SYNTHESIS AND EVALUATION OF POLYMERS FOR SELECTIVE SEPARATION OF CHOLESTEROL

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SYNTHESIS AND EVALUATION OF POLYMERS FOR SELECTIVE SEPARATION OF CHOLESTEROL

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BY

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DEDICATED TO MY FAMILY

CERTIFICATE

This is to certified that the work incorporated in the thesis entitled **"Synthesis and Evaluation of Polymers for Selective Separation of Cholesterol"** submitted by Mr. Mahesh Ambadas Gore, was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

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DECLARATION

I hereby declare that the work embodied in the thesis entitled **"Synthesis and Evaluation of Polymers for Selective Separation of Cholesterol"** submitted for Ph. D. degree to the University of Pune, has been carried out by me at the National Chemical Laboratory, Pune, under the supervision of Dr. M.G. Kulkarni. The work is original and has not been submitted in part or full by me for any degree or diploma to this or any other University. Whenever references have been made to previous works of other, it has been clearly indicate as such and included in the Bibliography.

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Mr. Mahesh Ambadas Gore

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ABSTRACT

Introduction

Cholesterol is a very important fatty steroid produced by liver and many other cells in the body. It helps to build structure of cell membranes and hormones like oestrogen, testosterone and adrenaline. Cholesterol produces bile acids, which help to digest fat and absorb important nutrients. But when the total amount of cholesterol synthesized by the body and obtained from diet, exceeds the amount necessary for the synthesis of membrane, bile acids and steroids, it leads to atherosclerotic plaque leading to heart attack or stork [Holtmeier, 1996]. Efforts are therefore under way to develop materials and methods to sequester cholesterol.

Attempts have been made to remove excess cholesterol using materials, which are biocompatible and clinically efficient. Some of these are statins, bile acid sequestrants [BAS], Fibrates, Niacin etc. However, each of these materials has its own drawbacks e.g statins cause anaemia, acidosis etc, BAS are needed in high dose, Fibrates cause rhabdomyolysis, abdominal pain while use of Niacin leads to hepatotoxicity, hyperglycemia etc.

New techniques for efficient separation of cholesterol from body continue to be explored. One of the techniques used extensively for the separation of cholesterol is the use of molecularly imprinted polymers [MIPs] as adsorbents [Davidson et al., 2002]. The basic principle used in molecular imprinting is to form an assembly comprising functional monomer and template molecule involving either covalent or non-covalent interactions in the presence of excess of crosslinker. Polymerization of this assembly and subsequent removal of template molecule leads to the formation of the cavity, which resembles in shape and size the template molecule and can be used for separation of template molecule *vis a vis* a molecule which has the same functional groups but different structure e.g. racemic mixtures of drugs. Broadly the researchers have exploited hydrogen bonding [Whitcombe et al., 1995] for rebinding of cholesterol from non aqueous media, and hydrophobic binding as well as inclusion complexes with cyclodextrin for rebinding from aqueous media [Asanuma et al., 1997]. One of the limitations of these polymers is their low capacity and selectivity resulting from non-specific binding of cholesterol on to the

hydrophobic crosslinking monomer used in polymerization [Zhong et al., 2001, Davidson et al., 2002].

In this work we have investigated molecular imprinting and affinity precipitation approaches for the selective separation of cholesterol and its derivatives like bile salts. MIPs developed hitherto have poor binding capacities and low selectivities.

To overcome the limitations of MIPs mentioned above, we explored newer approaches like incorporation of hydrophilic crosslinkers to minimize non-specific adsorption and use of cholesterol containing crosslinkers rather than cholesterol bearing monomers to retain the degree of crosslinking when the extent of cholesterol bearing monomer is increased [Gore et al., 2004].

The conventional molecular imprinting technique leads to the shrinkage of imprinted cavity and hence low degree of utilization of active site. The two stage approach involving sequential polymerization / crosslinking method has been shown to result in enhanced active site utilization [Gore et al., 2005a, 2005b].

In affinity precipitation approach, an affinity ligand is coupled to a water-soluble smart polymer to form a macroligand. This macroligand when mixed with protein solution forms a complex with the target protein. Phase separation occurs on change of the environment, which makes the polymer backbone insoluble [Mattiasson et al., 1998, Galaev et al., 1996, Gupta et al., 1994]. The present investigation has been undertaken to design and synthesize new affinity polymers that would enhance the binding between the ligand and the sorbate. β-Cyclodextrin is well known to form inclusion complexes with cholesterol [Szjtli, 1982]; and was therefore selected as an affinity ligand for the separation of cholesterol.

To overcome the crowding effect, various β-cyclodextrin monomers and dimers of β-cyclodextrin have been synthesized using spacers and linkages of β-cyclodextrin to vinyl group through primary or secondary binding sites of β-cyclodextrin and binding between cholesterol and monomers was studied by the solubility method [Breslow et al., 1996]. The monomers have been co-polymerized with N-isopropylacrylamide to yield hetero-bifunctional affinity polymers. The study demonstrated that as the degree of substitution [DS] of β-cyclodextrin increased, the binding constant increased but in case of dimer β-cyclodextrin conjugated polymers, it was independent of dimer β-cyclodextrin content.

The mechanism and stoichiometry of binding has been investigated by fluorescence technique using pyrene as a probe.

The work is presented in seven chapters and a brief outline of each is given below.

Chapter 1: Literature survey

In this chapter prior literature on MIPs for cholesterol removal, development of new BAS and ligand-complex formation for affinity precipitation has been reviewed.

Methodologies developed in the past based on covalent and noncovalent molecular imprinting techniques used for the separation of cholesterol have been described along with merits and limitations of each. Various approaches developed for the synthesis of BAS have been illustrated with examples. A detailed search of marketed BAS and criteria to develop BAS such as selection of functional monomer, conditions used for the rebinding of bile salts etc. has been made.

The discussion on affinity precipitation includes both homo and hetero-bifunctional precipitations. Merits and limitations of each method are summarized. Affinity thermoprecipitation is described with examples from the literature so far. The prerequisites for an ideal polymer to be used in affinity precipitation are summarized.

Applications of β-cyclodextrin and its polymers in separation and recovery of cholesterol using cyclodextrin containing monomers and dimers have been reviewed. Various techniques used to characterize complexes comprising cholesterol and cyclodextrin have been discussed.

Formation of inclusion complexes between cyclodextrin and various fluorescent molecules using fluorescence spectroscopy has been reviewed. The mode of binding and stoichiometry between these complexes are highlighted.

Chapter 2: Objectives and scope of the work

This work is directed towards the design, synthesis and evaluation of polymers that will overcome some of the limitations of MIPs and affinity based separation of cholesterol. The investigation has been undertaken with following objectives.

1. To design and synthesize MIPs, which exhibit enhanced capacities as well as selectivities for cholesterol. Cholesterol bearing crosslinkers will be synthesized to enhance cholesterol moiety into the polymer without changing the degree of

crosslinking. The role of hydrophilic crosslinker in minimizing nonspecific adsorption would be investigated to enhance the capacity.

2. To design and synthesize MIPs to minimize shrinkage and yield high percentage utilization of binding sites during BAS rebinding. A two stage approach involving sequential polymerization / crosslinking developed in our laboratory will be extended to validate its utility in enhancing binding site utilization. The approach involves selective polymerization of one vinyl group from divinyl monomer, with a functional monomer and subsequent crosslinking in the presence of the template
3. To design and synthesize new affinity thermoprecipitating polymers containing β-cyclodextrin ligand for the separation of cholesterol from aqueous medium. As a first step binding constant between β-cyclodextrin monomers, homopolymers, dimers and copolymers with cholesterol will be estimated by the solubility method. Study of binding constant between β-cyclodextrin or dimer β-cyclodextrin conjugated polymers and cholesterol as a function of DS of β-cyclodextrin to the polymer will be taken up.
4. To study mode of binding and stoichiometry between β-cyclodextrin or dimer β-cyclodextrin conjugated polymers, using pyrene as a fluorescent probe by fluorescence spectroscopy.

Chapter 3: Enhanced capacities and selectivities for cholesterol in aqueous media by molecular imprinting: role of novel cross linkers

MIPs for the binding of cholesterol reported so far, offer limited capacities. In this chapter we report cholesterol bearing monomers and crosslinkers for the synthesis of MIPs. To enhance the capacity and selectivity, cholesterol bearing monomers monocholesteryl itaconate and cholesteryl methacrylate as well as crosslinker glyceryldicholesteryl itaconate and monocholesteryl itaconate glycerol methacrylate have been synthesized. Binding sites are created by hydrophobic interactions between cholesterol bearing monomers and the template added during polymerization. Imprinted polymers prepared using equivalent amounts of functional monomer and template exhibited enhanced capacities [43.7 mg/g] in aqueous medium. The non specific binding is suppressed when crosslinkers containing hydrophilic groups *viz* glyceroldimethacrylate and monocholesteryl itaconate glycerol methacrylate are used. The ratio of binding

capacities for imprinted versus non imprinted polymers is 3.7 for cholesterol, 1.89 for stigmasterol and 2.3 for testosterone. The imprinting effect is reflected in higher capacity and selectivity values for cholesterol *vis a vis* other sorbates.

Chapter 4: Enhancing active site utilization in molecularly imprinted polymers: a new synthetic approach

In the conventional approach, the supramolecular assembly comprising functional monomer, crosslinker and the template is polymerized. The template is leached out and the polymer is used for the rebinding of template molecule. The template molecule thus finds itself in different environments during polymerization and rebinding stages. This is probably one of the reasons for low rebinding capacity and selectivity of MIPs. This chapter presents a two stage approach, which involves sequential polymerization / crosslinking for the synthesis of MIPs which minimizes shrinkage of imprinted cavity and enhances percentage utilization of binding site. In this approach one of the vinyl group of divinyl monomer is protected by complexation with dimethyl β-cyclodextrin [DMCD]. The selective polymerization of a single vinyl group of divinyl monomer, methylene bis acrylamide with allylamine hydrochloride yields a water soluble polymer which is followed by crosslinking in the presence of the template sodium cholate [NaC]. This leads to enhanced active site utilization to 84% as compared to the 33% by conventional method. This method results in enhanced binding capacity [587 mg/g] as well as selectivity [$\propto$ = 1.72] for NaC. The corresponding values for imprinted polymers prepared by conventional simultaneous polymerization, imprinting and crosslinking technique are [475 mg/g, $\propto$ = 1.35]. Selectivity study reveals that with increasing ratio of template to functional monomer, selectivity for NaC over sodium taurocholate [NaT] increased systematically from 1.64 to 3.55.

Chapter 5: Enhancing cholesterol-β-cyclodextrin binding: role of polymer architecture

In this chapter binding between cholesterol and β-cyclodextrin, polymers containing β-cyclodextrin, dimer β-cyclodextrin and polymer conjugates of dimer β-cyclodextrin has been investigated by solubility method. Monomers containing β-cyclodextrin and its dimers have been synthesized by varying spacer length and conjugation sites. The DS of β-cyclodextrin and dimer β-cyclodextrin on the polymer was varied from 2 to 67 mole %.

In case of polymers containing β-cyclodextrin, the association constant increased with β-cyclodextrin content. This indicates intermolecular stage wise binding between cyclodextrin and cholesterol at lower DS and chelate type binding at higher DS. Association constants between cholesterol and β-cyclodextrin dimers and polymer conjugates of dimer β-cyclodextrin are independent of DS, since in both cases only intramolecular chelate complexes are formed.

Chapter 6: Complexation between pyrene and β-cyclodextrin polymers: fluorescence study

The mode of binding and stoichiometry between β-cyclodextrin and pyrene has been investigated using fluorescence spectroscopy. A stagewise formation of 1:1 [β-cyclodextrin: pyrene] and intermolecular 2:1 complexes at low DS [below 5%] and 2:1 intramolecular chelate type complexes at higher DS [above 5%] is confirmed. On the other hand dimer β-cyclodextrin and dimer β-cyclodextrin conjugated polymers always formed 2:1 intramolecular chelate type complex, independent of the DS. In the former case the association constant increases with DS while in the latter case it is independent of DS.

Chapter 7: Conclusions and suggestions for future work

Our studies on recovery of cholesterol by MIPs and affinity precipitation technique lead to the following conclusions.

1. Use of cholesterol bearing monomers and crosslinkers enhances capacities as well as selectivities.
2. Hydrophilic crosslinker minimizes non specific adsorption of cholesterol.
3. The sequential polymerization / crosslinking technique leads to enhanced binding capacities and selectivities.
4. In affinity precipitation technique, crowding effect is minimized when spacer between polymer backbone and ligand, whereas stability of complex can be increased by incorporating more number of ligands.
5. The investigations on mode of binding and stoichiometry between β-cyclodextrin and pyrene by fluorescence spectroscopy, demonstrate 2:1 intermolecular binding at low [below 5%] DS, whereas intramolecular chelate type binding results at high [above 5%] DS.

Based on the conclusions arrived at, suggestions for future work are to validate the consequences resulting from this work and to enhance the capacities and selectivities of cholesterol or bile salts during recovery from aqueous media are summarized below.

1. Synthesis of bile salt bearing monomers and crosslinkers for the MIPs.
2. Synthesis of cholesterol bearing monomers for the sequential polymerization / crosslinking technique.
3. Use of two stage MIP approach with partial alkylation and quaterization of allylamine hydrochloride.
4. Application of two stage MIP approach with other functional monomers like 2-amino ethyl acrylate, since the incorporation of allylamine hydrochloride into the polymer is very low.
5. Investigation of the effect of pH and temperature on binding between cyclodextrin based polymers and pyrene using fluorescence spectroscopy for better understanding and designing of polymers for drug delivery systems.

References:

1. Asanuma H, Kakaza M, Shibata M, Hishiya T, Komiyama M, Chemical Communications, 1971, 1997.
2. Breslow R, Zhang B. J. Am. Chem. Soc., **118**, 8495, 1996.
3. Davidson L, Hayes W, Curr. Org. Chem., **6**, 265, 2002.
4. Galaev I. Y, Gupta M. N, Mattiassson B, Chemtech, December, 19, 1996.
5. Gore M. A, Karmalkar R. N, Kulkarni M. G, J. of Chromatography B, **804**, 211, 2004.
6. Gore M. A, Kulkarni M. G, Communicating shortly, 2005a.
7. Gore M. A, Kulkarni M. G, US Patent filed No.: NCL/NF 218/ 2005b.
8. Gupta, M. N, Mattiasson, B, Affinity Precipitation in Highly Selective Separations in Biotechnology, G. Street ed., Blackie Academic and Professional, Glasgow, UK, pp. 7-33, 1994.
9. Holtmeier H. J, Cholesterin-Zur physiologie, Pathophysiologie and KliniL: Springer-Verlag, Berlin, 1996.
10. Mattiasson B, Dainyak M. B, Galaev I. Y, Polym. Plast. Technol. Eng., **37**, 303, 1998.

