol was used, nearly two fold reductions in volume could be achieved. There was quantitative recovery at all the concentrations of glycerol (Fig. 4.24). There is possibly that glycerol can cross dialysis membrane and may contaminate protein sample. The sample concentrated in this way will suit in SDS-PAGE since sample buffer in SDS-PAGE contains glycerol as one of the ingredient to make sample heavier. The efficiency of device was also checked with other water binding material such as Sephadex G-100 (Fig. 4.25). When 50 mg Sephadex beads were placed in holed-tube, 150 µl protein sample was concentrated to 23 µl in 5 h with quantitative recovery (Fig. 4.25). Since Sephadex cannot cross pores of membrane, sample is concentrated without contaminating sample with Sephadex and, therefore, such materials are recommended for their use where possible contamination of glycerol is not desired. As compared to Sephadex, glycerol is more efficient in removing water from protein sample and, therefore, sample concentration through use of glycerol will be method of choice if sample is to be used for SDS-PAGE. The latter technique is widely used in research laboratories. Efficiency of device for dialysis was checked by placing 200 µl of 5 percent potassium dichromate solution in recessed face of lid and 1.5 ml distilled water to holed-tube. Absorbance of solution was measured after 20 times dilution with water at different time. About 60 and 86 percent potassium dichromate molecules could be dialyzed out in 30 and 60 min, respectively, indicating efficient dialysis (Fig. 4.26). There was not much additional change in absorbance after 60 min. Therefore, it is advised to replace water in holed-tube after 60 min. Similarly buffer exchange can be achieved in 60 min by placing buffer in holed-tube.

During concentration and dialysis of protein sample, the device is rotated vertically. In each round of rotation, buffer / water strikes membrane. This can cause displacement of protein molecules adhering to membrane and

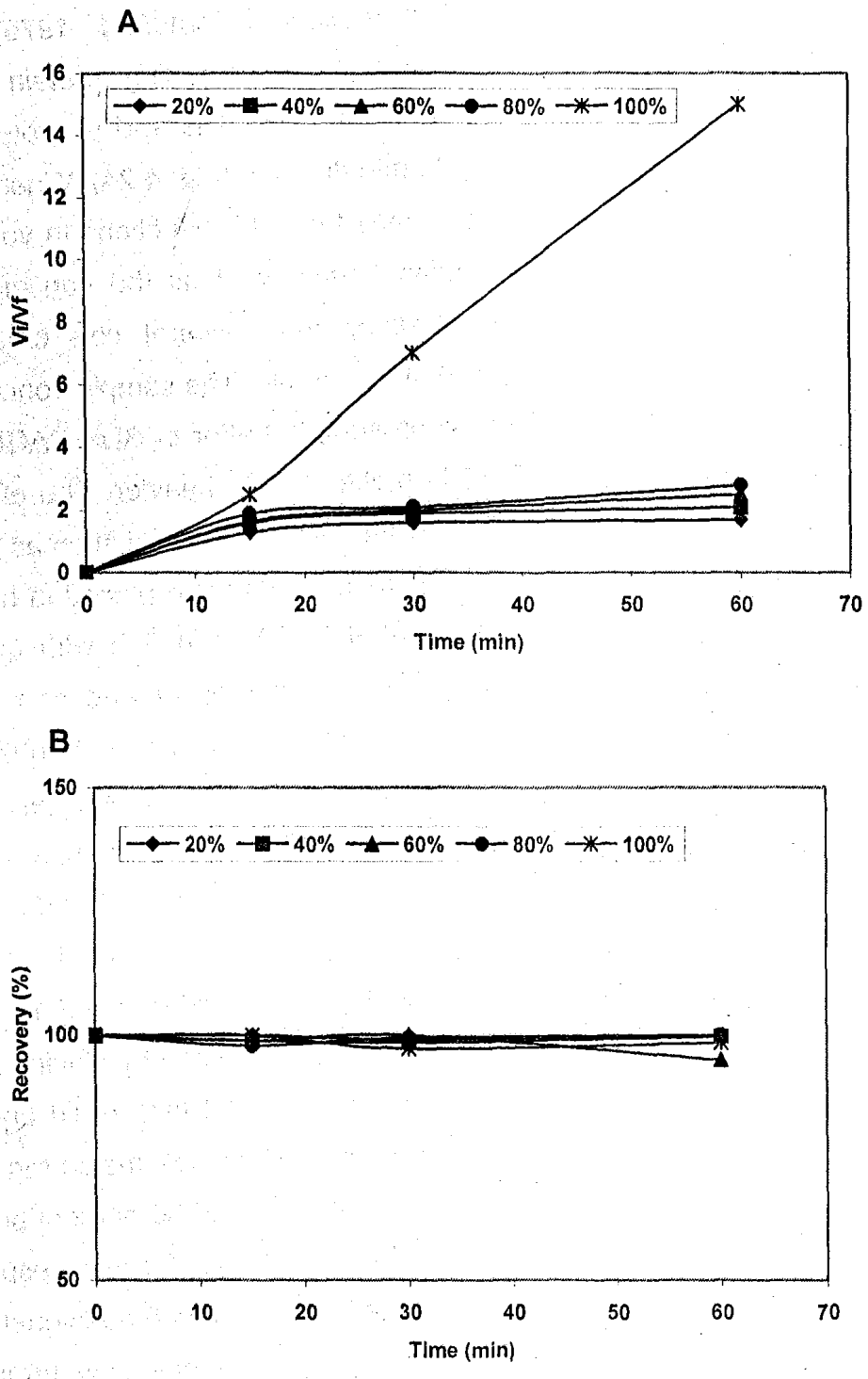


Fig. 4.24 Volume reduction (in folds) and recovery of protein using glycerol in holed-tube of multipurpose device. 150 µl (initial volume V_i) BSA (1 mg/ml) solution was subjected to concentration using different concentrations (20, 40, 60, 80 and 100%) of glycerol. At indicated time, final volume (V_f) and protein content of recovered sample were measured. (A) Volume reduction in folds (V_i/V_f), and (B) Protein recovery in percentage