11. Szjtli J, Cyclodextrins and their inclusion complexes, Academiai Kiado, Budapest, 1982.
12. Whitcombe M. J, Rodriguez E, Villar P, Vulfson E.N, J. Am. Chem. Soc., **117**, 7105, 1995.
13. Zhong N, Byun H. S, Bittman R, Teterahedron Letters, **42**, 1839, 2001.

Chapter 1

Literature Survey

1.0.0 Introduction

Cholesterol is a very important fatty steroid produced by liver and many other cells in the body. It helps to build structure of cell membranes and hormones like oestrogen, testosterone and adrenaline. Cholesterol produces bile acids, which help to digest fat and absorb important nutrients. But when the total amount of cholesterol synthesized by the body and obtained from diet, exceeds the amount necessary for the synthesis of membrane, bile acids and steroids, it leads to atherosclerotic plaque leading to heart attack or stork [Holtmeier, 1996]. Efforts are therefore under way to develop materials and methods to sequester cholesterol.

Attempts have been made to remove excess cholesterol using materials, which are biocompatible and clinically efficient. Some of these are statins, bile acid sequestrants [BAS], Fibrates, Niacin etc. However, each of these materials has its own drawbacks e.g statins cause anaemia, acidosis etc, BAS are needed in high dose, Fibrates cause rhabdomyolysis and abdominal pain, while use of Niacin leads to hepatotoxicity, hyperglycemia etc.

New techniques for efficient separation of cholesterol from body continue to be explored. One of the techniques used extensively for the separation of cholesterol, is the use of molecularly imprinted polymers [MIPs] as adsorbents [Davidson et al., 2002]. The basic principle used in molecular imprinting is to form an assembly comprising functional monomer and template molecule involving either covalent or non covalent interactions in the presence of excess of crosslinker. Polymerization of this assembly and subsequent removal of template molecule leads to the formation of the cavity, which resembles in shape and size, the template molecule and can be used for separation of template molecule *vis a vis* a molecule which has the same functional groups but different structure e.g. racemic mixtures of drugs. The researchers have exploited hydrogen bonding [Whitcombe et al., 1995] for rebinding of cholesterol from non aqueous media, and hydrophobic binding as well as inclusion complexes with cyclodextrin for rebinding from aqueous media [Asanuma et al., 1997]. One of the limitations of these polymers is their low capacity and selectivity resulting from non-specific binding of cholesterol on to the hydrophobic crosslinking monomer used in polymerization [Zhong et al., 2001, Davidson et al., 2002].

In affinity precipitation approach, an affinity ligand is coupled to a water-soluble smart polymer to form a macroligand. This macroligand when mixed with protein solution forms a complex with the target protein. Phase separation occurs on change of the environment, which makes the polymer backbone insoluble [Mattiasson et al., 1998, Galaev et al., 1996, Gupta et al., 1994]. The present investigation has been undertaken to design and synthesize new affinity polymers that would enhance the binding between the ligand and the substrate. β-Cyclodextrin is well known to form inclusion complexes with cholesterol [Szejtli, 1996]; and was therefore selected as an affinity ligand for the separation of cholesterol.

In the present work we have directed our efforts to design and synthesize polymers based on molecular imprinting and affinity precipitation technique for the selective separation of cholesterol and its derivatives *viz* bile acids. For the molecular imprinting technique, we have developed new cholesterol bearing monomers and crosslinkers, which would lead to enhanced capacity and selectivity. In addition, a two-step polymerization technique has been developed for the synthesis of molecularly imprinted polymers. This technique was used for the selective separation of bile salts. For the affinity precipitation technique, β-cyclodextrin was used as an affinity ligand for cholesterol and N-isopropylacrylamide [NIPA] as the thermosensitive monomer. We designed and synthesized various monomers and dimers containing β-cyclodextrin to enhance the separation as well as binding constant for cholesterol. For the better separation, binding constant between cyclodextrin containing monomers, dimers, homopolymers and copolymers were studied with cholesterol using solubility method. In addition, binding between β-cyclodextrin and cholesterol was investigated using pyrene as a fluorescent probe.

The present work has been undertaken after a systematic review of the literature available. As mentioned above, our work is focused on designing polymers for the separation of cholesterol from aqueous medium. We have confined our literature survey mainly to the various molecular imprinting and affinity based separation techniques and polymers used therein.

The literature survey starts with a brief description of conventional techniques used for the separation and recovery of biomolecules. This is followed by a description of the

merits of molecular imprinting and affinity techniques over earlier techniques. Various examples cited in the literature for each of the separation technique have been described. Finally, the unresolved issues have been highlighted which form the basis of our investigation.

1.1.0 Molecular imprinting

1.1.1 Principle of molecular imprinting

Following important steps are involved in molecular imprinting.

1. Pre-organiztion of functional monomers around the template molecule, which resembles the shape and size of the substrate molecule, by different types of interactions such as covalent, non covalent and coordination interactions.

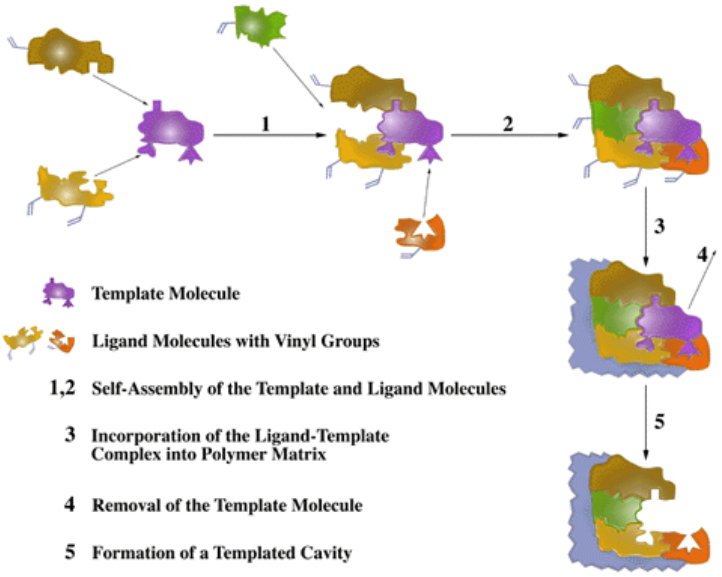
2. Polymerization of the macromolecular assembly with excess of crosslinker and subsequent removal of template so as to retain the specific orientation of functional groups around the cavity created by the template molecule [Figure 1.1].

Two basic approaches have been used for molecular imprinting 1. In the pre-organized approach, developed by Wulff and coworkers [1972] the aggregates in solution prior to polymerization are maintained by [reversible] covalent bonds. 2. In the self-assembly approach, developed by Mosbach and coworkers [1989] a supramolecular assembly comprising the print molecule and the functional monomers is formed by non covalent interactions. These approaches are summarized in the following paragraphs.

1.1.2 Pre-organized approach: Covalent imprinting

In this approach functional monomer and template are bound to each other by covalent linkages. Polymerization of monomer-template assembly with an excess crosslinker and subsequent removal of template by hydrolysis creates the cavity of shape and size complementary to the template molecule. Cavity formed by this method is much more specific than that formed by non covalent interactions. Wulff and Sarahan [1972] developed the concept of molecular imprinting in crosslinked polymers using polymerizable template-monomer assembly. A template molecule α-D mannopyranoside was esterified with para vinylphenyl boronic acid. Polymerization of this assembly, with divinylbenzene gave a highly crosslinked polymer with boronate ester covalently linked to the polymer [Figure 1.2]. Template molecule was then removed from the network by the hydrolysis of the ester. The free boronic acid groups in the polymer exhibited specific

Figure 1.1: Principle of molecular imprinting technique



binding for the template used in imprinting. The template used was optically active. Selectivity was studied by equilibrating the polymers with racemates of α D-mannopyranoside. Polymers exhibited selective sorption of the imprinted enantiomer with separation factor 1.2 to 6.0 depending upon the experimental conditions. It was shown that upto 70% α-D mannopyranoside could be obtained in batch process [Wulff et al., 1995]. Extensive research efforts on the covalent imprinting approach have been reported by Wulff and coworkers [1985, 1995], which highlight various parameters that enhance the performance of molecularly imprinted polymers [MIPs] as HPLC stationary phases. Schiff bases for covalent imprinting were investigated by Wulff et al., [1978], but slow equilibration rates has prevented their use in chromatographic separations.

Advantages of covalent imprinting

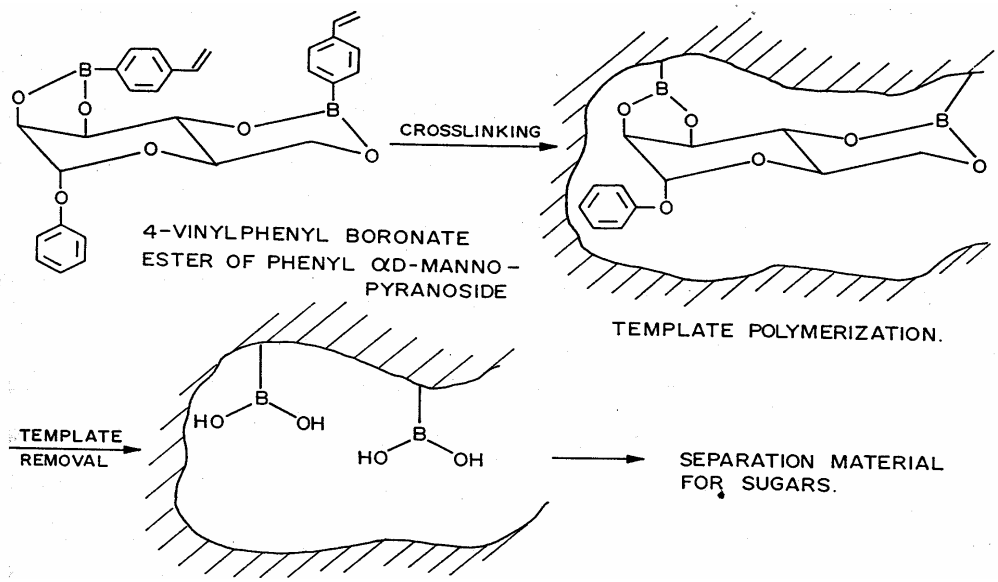
1. Monomer-template assembly is stable as well as guest-binding site in polymer is easily accessible

2. A variety of polymerization conditions e.g. high temperature, high or low pH, and highly polar solvent can be employed.

Disadvantages of covalent imprinting

1. Synthesis of the monomer-template assembly is complicated and less economical.

Figure 1.2: Boronate ester covalently linked to the polymer



2. The number of reversible covalent linkages available is limited.

3. Imprinting cavity may get disturbed during removal of template, as it requires severe conditions.

4. Guest binding and guest release is slow, since they involve the formation and breakdown of a covalent linkage.

1.1.3 Self-assembly approach: Non covalent imprinting

In this approach, functional monomer and template molecule are dissolved in suitable porogen in which weak interactions such as hydrogen bonding, ionic bonding and hydrophobic interactions between functional monomer and template are favored. Crosslinker is then added to this solution and polymerization is effected. The polymers so synthesized are subsequently worked up to elute the template. Due to the above-mentioned interactions amongst monomers and template, a cavity, which is complementary to the size and shape of the template molecule, is created incorporating the functional monomers in the polymer.

Ramstrom et al., [1993] used 2-vinyl pyridine and methacrylic acid [MAA] in the same polymer network. Dansyl phenyl alanine, which is capable of hydrogen bonding interactions with MAA and ionic interactions with 2-vinyl pyridine, was used as

template. This monomer-template assembly was polymerized with excess of crosslinker ethylene glycol dimethacrylate [EGDMA] to synthesize the imprinted polymers. This bifunctional MIP always exhibited higher separation factor [α] than those exhibited by monofunctional MIPs, containing either MAA or 2-vinyl pyridine. Mosbach and coworkers further extended this approach in developing various separations for drugs and peptides [Ekberg et al., 1989, Mosbach et al., 1996, 1994].

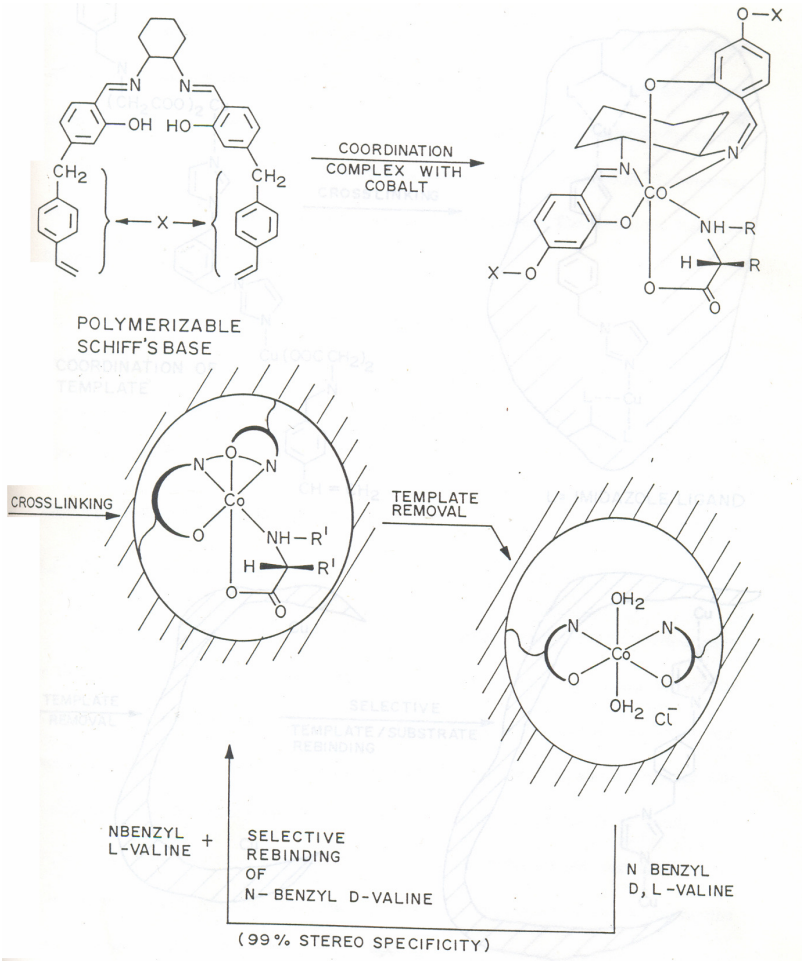
Metal ion coordination interactions have also been used extensively in separation. In these interactions functional monomers and template molecules are complexed with transition metal ion such as Co [II], Cu [II] etc. Due to complexation, monomers and template are oriented towards one another around the metal ion in such a way that cooperative effect as in the case of enzymes is possible [Belokon et al., 1980, 1982]. Fujii et al., [1985] used metal ion coordination for molecular imprinting. The 4-[p-vinyl benzyloxy] salicylaldehyde was complexed with Co [II] and N-benzyl, L, D-valine [Figure 1.3]. This assembly was polymerized with styrene and divinylbenzene and subsequently worked up to remove valine template. The polymers containing cobalt complexes exhibited high separation factor [~ 680] for the racemic mixture of L and D-valine.