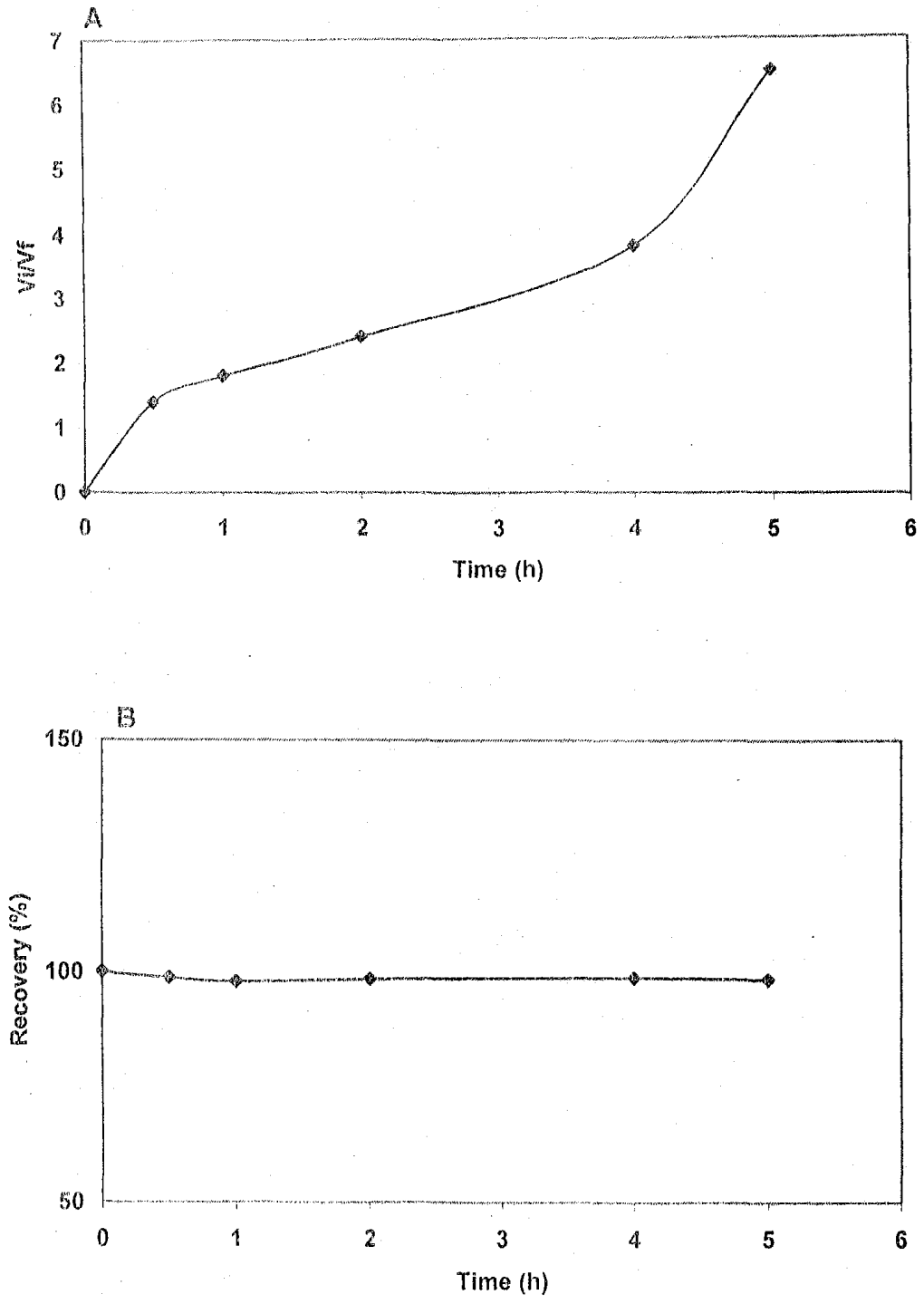


Fig. 4.25 Volume reduction (in folds) and recovery of protein using 50 mg Sephadex G-100 in holed-tube of multipurpose device. 150 µl (initial volume V_i) BSA (1 mg/ml) solution was subjected to concentration. At indicated time, final volume (V_f) and protein content of recovered sample were measured. (A) Protein concentration in folds (V_i/V_f), and (B) Protein recovery in percentage