Molecular imprinting using metal ion interaction was also reported by Dhal et al., [1991, 1992]. In this approach coordination complex of Cu [II] with [N-4 [vinyl benzyl imino] diacetic acid and various bisimidazoles as templates was studied and this assembly was then crosslinked in presence of EGDMA. The polymers obtained after removal of template and copper were reloaded with copper. Copper loaded polymers exhibited selective binding for the templates used in the formation of coordination complex. This approach was further extended by Dhal et al., [1995] for synthesis of polymeric support by grafting Cu [II] [N-4-vinylbenzylimino] diacetic acid onto surface of macroporous poly [Trimethylol propane trimethacrylate], which exhibited selective rebinding of bisimidazole.

Advantages of non covalent imprinting

1. Synthesis of covalent monomer-template conjugate is not necessary.
2. Template is easily removed from the polymer under very mild conditions.

Figure 1.3: Metal-ion interaction in MIPs



3. Guest binding and release are fast.

Disadvantage of non covalent imprinting

1. Monomer-template assembly is labile and not stoichiometric.

2. The polymerization conditions must be suitable for the noncovalent interactions between monomer and template.

3. The functional monomer in large excess often leads to non-specific binding.

1.2.0 Separation of cholesterol by molecular imprinting

The harmful effects of excess cholesterol on the human health have been mentioned earlier. Hence attempts have been made to separate cholesterol using materials which are biocompatible and clinically efficient. One of the techniques used extensively for the

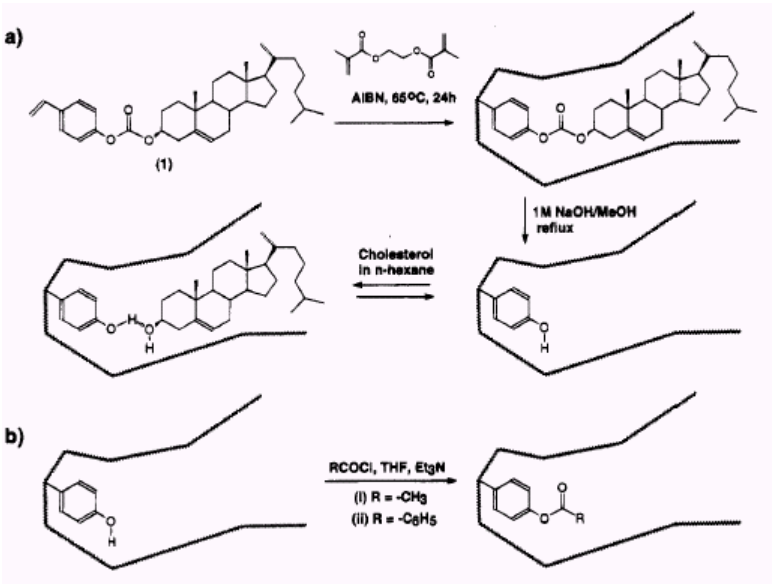
separation of cholesterol is molecular imprinting. Various approaches used for the separation of cholesterol are summarized below.

1.2.1. Molecular imprinting of cholesterol by covalent approach

A novel approach for the separation of cholesterol by covalent molecular imprinting was developed by Whitcombe and coworkers [1995, 2000, 2001]. This technique involved combination of covalent and non covalent imprinting technique. In this method covalent linkages were formed between cholesteryl [4-vinyl] phenyl carbonate and vinyl phenol through a hydrolysable carbonate ester linkage. After polymerization and subsequent removal of cholesterol by hydrolysis, rebinding was effected by non covalent interaction i.e. hydrogen bonding between the hydroxyl group of cholesterol and the phenolic group on the polymer [Figure 1.4]. The rebinding of cholesterol was evaluated in hexane and showed a fairly homogeneous population of binding sites [114 μmol/g] and dissociation constant 0.84 mM [Whitcombe et al., 1995].

Natalia et al., [2000] and Perez et al., [2001] developed core-shell emulsion polymerization for the synthesis of molecularly imprinted nanoparticles. In this technique, EGDMA and divinyl benzene [DVB] based imprinted beads were synthesized using cholesteryl [4-vinyl] phenyl carbonate as a template. Synthesis of submicrometer surface imprinted particles involved two-stage aqueous emulsion polymerization with a poly [DVB] shell over a crosslinked poly [styrene] core. The second polymerization was carried out using pyridinium 12-[4-vinylbenzyloxycarbonyl] dodecanesulfate and pyridinium12-[cholesteryloxycarbonyloxy] dodecanesulfate, which acts as template as well as surfactant [Perez et al., 2001]. The rebinding was studied from the mixture of 2-propanol: water and isohexane and was estimated to be 23 mg/g as against non imprinted polymer 7.7 mg/g. Selectivity study was not performed. In another approach, Wang et al. [2005] copolymerized a template cholesteryl 2-hydroxyethyl methacrylate carbonate and a crosslinker EGDMA followed by hydrolysis leading to the cavity complementary to the template molecule. The rebinding study was evaluated from hexane which showed higher capacity 290 μmole/g and dissociation constant [1.50 mM] as compared to earlier report [Whitcombe et al., 1995]. Selectivity studies were carried out in hexane using cholesterol, cholesterol acetate, progesterone and stigmasterol. The results showed that polymers did not bind to cholesterol acetate because the hydrogen-bonding site is blocked.

Figure 1.4: Covalent molecular imprinting for the separation of cholesterol



[a] Preparation of cholesterol imprinted polymers using cholesteryl [4-Viny1] phenyl carbonate [l], and [b] Chemical modification of the recognition site by acylation.

It exhibited similar binding towards both cholesterol and stigmasterol, but much higher binding towards progesterone.

Recently molecularly imprinted microspheres were synthesized by precipitation polymerization using a covalent bonding approach [Boonpangrak et al., 2006]. Cholesteryl [4-vinyl] phenyl carbonate was used as a template monomer. The imprinted microspheres were prepared using EGDMA and DVB as crosslinkers. The carbonate ester bond was hydrolyzed to create imprinted cavities in the resulting polymers. Rebinding was evaluated from mixture of acetonitrile: toluene [2:1 v/v] which demonstrated that imprinted microspheres prepared from DVB crosslinker had larger and more defined spherical shape, and displayed better imprinting effect [3.16] than EGDMA based microparticles [1.15]. For comparison, imprinted bulk polymers were also prepared in the same reaction solvent as that used in precipitation polymerization. Elemental analysis results indicated that imprinted microspheres prepared by precipitation polymerization contained 12 mole % template monomer, while that prepared by bulk polymerization, contained only 8 mole % template monomer. The efficiency of template

removal by hydrolysis for microspheres prepared by precipitation polymerization was 50 mole % while that for bulk polymerization materials was only 31 mole %. For DVB-based polymers, synthesized by precipitation polymerization imprinted microspheres displayed higher imprinting effect [3.16] than corresponding bulk polymer [2.1].

A review of molecular imprinting by covalent approach for the separation of cholesterol reveals following limitations. Synthesis of monomer template assembly by the covalent approach is complicated and number of reversible covalent linkages available is limited. Template removal by hydrolysis of carbonate ester bond may lead to distorted imprint cavity, as hydrolysis requires severe conditions. Rebinding study was limited to non aqueous solutions and template binding to the cavity could be very slow as it involves breaking of a covalent bond and formation of hydrogen bonding between functional monomer and template. These limitations lead to low capacity and selectivity of these materials for separation of cholesterol.

1.2.2 Molecular imprinting of cholesterol by non covalent approach

Sellergren and coworkers [1998] synthesized polymerizable derivatives of cholesterol and bile acids to be used as amphiphilic monomers in the imprinting of highly crosslinked methacrylates with cholesterol. The polymers were prepared under conditions favoring apolar intermolecular interactions and cholesterol rebinding was evaluated from intestinal mimicking fluids. The capacity of molecularly imprinted polymer for cholesterol was 17 mg/g as against 13 mg/g exhibited by the non-imprinted polymer.

Sreenivasan [1998, 2001a, 2001b, 1997] reported various routes for the separation of cholesterol by molecular imprinting. MIPs based on 2-hydroxy ethyl methacrylate [HEMA] and N-vinyl pyrolidone imprinted for cholesterol and testosterone were synthesized [1998]. Polymers were evaluated for the rebinding from methanolic solution of cholesterol and testosterone. Cholesterol imprinted polymers showed 4.86 mg/g capacity as compared to non imprinted polymer [0.16 mg/g], whereas testosterone imprinted showed 6.25 mg/g as compared to non imprinted [0.18 mg/g]. In another approach, imprinted polymer based on acrylic acid and EGDMA containing multiple recognition sites for steroids was discussed. Three steroids namely cholesterol, testosterone and hydrocortisone were used as template [2001b]. Polymers were tested for rebinding from dichloromethane containing mixture of cholesterol, testosterone and

hydrocortisone. The MIP showed 2.89 mg/g capacity for cholesterol as compared to the non-imprinted polymer [0.22 mg/g]. These polymers also exhibited good selectivity [defined as the ratio of binding capacity of imprinted versus non imprinted polymers] i.e. 13.5, 11.8 and 10 for cholesterol, testosterone and hydrocortisone respectively. Sreenivasan further employed metal-ion interaction for the separation of cholesterol [2001a]. Crosslinked polymer of EGDMA and Cu [II] acrylate monomer was synthesized in presence of cholesterol and rebinding was studied in dichloromethane containing cholesterol or testosterone. Results showed enhanced capacity [12.17 mg/g] as well as selectivity [$\propto$ = 9.9] compared to testosterone [1.02 mg/g, $\propto$ = 1.5]. Crosslinked polymers containing HEMA and EGDMA in presence of template cholesterol were synthesized using irradiation from ^{60}Co source [1997]. Rebinding was studied from methanolic solution of cholesterol or testosterone and demonstrated good selectivity [24.5] for cholesterol as compared to testosterone [1.07].

Davidson et al., [2003] reported for the first time 'tweezer' receptor that exhibits specificity for cholesterol. This tweezer functional monomer was crosslinked by EGDMA in presence of cholesterol. Cholesterol was removed by washing and resulting polymers were tested for the rebinding of cholesterol by HPLC. These polymers showed very low capacity factor [1.36] but good selectivity [5.4]. Hwang et al., [2002] synthesized cholesterol imprinted polymers by bulk polymerization exploiting both covalent and non covalent imprinting approaches. For covalent imprinting cholesteryl [4-vinyl] phenyl carbonate was used and crosslinked using EGMDA. Template molecule was removed by hydrolysis. In non covalent approach, MAA and 4-vinylpyridine were used as functional monomer and crosslinked using EGDMA in presence of cholesterol as a template. These polymers were packed in a column and separation of cholesterol was studied in chromatographic mode. The adsorption capacity factor for covalent imprinting was 11.3, MAA based polymer was 10.2, whereas 4-vinylpyridine based polymer showed 13.0 . Selectivity was in the range between 2 to 2.1 compared to β-estradiol.

Kugimiya et al., [2001] synthesized cholesterol imprinted polymer with 2-[Methacryloyloxy] ethyl phosphate [MEP] and affinity and selectivity for the polymers were evaluated from hexane solution by liquid chromatography. Polymer showed high capacity factor [15.8] for cholesterol as compared to cholesterol acetate [1.2] and

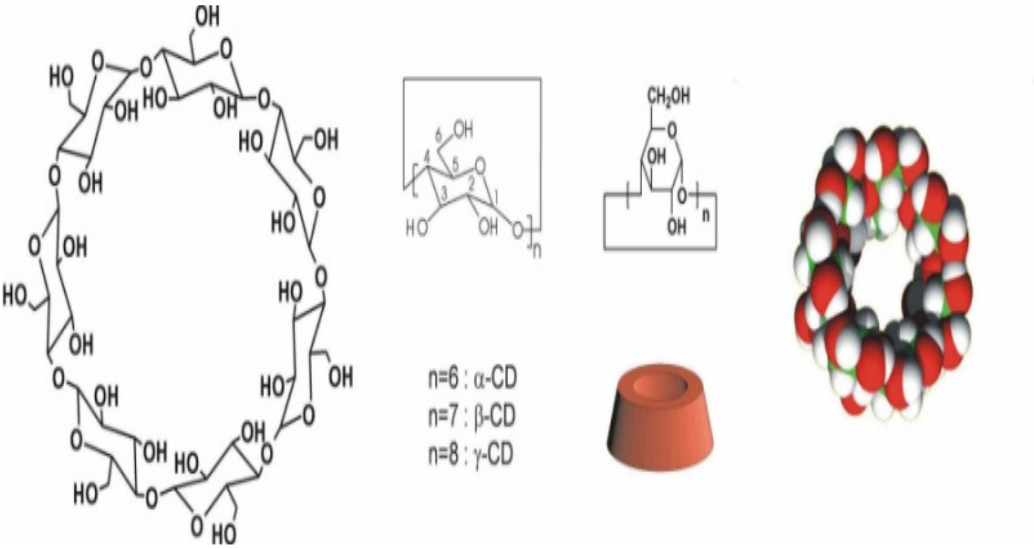
stigmasterol [8.2]. Further, 2-[Trifluoromethyl] acrylic acid [TFMAA] was used as functional monomer and binding ability between MEP and TFMAA with cholesterol was studied. TFMAA based polymers showed better [11.3] than MEP based polymers [6.2]. Based on these experiments the authors concluded that TFMAA was a better functional monomer as compared to MEP due to its ability to form strong hydrogen bond with the hydroxyl group of cholesterol. Ciardelli et al., [2006] prepared membrane based on copolymer of methylmethacrylate-co-acrylic acid modified by adding cholesterol imprinted nanoparticles in the membrane matrix. Rebinding capacity was estimated in phosphate buffer [pH 6.9] and ethanol. Results showed higher capacity [115.4 mg/g] in buffer solution as compared to ethanol [57 mg/g].