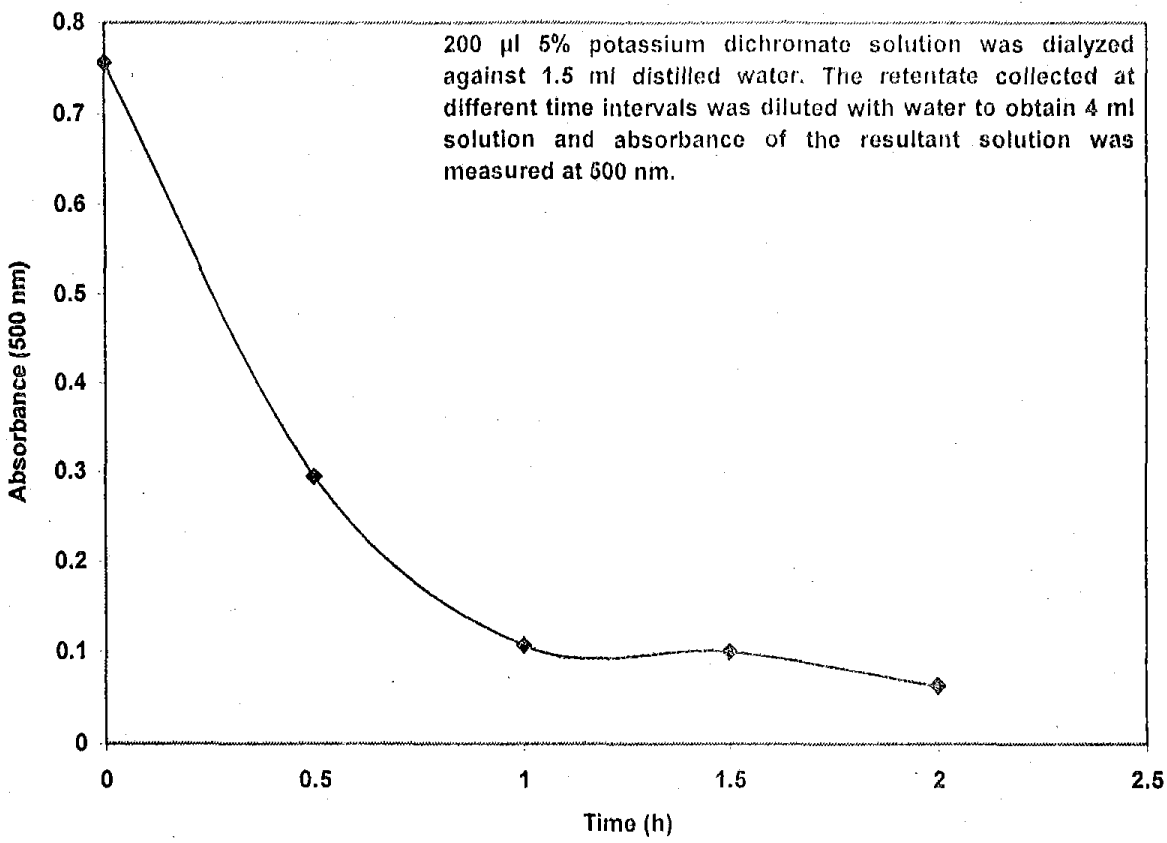


Fig. 4.26 Dialysis of potassium dichromate solution against distilled water

thereby free flow of small molecules across the membrane will be maintained. This is in contrast to most commonly available assembly for protein concentration / dialysis wherein centrifugal force is commonly used for pushing out small molecules across the membrane. Because of unidirectional force, large molecules, if bound to membrane, will not diffuse easily and result in membrane blockage. Over the time, these assemblies in fact work with decreased efficiency. During rotation, water binding materials in holed-tube mixes in each round of rotation and this allows new water-binding-molecules to come in contact with membrane thereby facilitating removal of dialyzed out water molecules. The process of protein concentration, dialysis and buffer exchange is simple and efficient, and recovery of protein is quantitative. The device and method is suitable for microlitre volumes and can be repeatedly used.

4.2 DISCUSSION

Large number of monomers and cross linkers are commercially available and this has helped in preparation of imprinted polymers at a faster rate. In present study, the efforts were made to prepare imprinted polymers against six different model molecules like tetracycline, lactic acid, retinyl acetate, ciprofloxacin, ascorbic acid and lysozyme. However, three of these preparations could be proved to contain cavities specific to target molecule. The preparation of polymer is not difficult and can be achieved in few days. But binding experiments had to be carefully designed for proving presence of cavities in imprinted polymer. Therefore, binding of the target molecule to the imprinted polymer has to be studied under different conditions such as (i) solvents of different polarity (ii) buffers of different pH) iii) different temperatures and (iv) different ionic strength. Only through careful binding studies one can prove presence of imprints in polymer. It has been observed that there was no selectivity of tetracycline to imprinted polymer at pH 4.0 (acetate buffer) whereas selectivity could be established in pure water, 1 M NaCl, methanol and acetonitrile. Similarly retinyl acetate imprinted polymer did not bind in methanol or acetonitrile; one could have believed as if the prepared polymer did not have affinity for retinyl acetate. However, the

binding studies in methanol-water mixture and acetonitrile-water mixture clearly demonstrated that imprinted polymer infact has imprints of retinyl acetate. To support it further, lactate imprinted polymer did show selectivity in pure water and the selectivity abolished in 1M NaCl. The selectivity of imprinted polymer over non-imprinted polymer will be achieved under the binding conditions where interaction between targeted molecule and polymer is favorable. The selectivity will be abolished under the conditions, which weakens these interactions. In the prepared polymers, hydrophobic interactions appear to be a major force in polymers prepared against tetracycline and retinyl acetate. The interaction of lithium lactate to PAA.HCl based polymer was ionic in nature.