Non covalent interactions between cyclodextrins and cholesterol have also been explored for the separation of cholesterol. Cyclodextrins are enzymatically modified starches formed by the action of the enzyme glucosyltransferase on starch. Three cyclodextrins *viz* alpha [α], beta [β] and gamma [γ] cyclodextrin, which contain 6, 7 and 8 α-1,4 linked glucose units respectively [Figure 1.5] are formed. Cyclodextrins are ring or torus shaped molecules and possess a hydrophobic cavity and a hydrophilic exterior.

β−cyclodextrin was used as a functional monomer or receptor in molecular imprinting by Asanuma and coworkers [1997, 1998, 1999, 2000, 2002, 2003]. These approaches are described below. β−cyclodextrin was crosslinked with either hexamethylene diisocynates [HMDI] or toluene 2, 4 diisocynate [TDI] in the presence of template cholesterol or stigmasterol. The template was eluted and polymers were tested for rebinding in water: THF mixture [5:6 v/v]. The polymers based on TDI crosslinker showed two fold greater binding activity [0.70] than those based on HMDI [0.15], whereas those based on epichlorohydrin showed only < 0.01 binding activity [1997].

Extending the approach further complex comprising β−cyclodextrin and cholesterol [3:1] was synthesized and crosslinked using HMDI, TDI or epichlorohydrin. Subsequent removal of template led the cavity specific for cholesterol. These polymers were characterized by solid ^{13}C-CP/MAS NMR spectra. Rebinding study from water: THF [5:6 v/v] mixture demonstrated that TDI crosslinked polymers showed greater binding activity [0.70] than those based on HMDI [0.15] or epichlorohydrin [< 0.01] [1998]. Hishiya et al [1999] compared the performance of preformed and in situ formed β−cyclodextrin and

Figure 1.5: Cyclodextrin



cholesterol complexes. However imprinting using first approach was found to be less efficient and showed lower capacity compared to the latter.

Asanuma et al., [2000] demonstrated that DMSO was the best solvent for the synthesis of β–cyclodextrin polymers. Imprinting of cholesterol and stigmasterol in the presence of β–cyclodextrin using crosslinking agent diisocynates was also analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy [Hishiya et al., 2002]. It was demonstrated that template promoted the formation of dimers and trimers of β–cyclodextrin, which are not formed in absence of template. Ordered assemblies were formed due to the guest molecules, in which two or three β–cyclodextrin molecules cooperated to bind large steroids. Recently Hishiya et al., [2003] reported imprinted polymers comprising β–cyclodextrin crosslinked with TDI in presence of cholesterol. The complete extraction of template was confirmed by ^{13}C CP/MAS NMR. Imprinted polymers were packed in column and rebinding was studied in chromatographic mode by HPLC. Retention time for cholesterol was found to be higher [3.13] as compared to that for stigmasterol [0.91], progenolone [3.01], progesterone [1.83] and 4-cholesten-3-one [0.80]. The imprinted β–cyclodextrin polymers efficiently and selectively retained the template molecules.

Sreenivasan [1998a] synthesized imprinted copolymer of HEMA and β-cyclodextrin coupled HEMA monomer in presence of cholesterol. The imprinted polymers showed 46.8 mg/g capacity, which was attributed to the presence of cyclodextrin moieties in the polymer. Selectivity was investigated using testosterone and demonstrated 13.7 mg/g as compared to non-imprinted 14.3 mg/g. Zhong et al., [2001] prepared polymers comprising acryloyl derivatives of cyclodextrins which were imprinted using cholesteryl acrylate and N, N' diacryloyl piperazine as a crosslinker. Since the high degree of crosslinker made rebinding from aqueous media difficult, hydrophilic monomers such as HEMA were incorporated. The rebinding capacity was estimated from propanol solution. Higher capacity [14-19 mg/g] for cholesterol imprinted polymer was observed as compared to that for non imprinted polymer [2-4 mg/g]. The selectivity was illustrated with estrone, which was bound by MIPs in the range 2-13 mg/g.

The microspheres comprising molecularly imprinted cyclodextrin were synthesized in dimethylsulfoxide / poly [dimethylsiloxane] emulsion [Egawa et al., 2005]. The rebinding was investigated from water /THF mixture [5/6, v/v] containing guest molecule cholesterol. Maximum binding capacity for cholesterol imprinted polymer was 61% as against to non imprinted [46%] polymer. The polymers prepared in poly [dimethylsiloxane] showed lower capacity for cholesterol [51%] than in absence of poly [dimethylsiloxane] [60%].

Literature on separation of cholesterol by molecular imprinting technique highlights following limitations. Interaction between monomer and template molecule is not stoichiometric. The polymerization conditions and solvent used for polymerization must promote non covalent interactions. High degree of crosslinking leads to non specific adsorption of template to the crosslinker due to the hydrophobic interaction. Hence there is a need to devise new polymerization methodologies and design new functional monomers to enhance the capacity as well as selectivity for cholesterol by minimizing non specific adsorption.

1.3.0 Bile acid sequesterants [BAS] for reduction of cholesterol

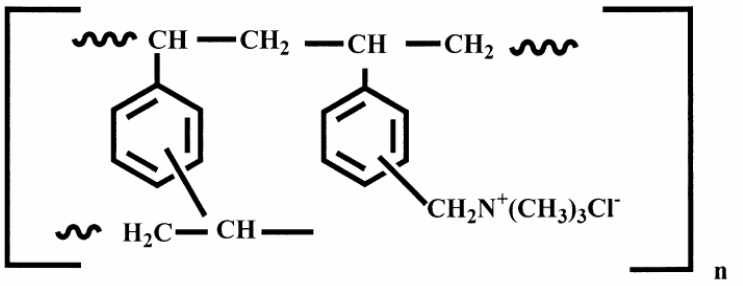
BAS act as anion exchange resins, for the binding of bile acids in the lumen of the small intestine. BAS disturb the enterohepatic circulation of bile acids. This results in an increased hepatic synthesis of bile acids from cholesterol because bile acids suppress the

microsomal hydroxylase that catalyzes the conversion of cholesterol to bile acids. Due to this reduction of the hepatic cholesterol, there is an increase in the activity of the low density lipoproteins [LDL] receptor in the liver. This stimulates the removal of LDL from plasma, resulting in decrease in the concentration of LDL cholesterol [Schneider et al., 1966]. Therefore attempts have been made to separate bile salts by various techniques for the reduction of cholesterol. The major BAS used for the reduction of cholesterol are discussed in the following sections.

1.3.1 BAS synthesized for the reduction of cholesterol

The most commonly used synthetic resin is a strongly basic anion-exchange resin in the chloride form that contains quaternary ammonium functional groups, which are attached to a styrene divinylbenzene crosslinked copolymer [Figure 1.6].

Figure 1.6: Cholestyramine

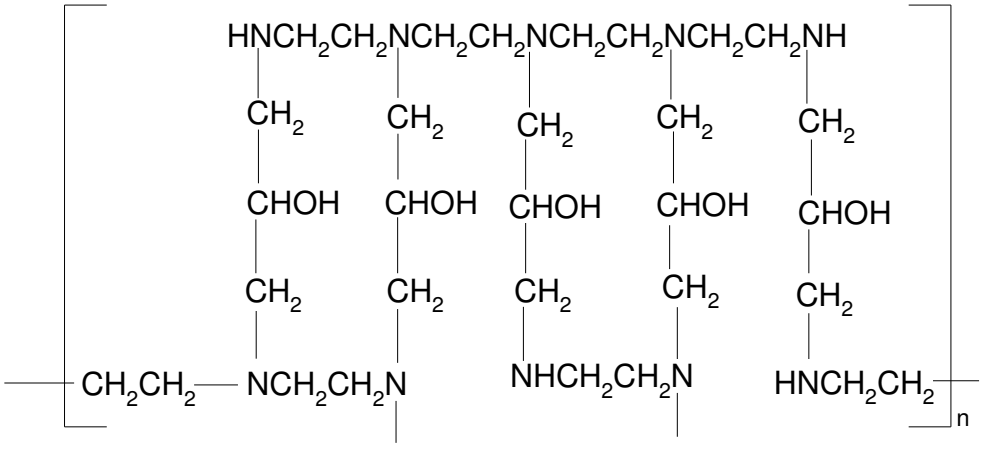


The trade names are Cholestyramin, Dowex 1-X2-Cl, Cuemid, Quantalan and Questran. [Donnelley et al., 1958, Terinent et al., 1960]. It is not affected by the digestive enzymes in the gastrointestinal tract and is not absorbed during use. Extensive studies were undertaken to determine its effectiveness and toxicity. Experimental animals such as dogs [Bergen et al., 1959], cockerels [Jansen et al., 1965, Asano et al., 1975], rats [Manes et al., 1971, Kempi et al., 1971], hamsters [DePalma et al., 1979] and monkeys [Havel et al., 1973, Hashim et al., 1965] have been subjected to Cholestyramine studies. These experimental studies have shown that Cholestyramine binds bile acids both *in-vitro* and during *in-vivo* tests. Cholestyramine has been shown to bind bile acids in the intestinal tract of experimental animals, which leads to conversion of cholesterol to bile acids. This

results in stimulation of cholesterol uptake reducing the serum cholesterol. Metabolic and clinical studies performed on humans [Thale et al., 1978, Zhu et al., 1992] have shown a significant reduction in the concentration of LDL cholesterol. Cholestyramine is currently the drug of choice administered to patients suffering from hyperlipoproteinemia. Originally used for the treatment of patients with elevated levels of plasma bile due to cholestasis, it has found extensive use in lowering plasma LDL cholesterol concentrations. Adverse effects of this drug include unpleasant sandy and gritty quality of drug preparation, nausea, abdominal discomfort, indigestion, constipation and bloating usually associated with the gastrointestinal tract [Kane et al., 1982].

Another widely used bile acid sequestering resin is Colestipol Hydrochloride [trade name Colestid]. This resin is a copolymer of diethyl pentamine and epichlorohydrin [Figure 1.7]. It is highly hydrophilic but insoluble in water. A typical method for the preparation of colestipol hydrochloride involves the reaction of polyethylene polyamine from 2 to 6 ethylene units with epichlorohydrin [Tkahashi et al., 1980, Class 1991]. It is usually given to the patients suffering from higher LDL concentrations. Both Cholestyramine and colestipol hydrochloride have been shown to reduce LDL concentrations in the range 50–60% when the drug is administrated with an inhibitor of HMG CoA reductase [lovastatin or nicotinic acid] [Kane et al., 1981, Bilheimer et al., 1983]. Due to the increase of triglyceride concentrations in the blood plasma, bile acid sequestering drugs

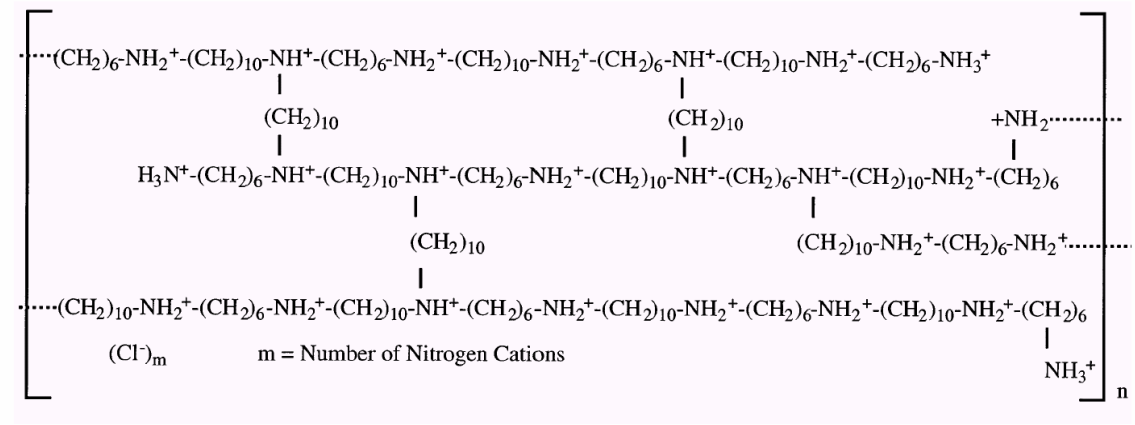
Figure 1.7: Colestipol



rapidly increase the activities of alkaline phosphates and hepatitic transaminases while hyperchloraemic acidosis can also occur as a result of these drugs being in the chloride form of an anion exchange resin. Dosages higher than recommended, have resulted in impaired fat-soluble vitamin absorption. Hence vitamin supplements are usually recommended along with BAS [Ilingsworth et al., 1987].

A novel poly [alkylammonium] based bile acid sequestrant DMP 504 [Raghavan et al., 1997, Schreiber et al., 2001] is a hygroscopic, amorphous crosslinked polymer [Figure 1.8]. Maximum binding capacity of DMP 504 for cholic acid in a phosphate buffered [pH 7.0] was 4.9 mmol/g.

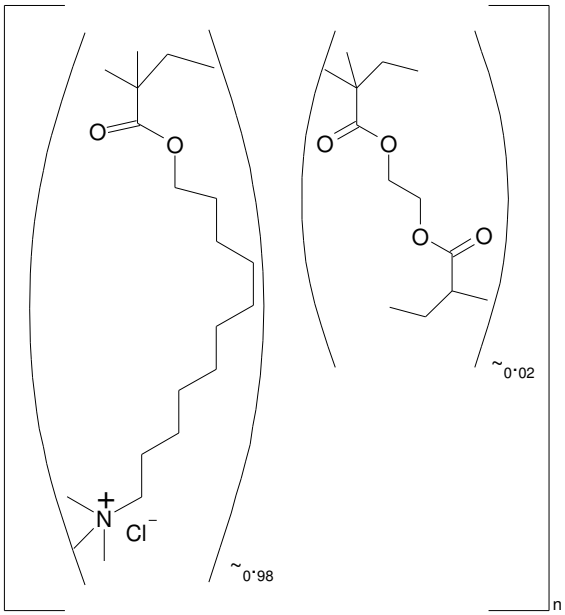
Figure 1.8: DMP 504



The bile acid sequestering agent, SKF 97426A, which is a quaternary alkylammonium polymethacrylate [Figure 1.9] showed a threefold increase in binding capacity than Cholestyramine in the hamster [Benson et al., 1997]. Association, disassociation, affinity and capacity experiments were performed under physiological conditions. The bile acid equilibrium was reached with SKF 97426A and Cholestyramine within 30 and 6 min respectively. SKF 97426A and Cholestyramine had similar capacities for all the bile acids. The values ranged from 2.5 to 4 mmol/g and both had very high affinities and slow dissociation rates for the dihydroxy bile acids. SKF 97426A had much higher affinities for the trihydroxy bile acids glycocholic and taurocholic acids than Cholestyramine.

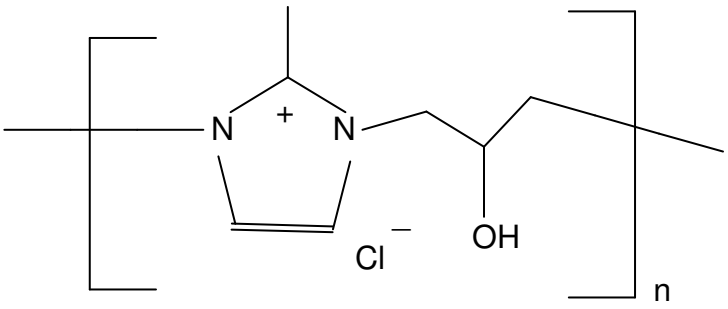
Disassociation of glycocholic and taurocholic acids from SKF 97426A was also much slower, 27 and 25% respectively after 60 min than Cholestyramine.

Figure 1.9: SKF 97426A



Polymer 2-Methyl-1 H-imidazole with chloromethyl oxirane [MCI-196] [Figure 1.10] exhibited *in-vitro* bile acid binding capacity four times greater than that of Cholestyramine [Homma et al., 1996]. The effects of treatment with MCI-196 tablets on plasma levels of lipids and apolipoproteins were investigated and showed a significant reduction in plasma levels of total cholesterol, LDL cholesterol and

Figure 1.10: MCI-196



apolipoprotein B by 11.1, 17.9 and 11.1%, respectively. MCI-196 has demonstrated ability in the treatment of hypercholesterolemia with ease of administration and safe use [Drugs of the Future 1993].

Zhu and coworkers [1996, 2001, 2001a, 2000, 2000a] used various approaches for the study of BAS and are summarized below. Zhu et al., [1996] synthesized 3-α-methacrylate monomer of cholic acid to achieve hydrophilicity and monomers were polymerized with MAA and HEMA. Methyl ester groups on cholic acid of homopolymers were removed by hydrolysis. The copolymers were found to be random and the incorporation of monomers close to the feed ratio. It was also found that methacrylamide bond in the polymers was more resistant to hydrolysis than the methacrylate bond. In another approach [Benrebouh et al., 2001] thermo and pH sensitive polymers containing cholic acid derivatives were synthesized. In this, copolymers of NIPA and methacrylate monomers containing cholic acid with ethylene glycol and oligo [ethylene glycol] spacers were synthesized. The copolymers contained 1-5% of cholic derivatives. The presence of NIPA imparted thermo sensitivity, while copolymer responds to change in pH when the carboxylic acid group of cholic acid was liberated by a selective hydrolysis of ester protecting group. The effects of common surfactant sodium do-decylsulphate and bile salt NaC were compared. In another approach [Zhu et al., 2000] Cholestyramine and crosslinked polyacrylamide resin with lateral alkyl [C_{12}] quaternary ammonium groups [QPDA12] were used to investigate their ability to bind sodium cholate [NaC], sodium glycocholate [NaGC], sodium taurocholate [NaT] and sodium chenodeoxycholate [NaCDC]. The bile salts were evaluated individually and competitively from phosphate buffer [pH 7.4] at room temperature. The QPDA12 showed high affinities for all bile salts, while Cholestyramine exhibited high affinity to NaCDC. The binding constants of QPDA12 and Cholestyramine for NaCDC were 13.7×10^3 M^{-1} and 5.82×10^3 respectively. Binding of QPDA12 for NaC was very high [6.50×10^3 M^{-1}] as compared to that of Cholestyramine [0.523×10^3 M^{-1}]. The binding capacity for all bile salt anions was in the range of 1.35 to 1.73 mmol/g, while that of Cholestyramine was 2.75 to 3.72 mmol/g. Nichifor et al., [2001a] synthesized cationic dextran hydrogel microspheres with pendant quaternary ammonium groups having alkyl substituents [C_2-C_{12}] at quaternary nitrogen. The *in-vitro* sorption of NaGC, NaC, NaT

and NaCDC with these hydrogel was studied as a function of substituent alkyl chain length and bile acid hydrophobicity. Sorption experiments were performed in phosphate buffer [pH 7.4] either in individual or competitive binding mode. The individual binding experiments showed that increase in the length of alkyl chain of the substituents leads to an increase in both ionization constant [5.39 L/mmol] and overall stability constant [8.95 L/mmol] but cooperativity decreases to 1.65. The competitive sorption studies indicate that the hydrogels display good affinity for both dihydroxy and trihydroxy bile salts. The binding constant for the sorption of bile salts by dextran hydrogel was 20-30 times higher than that for cholestyramine under similar conditions. Baille et al., [2000] synthesized poly [β-cyclodextrin] resins by crosslinking of β-cyclodextrin with different amounts of epichlorohydrin. Some hydroxy groups of these polymers were functionalized with alkyl quaternary ammonium groups. The polymers were tested for their ability to bind NaC, NaGC and NaCDC individually and competitively from phosphate buffer [pH 7.4]. Binding isotherm showed higher capacity for NaCDC [0.34 mmol/g] as compared to NaC [0.25 mmol/g] and NaGC [0.33 mmol/g], whereas binding constant for NaCDC was higher [0.51×10^{-3} M^{-1}] than for NaC [0.41×10^{-3} M^{-1}] and NaGC [0.25×10^{-3} M^{-1}]

Other anion exchange resins based on copolymers of styrene and DVB and quaternized with various functional groups [trimethylamine, imidazoles, pyridinium groups etc.] have been investigated as bile acid sequestering agents [Cook et al., 1978, Wagner et al., 1978, Jaxa-Chamiec et al., 1990, Pierre et al., 1992]. In addition to the styrene based polymers, anion-exchange resins based on acrylic monomers have been prepared and investigated for the treatment of hyperlipoproteinemia [Borzatta et al., 1980, Grier et al., 1980, Pierre et al., 1986, Zhu et al., 1992a, Kobayashi et al., 1995, 1995a, Gaoming et al., 1996].

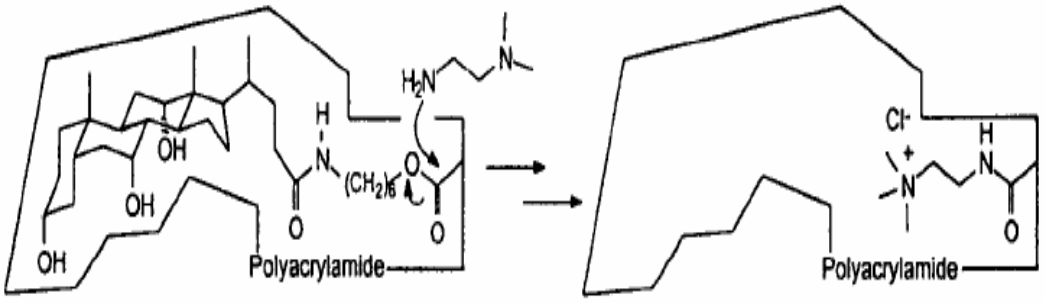
Other resins that have been explored in the past as bile acid sequestering agents are the poly [dialkyl and hydroxydialkylimino] ethylene halides [Wagner et al., 1979], charcoal coated glass beads containing anions [Lauterburg et al., 1979] and novel ingestible polymeric phosphonium salts [Pierre et al., 1993].

1.3.2 BAS by molecular imprinting technique

Idziak et al., [1999] used the covalent approach for the synthesis of MIPs. In this approach polymerizable derivative of cholic acid was used as template and crosslinked using N, N-diethylenebisacrylamide. The cleavage of the template from polymer matrix

was effected by aminolysis of the ester function [Figure 1.11] using N, N – dimethylethylenediamine as reagent and tertiary amino group subsequently quaternized creating a site for binding of bile acid salts. The amount of purified template that was recovered from polymers after aminolysis varied from 32 to 75% of the theoretical value. The rebinding of template molecule was not studied but the aminolysis rate for the various monomers was investigated.

Figure 1.11: Covalent imprinting for BAS synthesis



Chad et al., [2001] used non covalent approach for the synthesis of MIPs for BAS. In this approach poly [allylamine hydrochloride] was used as a functional polymer, due to its low toxicity and biocompatibility. The imprinted polymers were obtained by partially neutralizing poly [allylamine hydrochloride] and crosslinking with epichlorohydrin in the presence of NaC. Template was removed by extraction in methanol and led to an imprinted polymer network containing binding sites complementary to the template molecule. The imprinted and non imprinted polymers were tested for the rebinding of NaC *in-vitro* and *in-vivo*. In vitro bile acid experiments were performed by a batch equilibrium binding procedure. Binding parameters such as association constants, maximum binding capacities and co-operativity for the imprinted and non imprinted polymers were studied using binding isotherm. The association constants were calculated by the Hill's equation. Results demonstrated that the imprinted polymers exhibit higher capacity [1.97 mmol/g] and co-operativity [n = 3.58] as compared to non imprinted polymers [1.30 mmol/g, n = 1.92]. In vivo study was performed using hamsters as an animal model. The hamsters were fed the polymers in their diet and the bile acid excreted

in their feces was analyzed. Results demonstrate higher binding capacity [1.7 μmole/g] as compared to non imprinted polymer [1.4 μmole/g].

This review reveals that the utilization of active site was very low, which is reflected in low capacity as well as selectivity. Hence there is a need to devise new imprinting approaches for the synthesis of BAS.

1.4.0 Smart polymers for the separation of biomolecules

Smart polymers undergo fast and reversible changes in the microstructure in response to the small changes in the environment e.g. pH, temperature, ionic strength, etc. The microscopic changes in polymer structure result in precipitation in solution or sharp volume transitions in hydrogels. Smart polymers have been exploited for the development of new protein purification techniques, *viz* affinity precipitation [Mattiasson et al., 1998, Gisser et al., 1994].

Classification of smart polymers

Smart polymers are generally divided into different categories depending on the stimuli to which they respond.

1.4.1 pH sensitive polymers

Polymers containing ionizable functional groups that respond to a change in pH are termed as pH sensitive polymers. Charge can be generated along the polymer backbone, which results in an increase in the hydrodynamic volume of the polymer. Many polymers such as polyacrylic acid, polymethacrylic acid [PMAA], poly [ethylene imine], poly [L-lysine], and poly [*N*, *N*-dimethyl aminoethyl methacrylate] are typical examples of pH-sensitive polymers [Table 1.1]. For example copolymer of methyl methacrylate – MAA precipitate from aqueous solution at pH 5.0. This is due to protonation of carboxylic groups, which reduces the net charge on the polymer. Therefore hydrophobicity of the polymer increases as compared to the surrounding water, which causes polymer to precipitate. Replacing MAA by dimethylaminethyl methacrylate precipitates the polymer from alkaline condition [pH 9.0] [Galaev et al., 1996]. The net charge on the polymer can also be neutralized by adding low molecular weight counter ion or a polymer bearing opposite charge as to form polycomplexes which are very sensitive to changes in pH or ionic strength [Dublin et al., 1994].

Table 1.1: Monomers for pH responsive polymers

pH	Monomer	pH sensitive group
Acidic	Acrylic acid	-COOH
	Methacrylic acid	-COOH
	Sodium styrenesulphonate	$-SO_3^- Na^+$
	Sulfoxymethacrylate	$-SO_3H$
Basic	Aminoethyl methacrylate	$-NH_2$
	N, N-dimethylaminoethyl methacrylate	$-N[CH_3]_2$
	N,N-diethylaminoethyl methacrylate	$-N[CH_2CH_3]_2$
	Vinylpyridine	-pyridine ring
	Vinylbenyltrimethylammonium chloride	$-N[CH_3]3_+,Cl^-$

1.4.2 Temperature sensitive polymer

Polymers that undergo temperature dependent phase transition or exhibit lower critical solution temperature [LCST] are employed in affinity thermo-precipitation. Thermoprecipitation is very attractive technique since it requires only the input of energy [Eggerts et al., 1998]. The polymers used in affinity thermoprecipitation are exclusively synthetic. Galaev and Mattiasson [1993] reviewed a large number of such polymers. The affinity thermoprecipitation technique has been developed over last two decades but now it is fast emerging method for the separation of various proteins as seen from Table 1.2.

Gupta and Mattiasson [1994] enlisted various criteria for the use of thermoprecipitating polymers in affinity thermoprecipitation. These are listed below.

a) The temperature range of phase transition should not be extreme. In general the polymers exhibiting LCST close to physiological temperature i.e. 37 ^{0}C are suitable. Also the phase transition should be abrupt i.e. maximum 2-3 ^{0}C. Otherwise a temperature shock of 5-10 ^{0}C could lead to enzyme deactivation [Ghose and Mattiasson 1993].

Table 1.2: Affinity thermoprecipitation of various proteins

No.	Affinity ligand	Thermoprecipitating polymer	Recovered protein	Reference
1	Human monoclonal antibody	Poly[N-isopropylacrylamide-co-N-acryloylsuccinimide]	Human antigen	Monji et al., 1987
2	Protein A	--"--	Human IgG	Chen et al., 1990
3	p-amino benzamidine	Poly[N-isopropylacrylamide-co-glycidyl methacrylate or N-acryloyl succinimide]	Trypsin	Nguyen et al., 1989
4	p-amino benzamidine	Poly[N-isopropylacrylamide]	Trypsin	Galaev et al., 1993a
5	p-amino benzamidine	Poly[N-isopropylacrylamide-co-acryloyl spacer	Trypsin	Vaidya et al., 1999
6	IgG	Poly[N-isopropylacrylamide-co-glycidyl methacrylate]	Protein A	Galaev et al., 1992
7	Cibacron blue	Eudragit S-100	Lactate dehydrogenase and Pyruvate Kinase	Guoquiang et al., 1994
8	Cysteamine-maltose	Poly [N-acryloyl piperidine]	α-glucosidase and Con.A	Hoshino et al., 1998
9	Cu[II]-1, vinyl imidazole complex	Poly[N-isopropylacrylamide]	α-amylase inhibitor	Kumar et al., 1998
10	Psoralen	Poly[N-isopropylacrylamide]	Eco.R.I	Umeno et al., 1998

b) The attachment of the affinity ligand should not drastically alter the transition temperature of the thermosensitive polymer.
c) The affinity of the affinity ligand towards target protein or enzyme should not be altered conjugation with the polymer.