In spite of number of different monomers used for preparation of imprinted polymer against ciprofloxacin, it has not been possible to have a polymer preparation having affinity to ciprofloxacin. Although we do not have a direct evidence of participation of ciprofloxacin in polymerization reaction, the possibility of such reaction cannot be ruled out owing to the presence of C-F bond in the molecule. For such molecules, it may be better to attempt preparation in presence of molecules similar to ciprofloxacin but lacking the bonds capable of participation in free radical reactions. It is being observed that ciprofloxacin can interact with tetracycline-imprinted polymer.

The work reported in thesis will allow concentration of small molecules such as tetracycline, ciprofloxacin and lactic acid present at very low concentrations and will therefore improve the sensitivity of methods used for estimation of these molecules. Further, because of the high specificity of the imprinted polymer, the interferences caused by the milk constituents in the estimation of small molecules will be largely eliminated.

The preparation of imprinted polymer against macromolecules has not been successful and also in present work, a preparation having imprints against lysozyme could not be achieved. The large size molecule will find difficulty in correctly orienting itself in a way to be able to interact with imprinted polymer. Some possibility does exist if intended cavities are present in the surface of the polymer. But these cannot create much difference between binding of macromolecule to imprinted and non-imprinted polymer.

Summary and Conclusions

5. SUMMARY AND CONCLUSIONS

Molecular imprinted polymers have tremendous scope in analytical chemistry and art of preparation of these polymers is actively pursued in many research laboratories in the world. Also, the preparation methods are still in infancy. The focus of the preparation methods for imprinted polymer is concentrated towards small molecules, the reason being very little steric hindrance or diffusion constraints in the process of interaction of target molecule with imprinted polymer. In view of it, in proposed work, efforts were made to prepare molecular imprinted polymers against small molecules such as tetracycline, ciprofloxacin, lactic acid, vitamin A and ascorbic acid. Although in general, there has not been any success in preparing molecular imprinted polymers against large molecules, efforts were made to prepare imprinted polymer against one such macromolecule, namely, lysozyme. The work reported in the thesis is largely confined to preparation protocols against targeted analytes. The equal emphasis has been made to prove or disprove whether prepared polymers have affinity (imprint) with target molecule. This required binding of target molecules under different solvents, varying in their polarity or at different pH or at different ionic strength. The prepared polymers were also evaluated for their specificity by evaluating the binding behaviour of similar or distinctly unrelated molecules from target molecules.

During the course of study, a multipurpose device for protein concentration, dialysis and buffer exchange was also designed.

The summary and conclusions from the present study carried out under the title "Preparation of molecular imprinted polymers against model molecules" are given below :

5.1 PREPARATION AND EVALUATION OF TETRACYCLINE IMPRINTED POLYMER

Tetracycline imprinted polymer was prepared using methacrylic acid (monomer), ethylene glycol dimethacrylate (cross-linker) and benzoyl peroxide (initiator). The polymerization reaction was carried out in acetonitrile and the polymer was prepared in presence (imprinted) or absence (non-imprinted) of tetracycline. The ratio of monomer : cross-linker : template was 3:25:0.25. The cross-linker concentration (on mole basis) was kept high for providing a close contact of tetracycline with atoms / groups of polymerization matrix. The final preparation obtained was hard and grainish in texture and was light brown in colour. From 262 µl methacrylic acid and 4.76 ml of ethylene glycol dimethacrylate, approximately 2.5 g of polymer matrix was obtained. The polymer prepared in absence of tetracycline was white in colour and was of similar in appearance and hardness as that of polymer prepared in presence of tetracycline.