1.4.3 Ion sensitive polymers

The solubility of a protein progressively decreases with increase in ionic strength. The salting out of a desired protein can be achieved by increasing the ionic strength of the solution so as to precipitate the protein. The salt concentration required to salt out the desired protein depends upon the total number of ionic/polar amino acids. At high salt concentration the ionic interactions between salt and polar amino acids predominate over hydrogen bonding interactions with water, which precipitates the protein. Salting out is a complex phenomenon and complete physico-chemical understanding of the process is not achieved. Ammonium sulfate [$[NH_4]_2SO_4$] is frequently used for salting out as it provides high ionic strength without denaturation of protein and its solubility in water is high. This technique of protein precipitation is generally used in initial stages of protein separation in bulk, as it is easy to scale up and yields are often high. A major disadvantage is the product obtained by this method is crude which requires further processing by other techniques such as gel filtration, dialysis or solvent fractionation [Gupta et al., 1994].

1.5.0 Affinity based techniques in bioprocessing

1.5.1 Affinity chromatography

The affinity between ligand and ligate [biomolecule] is exploited in this technique. When a biomolecule comes in contact with the affinity ligand, a complex is formed between the two. The strength of this complex depends upon different interactive forces e.g. cationic, anionic, hydrophobic, covalent etc. The unbound biomolecules are eluted more easily from the column as compared to bound one. The affinity complex of biomolecule and ligand that is retained strongly on the column is then dissociated either by pH, ionic strength or addition of other more potent affinity ligand. Thus, the technique provides many advantages such as one-step purification of enzymes. Overall it is a mild process used for separation of enzymes and recoveries from a complex mixture. However this technique suffers from disadvantages such as, the resistance to mass transfer is a common

problem, which retards binding rate and thereby reduces the available capacity of the column [Ramirez-Vick et al., 1997].

1.5.2 Affinity ultrafiltration

In this technique, the biomolecule to be purified is allowed to bind to an immobilized ligand, attached either to water insoluble crosslinked polymer membrane or to a high molecular weight water-soluble polymeric carrier. Thus in this technique the biomolecule is retained on the membrane, whereas in the latter case the biomolecule binds to an affinity ligand attached to a high molecular weight water-soluble polymer. When affinity ligand is attached to a membrane the use of spacer provides better accessibility for ligand to the enzyme. On the other hand use of water-soluble polymeric ligand forms precipitates after binding with enzyme, which might cause drop in efficiency during elution step [Mattiasson et al., 1984]. This technique suffers from disadvantages such as; use of high molecular weight water-soluble carriers to which affinity ligands are attached, increases the cost of overall process. Particulate material in the initial protein mixture and rarely formed precipitate after ligand enzyme binding may cause plugging of the membrane, which will reduce the capacity of the membrane to separate desired biomolecule. Filtration has to be carried out in batch mode. Usually a second filtration step is necessary to concentrate the dissociated protein from the affinity ligand [Mattiasson et al., 1987].

1.5.3 Affinity partitioning

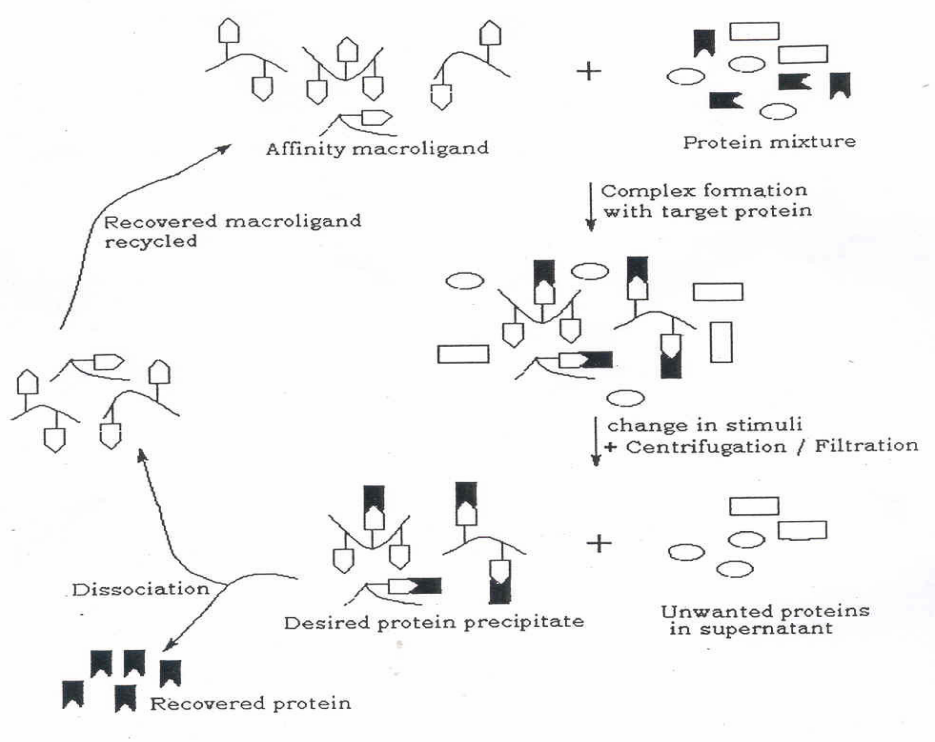
In the conventional two-phase aqueous extraction technique, a suitable affinity ligand is attached to a polymer in order to provide the selectivity during partitioning of desired protein or biomolecule. Thus during partitioning two polymers get concentrated in opposite phases and affinity interactions between enzyme and ligand help in dragging the desired enzyme predominantly into one phase. The purification of a desired protein in this magnitude could only be achieved by multiple extraction of the desired protein from the phase containing affinity ligand. Multiple extraction is achived using three to four washing phases containing a second affinity ligand either to counter extract the target protein or to remove contaminants effectively, depending upon the relative affinity of desired protein for the second ligand. In this technique the affinity ligand is conjugated to a phase forming polymer and the two-phase system is generated by either dissolving the

polymer with another immiscible polymer or with a salt in the mixture of protein. The two phases are separated and the target protein complex with the affinity ligand is dissociated using same eluents as used in conventional affinity chromatography. The dissociated protein is concentrated and further purified [Chen 1990a, b].

1.5.4 Affinity precipitation

In affinity precipitation, an affinity ligand is coupled to a water-soluble smart polymer or to a spacer to from a macroligand. This macroligand when mixed with protein solution forms a complex with the target protein [Figure 1.12]. Phase separation occurs with or without change in the environment, which makes the backbone insoluble. At this point one of the two methods is followed. In the first, the target protein is eluted from the insoluble macroligand-protein complex, which is precipitated. In the second, the precipitate is dissolved, the protein is dissociated from the macroligand and the

Figure 1.12: Affinity precipitation technique



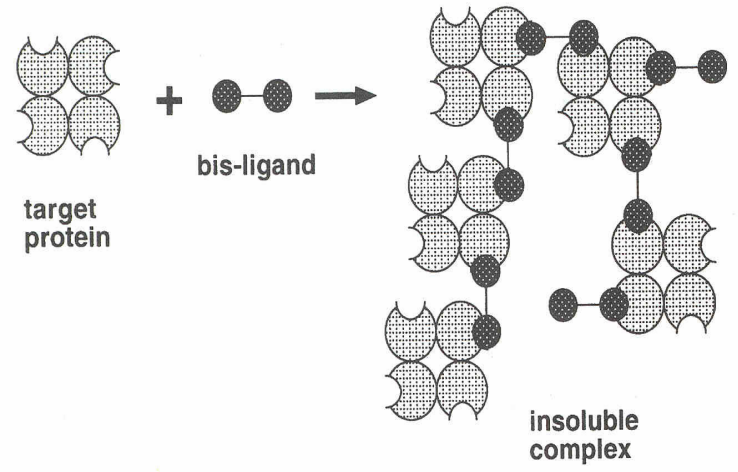
dissociated macroligand is precipitated again which can be reused for the next cycle of protein purification [Mattiasson et al., 1998, Galaev et al., 1996].

The affinity precipitation technique is classified depending upon the route followed to induce precipitation of the complex formed between affinity macroligand and the biomolecule. The nature and type of polymer chosen for the synthesis of an affinity macroligand indirectly affects the route by which this precipitation could be achieved. Accordingly the systems could be classified as, i] Homo-bifunctional or primary effect or bis-ligand precipitation and ii] Hetero-bifunctional or secondary effect affinity precipitation.

i] Homo-bifunctional or primary effect or bis-ligand precipitation

Homo-bifunctional precipitation involves synthesis of a bivalent ligand in which two identical ligands are linked via a spacer arm as shown in Figure 1.13. The bis ligand thus synthesized can bind to two proteins at two different binding sites, provided the interlinking spacer is long enough to cover the binding site of two different oligomeric proteins. The oligomeric proteins bind to more than one bis ligand and give rise to lattice

Figure 1.13: Bis-ligand precipitation



formation. After growing to a particular size the giant three dimensional protein aggregate precipitates from the solution. Dissociation of the complex and then centrifugation or filtration helps to recover the desired protein. The advantage of this technique is that it is a single step purification process and does not require chromatographic column or high-speed centrifugation [Irwin and Tipton 1995, 1996].

Irwin and Tipton formulated the conditions for the bis ligand precipitation which are as follows.

1. The protein or enzyme to be purified should be oligomeric i.e. contain more than one binding site

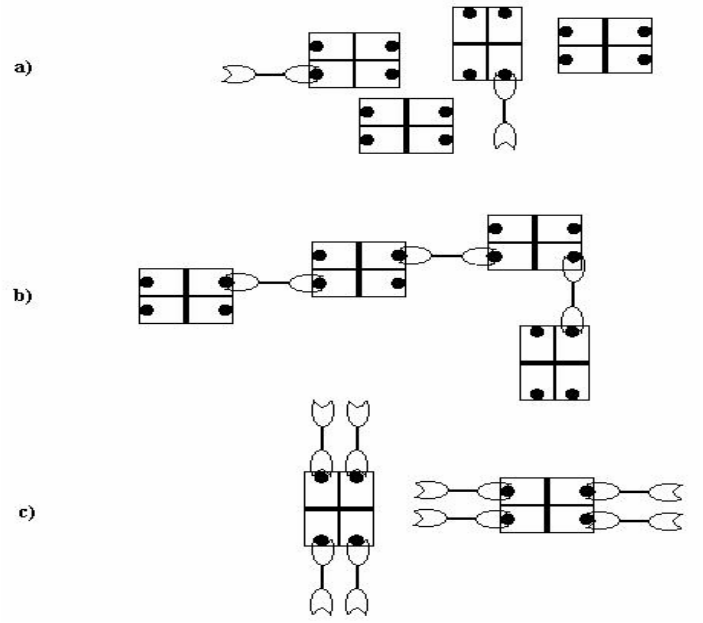
2. The bis-ligand should have a strong affinity for the enzyme.

3. The spacer connecting the two ligands has to be long enough to bridge the distance between two ligand-binding sites.

4. The ratio of bis-ligand to enzyme subunit should be optimum. If this is low, the concentration of coenzyme ligand will be less than the available subunit sites on the enzyme, which will not allow formation of the lattice structure, as there are not enough crosslinks available during complex formation. If this ratio is too high, each enzyme-binding site may be occupied by one end of a bis-ligand molecule but it will not lead to intermolecular crosslinking, which is essential for precipitation reaction.

The effect of bis-ligand concentration on affinity precipitation of tetrameric protein is schematically shown in Figure 1.14.

Figure 1.14: Effect of bis-ligand concentration on affinity precipitation of tetrameric protein



a) If the ratio of bis-ligand to tetrameric protein is low, affinity precipitation will not occur.
b) At an optimum ratio, maximum lattice formation and precipitation will occur.
c) When the bis-ligand is in excess over the tetrameric protein, a decrease in precipitation will occur because each binding site on protein is occupied by different bis-ligand molecule.

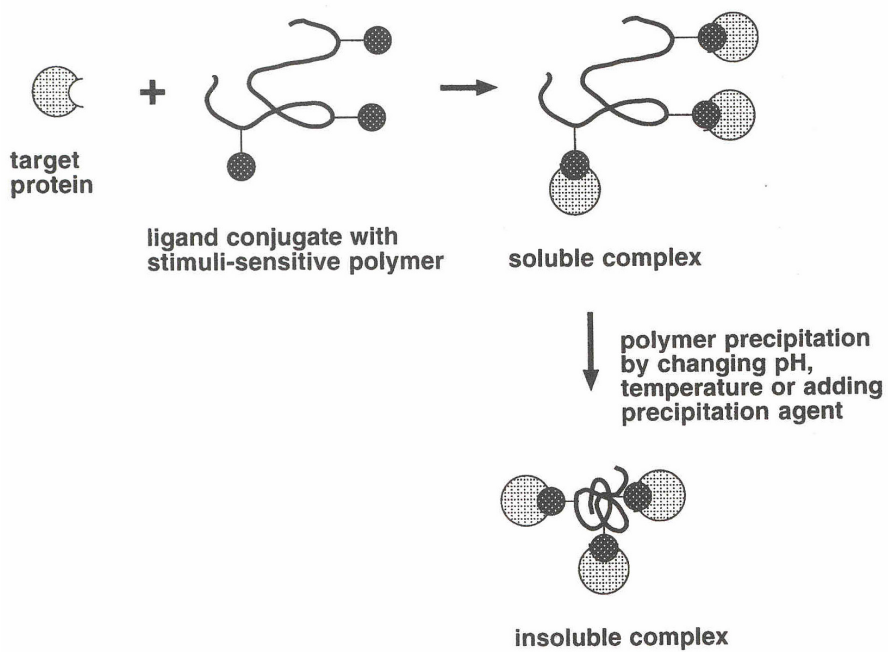
ii] Hetero-bifunctional affinity precipitation or secondary effect precipitation

This approach involves synthesis of an affinity macroligand by conjugating an affinity ligand to a smart polymer, which exhibits reversible solubility characteristics in response to the external stimuli. Thus, one segment of macroligand exhibits affinity for the target protein while the other controls the solubility of the complex [Figure 1.15].

Main steps included in this precipitation are summarized below.

1. Synthesis of a suitable hetero-bifunctional affinity macroligand.
2. Addition of the macroligand to the crude protein mixture, which contains the desired protein or enzyme.

Figure 15: Hetero-bifunctional affinity precipitation



3. Precipitation of macroligand-target protein complex by the choice of stimuli.

4. Recovery of macroligand-protein complex by centrifugation or filtration. In this step the target protein is separated from other contaminating proteins.

5. Dissociation of target protein from affinity macroligand by pH, ionic strength etc.

6. Elution of desired protein.

This technique offers certain merits over the homo-bifunctional approaches, which are summarized below.