The presence of imprints in prepared polymer was established by studying the binding of tetracycline to prepared polymer in water, 1 M NaCl, methanol, acetonitrile and buffers of different pH values. The values of partition coefficient, binding capacity and selectivity clearly established that compared to non-imprinted polymers, imprinted polymers have distinct affinity for tetracycline. The selectivity values were in the range of 1.5 to 3.9 and were dependent on the solvent used. The selectivity values were highest in water and at pH 7.0. The lower selectivity in less polar solvent (methanol or acetonitrile) compared to water indicates that interaction of tetracycline with imprinted polymer is hydrophobic in nature. The differences in selectivity in water and acetonitrile were exploited in binding and elution of tetracycline in a chromatographic column packed with imprinted polymer. The binding of tetracycline to non-imprinted polymer in water was nearly quantitative and bound tetracycline could be eluted out with increasing concentration of acetonitrile. The results clearly suggested that tetracycline-imprinted polymer could be effectively used in chromatographic column. The specificity of the

imprinted polymer was checked by evaluating the binding of ciprofloxacin and amoxicillin. Tetracycline imprinted polymer did have affinity for ciprofloxacin, but not to amoxicillin. The structure of amoxicillin is more open as compared to tetracycline and thus did not fit well into cavities created for tetracycline. Further, tetracycline imprinted polymer could interact with ciprofloxacin and, therefore, the prepared polymer can be used for concentration of ciprofloxacin as well. The performance of the prepared polymer was also evaluated in milk samples spiked with tetracycline and the polymer was able to recognize tetracycline in milk. Milk with tetracycline (100 µg/ml) provided nearly 9 fold absorbance of eluent coming out with 100% acetonitrile over the milk samples without tetracycline. Therefore, it can be concluded that tetracycline-imprinted polymer can be applied to milk samples. The prepared polymer was stable at room temperature at least up to 9 months, indicating that the binding pockets are very stable in network of polymer preparation. The prepared polymer could be used repeatedly (at least 5 times) without loosing efficiency.

5.2 PREPARATION AND EVALUATION OF LITHIUM LACTATE IMPRINTED POLYMER

Lithium lactate imprinted polymer was prepared by cross-linking poly-allylamine hydrochloride with epichlorohydrin in presence of lithium lactate. The selection of poly-allylamine hydrochloride as pre-formed polymer was based upon possible ionic interactions between amino groups in PAA.HCl and carboxyl groups present in lithium lactate. The prepared polymer was gelly in structure and transparent. For comparative purposes, polymer in absence of lithium lactate was also prepared. The partition coefficient, binding capacity and selectivity of the prepared polymer was studied in water, 1 M NaCl and buffers of different pH values ranging from 4.0 to 7.0.

The higher selectivity was noted in water and in BES buffer of pH 7.0. There was no selectivity in 1 M NaCl indicating that recognition of lithium lactate by prepared polymer is ionic in nature. The lower selectivity at pH 4.0, 5.0 and 5.6 was either due to existence of more molecules of acetic acid in undissociated state or inhibition of interaction by the ions present in the buffer.

It can be concluded that in the binding pocket, the interaction between the negatively charged carboxyl groups and positively charged amino group is the key force in recognition. The prepared polymer was also evaluated for their selectivity towards D(-) and L(+) lactic acid. It was found that imprinted polymer did not differentiate between D(-) lactic acid, L(+) lactic acid and lithium lactate. Therefore, the prepared imprinted polymer has potential to concentrate total lactic acid. The performance of the prepared polymer was further evaluated from binding and elution behaviour of lithium lactate in chromatographic column. There was quantitative binding of lithium lactate in water to imprinted polymer and the bound lactic acid could be eluted by application of 1 M NaCl. These results also confirm our earlier conclusion that NaCl can disrupt the ionic interaction between lithium lactate and polymer. The prepared polymer, when stored in water at room temperature, remained stable up to 9 months. This suggested that cavities for lithium lactate were highly stable in the network of polymer preparation. The experiments were also conducted for the applicability of imprinted polymer to milk. Since the interaction between lactate and polymer is ionic in nature, and also the fact that milk contains various ions at different concentration, the prepared polymer cannot be used for concentrating lactic acid molecule.