1. It does not require optimization of the concentration of affinity ligand and protein to achieve the best precipitation yield of the desired protein.
2. This approach can be used for monomeric as well as multimeric proteins.
3. It is more adaptable to scale-up for various ligand-protein systems.
4. The overall process requires less equipment.
5. The technique uses homogeneous condition for enzyme binding.
6. Thermosensitive and highly unstable enzymes can be easily separated.
7. The synthetic polymers can be tailored as per the specific application.

After discussing various possibilities of using affinity precipitation in conjunction with other techniques used for different applications, it would be appropriate to summarize the prerequisites for design and applications of new polymeric ligands for used in affinity precipitation.

1. The polymer must contain reactive functional groups to which affinity ligand could be attached.
2. The binding efficiency of the affinity ligand to the target protein should be high when conjugated with the polymer.
3. The polymer should not interact non specifically and coprecipitate impurities.
4. The phase separation of polymer should be complete.
5. Polymer should be inexpensive.
6. The phase separation should occur with a small change in stimuli.
7. The precipitated polymer should be compact for its easy separation at low-speed centrifugation or filtration.
8. Polymer should readily solubilize after the precipitate is formed.
9. The precipitation-solubilization cycles must be repetitive without losing much of the

efficiency of polymer.

The review of literature on affinity precipitation technique reveals limitations like crowding effect, stability of ligand and guest complex, etc. These limitations can be overcome by using β–cyclodextrin as an affinity ligand for cholesterol. It is well known that β–cyclodextrin forms inclusion complex with cholesterol [Szejli et al., 1998,Wen et al., 1996]. The literature available on inclusion complexes and their characterization is summarized in the following section.

1.6.0 Formation and characterization of β–cyclodextrin and cholesterol complex

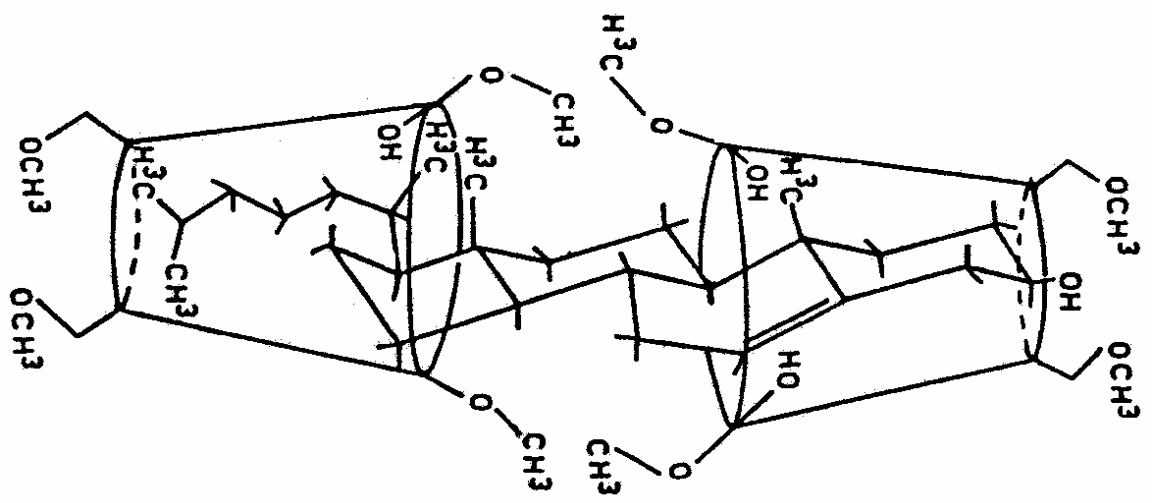
The first inclusion complex between β–cyclodextrin and cholesterol was reported by Schlenk et al., [1958] but the proof of the existence of complex was not given. The existence of β–cyclodextrin cholesterol inclusion complex and the physico-chemical properties of this complex were investigated by Claudy et al., [1990,1991], who prepared inclusion complexes between β–cyclodextrin and cholesterol containing 1:1, 2:1 and 3:1 mole ratios of [β–cyclodextrin: cholesterol] in feed. These complexes were prepared in the mixture of β–cyclodextrin in water [10 weight %] and 1 g cholesterol in 25 ml hexane. Complex was separated and washed repeatedly with water and hexane to remove uncomplexed β–cyclodextrin and cholesterol. These complexes were characterized by X-ray diffraction, thermogravimetric analysis [TG], differential scanning calorimetry [DSC] and nuclear magnetic resonance spectroscopy [^{13}C NMR]. Results demonstrate that irrespective of variation in molar ratio in feed a 3:1 complex was formed. Fromming et al., [1993] prepared complexes of β–cyclodextrin and cholesterol and characterized them by ^{1}H NMR spectroscopy, X-ray diffractometry, heat of fusion and contact angle measurements. Results indicated that 2:1 complex forms at lower β–cyclodextrin concentrations [2.2×10^{-3} Mole], whereas a 3:1 complex is formed at higher β–cyclodextrin concentrations [13.2×10^{-3} Mole].

Ramaswamy et al., [1998] reported the formation of inclusion complex between copolymer of β–cyclodextrin and epichlorohydrin, β–cyclodextrin and 2,6 dimethyl β-cyclodextrin [DMCD] with cholesterol in water or mixture of water benzene [1:1 v/v]. During the formation of inclusion complex cholesterol was oxidized by hydrogen peroxide [H_2O_2] in the presence of 1:1 copper complex of β–cyclodextrin [Cu [II]-

β–cyclodextrin], Cu $[OAc]_2$, $CuSO_4$ and $CuCl_2$. Greater proportion of cholest-4-ene-3-one was obtained in the presence of Cu [II]-β–cyclodextrin indicating that ring containing hydroxyl group of cholesterol was included inside the cavity. These complexes were further characterized by ^1H and ^{13}C NMR, one dimensional difference nuclear overhauser enhancement [1D NOE], rotating frame nuclear overhauser enhancement spectroscopy [ROSEY] and proton spin-lattice relaxation time [T1] measurements. UV visible spectroscopic studies were carried out to study the position of cholesterol inside the β–cyclodextrin cavity. These studies concluded that cholesterol forms 2:1 complex with β–cyclodextrin at lower β–cyclodextrin concentration [1.11×10^{-3} Mole] [Figure 1.16].

Sreenivasan [1995, 2001c] prepared complexes comprising β–cyclodextrin and salicylic acid or cholesterol and demonstrated replacement of less hydrophobic molecule salicylic

Figure 1.16: Inclusion complex between β–cyclodextrin and cholesterol



acid by the more hydrophobic molecule cholesterol using DSC. William et al., [1998] prepared an inclusion complex comprising cholesterol and hydroxypropyl-β–cyclodextrin [HPCD] by lyophilization method. Inclusion complex was characterized by FTIR, X-ray, DSC and confirmed the formation of inclusion complex. DSC thermograms indicated that the endothermic peak of cholesterol was absent in complex.

X-ray diffraction showed that pure cholesterol and HPCD exhibited crystalline nature whereas complex showed amorphous characteristics.

The stoichiometry between cholesterol and dimer β–cyclodextrins was investigated by molecular modeling using molecular mechanics and molecular dynamics stimulation [Bonnet et al., 2001, 2002]. The α, β, γ-cyclodextrin dimer were studied and the relative stability of dimer and molecular interactions involved were investigated. Three possible starting orientations were considered for the dimer i.e. head-to-head [head is the secondary hydroxyl side of cyclodextrin], head-to-tail [tail is the primary hydroxyl side of cyclodextrin] and tail-to-tail. The results demonstrated that head-to-head orientation was the most stable, whereas head-to-tail orientation was found most stable for α and dimer β–cyclodextrin and tail-to-tail orientation for the dimer β, γ-cyclodextrins. Choi et al., [2001] reported molecular modeling studies on the inclusion complex formation of cholesterol with β–cyclodextrin in monomer and dimer using Monte Carlo docking simulations, molecular dynamics and non-equilibrium molecular dynamics stimulation. These results demonstrated more favorable inclusion complex formation between cholesterol and β–cyclodextrin dimer.

Literature reveals the formation of complex between cyclodextrin as well as dimer cyclodextrin and cholesterol. The complex formation was confirmed by various techniques e.g. NMR, DSC, FTIR, X-ray, molecular modeling etc. The stability of complex can be investigated by studying binding constant.

1.7.0 Study of binding constant between β–cyclodextrin and cholesterol

Frijlink et al., [1991] developed a solubility method to study the binding constant between β–cyclodextrin and cholesterol. The formation of inclusion complex between intravenously administered HPCD or β–cyclodextrin with endogenous lipids was investigated. Formation of endogenous complex between cholesterol and β–cyclodextrin in the bloodstream leads to extraction of cholesterol from large lipoprotein particles. Binding constant between β–cyclodextrin and cholesterol was investigated as a function of pH. Binding constant between β–cyclodextrin and cholesterol at pH 6.4 was 1.7×10^4 M^{-1} whereas at pH 10.8 this was enhanced to 3.4×10^4 M^{-1}. Binding constant between HPCD and cholesterol at pH 6.4 was 1.9×10^4 M^{-1} whereas at pH 10.8 it was 2.3×10^4 M^{-1}.

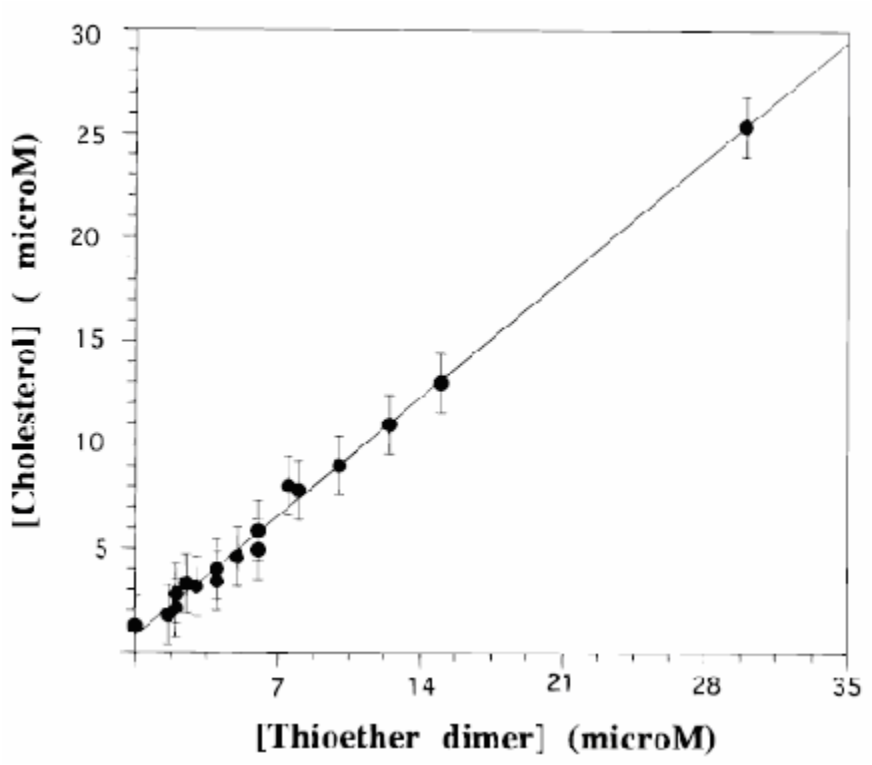
This increase in binding constant at higher pH was further explained by Bergeron et al., [1984]. He pointed out that charge and the hydration of hydroxyl group of β–cyclodextrin and guest molecules is important for the complex formation. An increase in the binding constant at higher pH is associated with the hydroxyl group of the guest molecule placed near the rim of β–cyclodextrin cavity. At higher pH, the interactions with β–cyclodextrin hydroxyl groups stabilize the complex. The marginal increase in the binding constant of HPCD may result from the hydroxyl groups being partially hydroxypropylated and consequently being unable to interact with the hydroxyl group of cholesterol. The binding constant between β–cyclodextrin and dimer β–cyclodextrins with various guest molecules was studied [Breslow et al., 1995, 1990, 1989, Nesanas et al., 2000, Zhang et al., 1993,Yang et al., 1997]. Breslow et al., [1996] reported that for the complete inclusion of cholesterol into the β–cyclodextrin cavity two or more cyclodextrin molecules are required. The height of β–Cyclodextrin cavity is 7.8 A^0 and cholesterol is 15 A^0 in length, therefore it is not possible to include cholesterol in one β–cyclodextrin cavity. The authors [Breslow et al., 1996] synthesized various dimer cyclodextrins and dimer cyclodextrins bearing polymers and studied the binding constant with cholesterol by the solubility method either by colorimetric or fluorimetric determination of cholesterol. In this method, solutions containing β-cyclodextrin in water were stirred with an excess of cholesterol for 24 hrs at 25 ^{0}C and the filtrate was analyzed for cholesterol. The plot of β-cyclodextrin concentration versus concentration of cholesterol in aqueous medium was plotted. The plot was linear [Figure 1.17] and from the slope and intercept [S_0], the association constant for the complexation between cyclodextrin and cholesterol was calculated according to the following equation 1.

$$\mathbf{K_a = Slope/S_0\ [1\text{-}Slope]} \qquad \mathbf{[Equation\ 1]}$$

The results demonstrated that the binding constant for the dimer β–cyclodextrin [3.30×10^6 M^{-1}] was higher than that for β–cyclodextrin [1.7×10^4 M^{-1}] or HPCD [1.9×10^4 M^{-1}]. This showed that there is a cooperative binding between dimer β–cyclodextrin and cholesterol. Binding between crosslinked β–cyclodextrin by epichlorohydrin polymer with cholesterol was also studied and the affinity for cholesterol was three times that of β–cyclodextrin but 100 times lower than that for dimer β–cyclodextrin.

Estimation of binding constants indicates the strength of complex, but does not give any insight into the mode of binding or stoichiometry between β–cyclodextrin and cholesterol. This can be investigated using fluorescence technique. Since cholesterol is non-fluorescent, pyrene molecule can be used as a model fluorescent probe and binding can be studied by fluorescence spectroscopy. Various fluorescent probes

Figure 1.17: Solubility graph for the study of binding constant



were used for the study of binding or stoichiometry between cyclodextrin and guest molecule. The findings are reviewed and summarized in the following sections.

1.8.0 Binding between cyclodextrin and guest by fluorescence spectroscopy

Fluorescence is one of the techniques exclusively used for the study of binding between β–cyclodextrin and guest molecules. It gives the information about binding and stoichiometry between host and guest. These approaches are reviewed and summarized in the following paragraphs.