5.3 PREPARATION AND EVALUATION OF IMPRINTED POLYMER OF VITAMIN A

Retinyl acetate imprinted polymer was prepared using methacrylic acid (monomer), ethylene glycol dimethacrylate (cros-linker) and benzoyl peroxide (initiator). The polymerization reaction was carried out in methanol and the polymer was prepared in presence (imprinted) or absence (non-imprinted) of retinyl acetate. The ratio of monomer : cross-linker : template was 3:25:0.25. The cross-linker concentration (on mole basis) was kept high for providing close contact of retinyl acetate with atoms / groups of polymerization matrix. The final preparation obtained was hard and grainish in texture and was white in colour. From 262 µl methacrylic acid and 4.76 ml of ethylene glycol dimethacrylate, approximately 2.5 g of polymer matrix was obtained. The polymer prepared in absence of retinyl acetate was also white in colour and

was of similar in appearance and hardness as that of polymer prepared in presence of retinyl acetate.

The binding of retinyl acetate to prepared polymer was evaluated in methanol, acetonitrile and water mixture of these solvents. The selectivity was highest in 1:1 acetonitrile-water mixture and there was no binding (selectivity) in acetonitrile or methanol. The results indicated that prepared polymers have imprints of retinyl acetate and the recognition were through hydrophobic interaction. The chromatographic profile of binding and elution of retinyl acetate to imprinted and non-imprinted polymers clearly established the presence of cavities specific to retinyl acetate in imprinted polymers and the abolition of recognition in less polar solvents such as acetonitrile. The prepared polymer was also evaluated for its binding to distinctly unrelated molecules such as tetracycline and ciprofloxacin. The results indicated that prepared polymer did not have any recognition for tetracycline and ciprofloxacin. The prepared polymer was stable at room temperature at least up to 7 months indicating that binding pockets are very stable in network of polymer preparation.

5.4 CIPROFLOXACIN IMPRINTED POLYMER

Using general methods of preparation of imprinted polymer, the imprinted polymers were prepared against ciprofloxacin. The monomers used were methacrylic acid, methyl methacrylate and 4-vinyl pyridine. The complete polymerization could not be achieved when methyl methacrylate was cross-linked with EGDMA in presence of ciprofloxacin. The polymers prepared with methacrylate, 4-vinyl pyridine and in presence of ciprofloxacin did not show any selectivity as compared to polymers prepared in absence of ciprofloxacin. Although, the preparation methods and the monomers were of similar in nature as that used in preparation of tetracycline imprinted polymers, the prepared polymers did not have cavities specific to ciprofloxacin. Although the reasons are not much clear, the possibility of participation of ciprofloxacin in free radical reaction cannot be ruled out owing

to the presence of C-F bond present in molecule. Further, poly-allylamine based imprinted polymers also did not exhibit any selectivity.

5.5 ASCORBIC ACID IMPRINTED POLYMER

The ascorbic acid imprinted polymer was also prepared from methacrylic acid, EGDMA and benzoyl peroxide. The prepared polymer was evaluated for its binding to ascorbic acid. The binding protocol required incubation of prepared polymer with ascorbic acid for 24 h. It was observed that during the incubation, ascorbic acid oxidized to dehydro ascorbic acid and accurate measurement of ascorbic acid could not be achieved. In such situation, it was not feasible to conclude whether prepared polymer has imprints of ascorbic acid or not.

5.6 LYSOZYME IMPRINTED POLYMER

Lysozyme is a large molecule having molecular weight of 15 kDa. The polymer was prepared from pre-formed polymer PAA.HCl. The preparation was gelly, but in contrast to lactate imprinted PAA.HCl based polymer, the preparation was translucent. Lysozyme binding studies were carried out in water, 1:1 methanol-water mixture and 1:1 acetonitrile-water mixture. The results did not support that the prepared polymer has cavities specific to lysozyme.

5.7 MULTIPURPOSE DEVICE FOR PROTEIN CONCENTRATION, DIALYSIS AND BUFFER EXCHANGE

During the course of study, a device for protein concentration, dialysis and buffer exchange was developed and a patent has been filed (1703/DEL/2006). This device could concentrate 150µl protein sample to 10µl in 60 min duration. The protein recovery was quantitative. This multi purpose device could also dialysed out 86% of potassium dichromate molecules in 60 min.

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