Hollas et al., [1998a] investigated binding between pyrene and β–cyclodextrin conjugated polymers as a function of degree of substitution [DS] of β–cyclodextrin after partial deprotection of poly [allylamine hydrochloride]. Stagewise binding of 1:1 [pyrene: β–cyclodextrin] and 1:2 complexes between pyrene and β–cyclodextrin was confirmed. Results demonstrated that at high DS [upto 23%] intramolecular 1: 2 chelate type complex was formed because of high β–cyclodextrin concentration, whereas at low DS [below 5%] intermolecular 1: 2 complex was formed. Binding constant was evaluated using fluorescence intensity peak at 373 nm [I_1] and 384 nm [I_3]. These results demonstrated that the binding constants for β–cyclodextrin polymers were increased [10^5 mole.l^{-1}] by two orders of magnitude with increasing DS as compared to β–cyclodextrin [10^3 mole.l^{-1}]. The effects of temperature, pH, ionic strength and addition of urea were also investigated [Jorg et al., 1999, Hollas et al., 1998b]. Binding constant increased [10^7 mole.l^{-1}] with increasing temperature as compared to β–cyclodextrin [10^5 mole.l^{-1}]. The chelate type binding was observed below 35 ^{0}C, whereas stagewise 1: 2 binding was observed above 35 ^{0}C. Intensity ratio [$R = I_1/ I_3$] resulting from titration experiments in neutral, acidic or salt solutions of polymers was investigated. This showed that in acidic and neutral solutions, pyrene was complexed but the R-values were significantly higher in acidic solutions indicating strong binding. This could be due to the higher solvent polarity and indicates formation of 1:1 complex. Hindrance in the formation of chelate type complexes in acidic medium was explained by the folding of polymer backbone around β–cyclodextrins. The complexation of pyrene shifted by two orders of magnitude to smaller β–cyclodextrin concentration in the presence of salt. This was attributed to the entropic contribution of the chain as a result of reduced distance between neighboring β–cyclodextrins. Similarly conformation study was performed in presence of denaturing agents like urea. To study the influence of hydrogen bonding in poly [allylamine hydrochloride] conjugated β–cyclodextrin, the distance between adjacent β–cyclodextrin moieties was controlled by the addition of urea. Results indicate hindered formation of chelate complexes in the presence of urea and were attributed to the stabilization of free pyrene in combination with a coil expansion due to decreasing hydrophobic interactions and a break up of hydrogen bonds.

Kusumoto et al., [1986] investigated pyrene-β–cyclodextrin-sodium dodecyl sulfate [SDS] system on the basis of the absorption and fluorescence behavior of pyrene in very dilute solutions. The formation of two types of aggregates below and above critical miceller concentration [CMC] in the presence of β–cyclodextrin and SDS was reported. Results showed that pyrene-β–cyclodextrin-SDS complex formed below CMC and mixed micelles containing ∝ or β–cyclodextrin were formed above CMC. In another approach Kusumoto et al., [1987] studied the binding constant of pyrene complexes with β–cyclodextrin, DMCD and heptakis [2,3,6-tri-o-methyl]- β–cyclodextrin [TMCD]. Intensity ratio [R] of pyrene increases with increasing medium polarity. The use of this ratio permits the determination of the binding constant of inclusion complexes since the β–cyclodextrin cavity presents hydrophobic environment. Therefore following equations were used for the study of binding constant.

Equation 2 was used for calculating binding constant.

$$\mathbf{1/R_0\text{-}R = 1/K_1\,[R_0\text{-}R_1]\,[\beta\text{–cyclodextrin}]_0 + 1/R_0\text{-}R_1} \qquad \textbf{[Equation 2]}$$

Where, R_0 and R_1 denote the intensity ratios for pyrene in water and in the complex respectively, R is the ratio of I_1/I_3 at given β–cyclodextrin concentration.

Equation 3 was used for calculating the binding constant.

$$\mathbf{1/R_0\text{-}R = 1/K_2\,[R_0\text{-}R_2]\,[\beta\text{–cyclodextrin}]_0^2 + 1/R_0\text{-}R_2} \qquad \textbf{[Equation 3]}$$

Where, R_0 and R_2 denote the intensity ratios for pyrene in water and in the complex respectively.

Binding constants for 1:1 complex [K_1] calculated from equation 2 were 128, 900, 640 mol^{-1} dm^3 for β–cyclodextrin, DMCD and TMCD respectively, whereas for 2:1 β–cyclodextrin complex [K_2] was found to be 79 mol^{-1} dm^3.

Warner and coworkers [1985, 1986, 1991a, 1991b] reported binding between β–cyclodextrin and pyrene. Patonay et al., [1985] compared various methods for the sample preparation that could be used for the study of fluorescence enhancement by micelles or β–cyclodextrins. The method prepared by the authors ensured that pyrene concentration is independent in the presence of micelles or β–cyclodextrin. Further [Patonay et al., 1986] investigation on inclusion complexes between α, β, γ-cyclodextrin and pyrene in aqueous solutions by modified Stern-Volmer [MSV] equation indicated

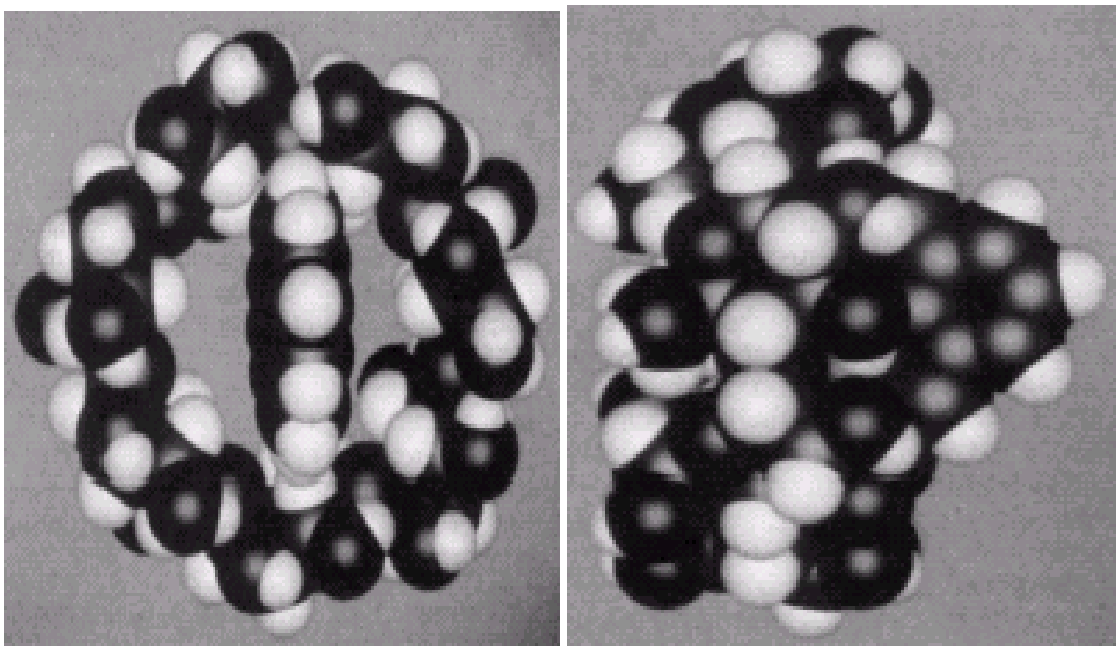
that MSV provides more accurate description of the quenching process of pyrene in the presence of β–cyclodextrin. The binding constants for α, β, γ-cyclodextrin were calculated in the temperature range from 3 to 52 ^{0}C. Modified MSV binding constants for γ–cyclodextrin pyrene system were between 513 to 330 M^{-1}, whereas in the presence of α-cyclodextrin [2×10^{-3} Mole] these varied between 531 to 102 M^{-1} in the temperature range 3 to 21 ^{0}C. MSV equation is useful for the interpretation of pyrene cyclodextrin systems at cyclodextrin concentration below 1×10^{-3} Mole. The steady state fluorescence measurements, which give, information about the stability of β–cyclodextrin-pyrene inclusion complex under chromatographic conditions [Pena et al., 1991a] were reported. The presence of t-butyl alcohol or cyclopentanol in the medium enhanced the strength of the β–cyclodextrin pyrene complex due to the formation of ternary complexes. The effect of increasing the concentration of methanol on the complex was evaluated using HPLC. The binding constant was calculated using Marquadt's iterative method. The binding constant of complex in the presence of methanol from 0 to 40% [v/v] was found to be in the range 5.93 to 4.44 M^{-2}. The binding constant decreased with increasing methanol concentration. Pena et al., [1991b] further studied the inclusion complexes of pyrene with β and γ–cyclodextrin in aqueous solutions. Also the stoichiometry and binding constants were investigated by method reported by Kusumoto et al., [1987] Binding constants for the γ–cyclodextrin and pyrene system was 250 M^{-1}. The value for β–cyclodextrin and pyrene was found to be 8.5×10^5 M^{-2} since the latter formed 1:2 complex. Examination of Corey-Pauling-Kolton [CPK] models revealed that only part of the pyrene actually entered the β–cyclodextrin due to the bulkiness of pyrene [Figure 1.18a]. Viewing β–cyclodextrin from secondary hydroxyl side revealed that pyrene was partially included inside the cavity. Similar results were obtained in the case of γ-cyclodextrin but pyrene exposed to the aqueous medium was very small as compared to β–cyclodextrin [Figure 1.18b].

Xu et al., [1993] investigated binding between β–cyclodextrin and polymers containing β–cyclodextrin with pyrene by fluorescence spectroscopy. Fluorescence titration experiments showed stagewise binding with β–cyclodextrin, whereas polymer containing

Figure 1.18: CPK models for the inclusion of pyrene in β and γ-cyclodextrin

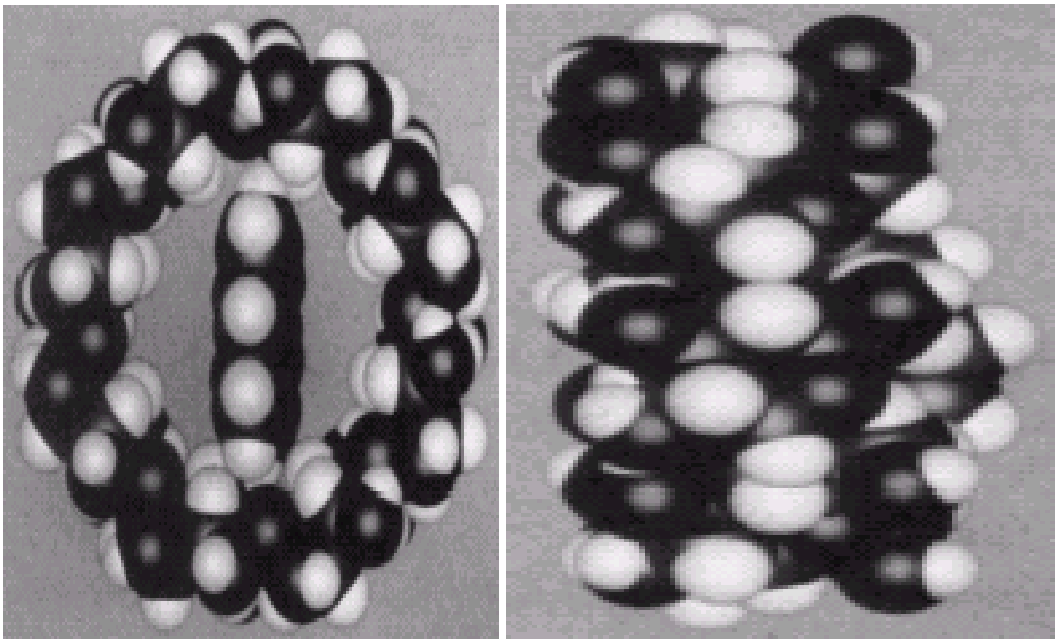
a: [I] Pyrene inside β–cyclodextrin as viewed from the secondary hydroxyl side [II] side view showing only part of the pyrene inside the β–cyclodextrin cavity.

I II



b: [I] Pyrene inside γ–cyclodextrin as viewed from the secondary hydroxyl side [II] side view showing pyrene inside the γ–cyclodextrin cavity

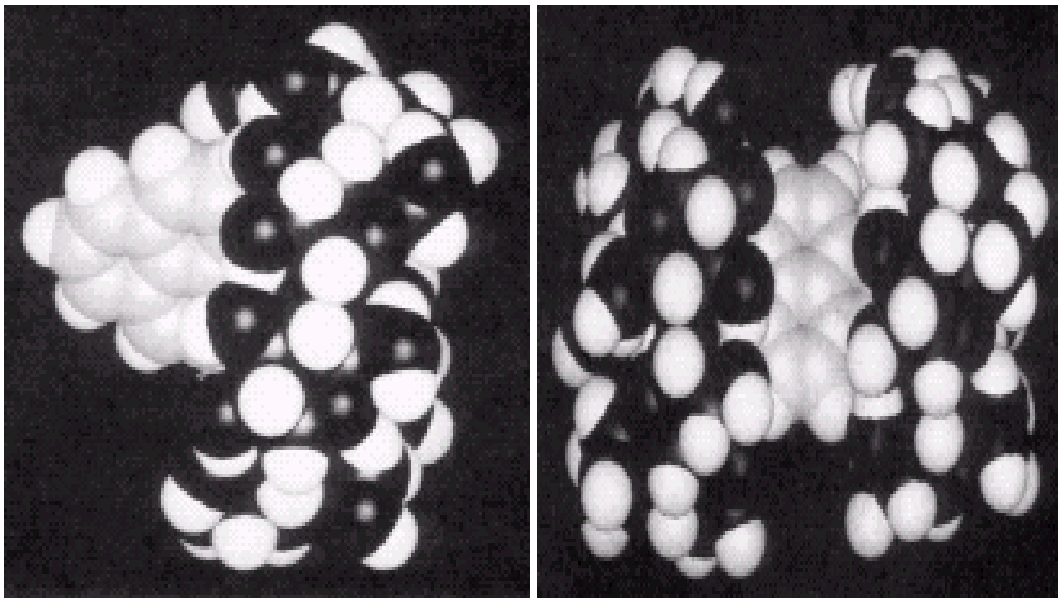
I **II**



β–cyclodextrin showed chelate binding. These results were analogous to those reported by Pena et al., [1991b] and Hollas et al., [1998]. Xu et al., [1993] also developed model for the sequential formation of 1:1 and 1: 2. The 1:1 complex [Figure 1.19] showed that 50% of pyrene was fitted into the cavity of one β–cyclodextrin. For the formation of complete inclusion complex, pyrene, which is projecting out of first β–cyclodextrin r, inserts itself into a second β–cyclodextrin. This double capped species can completely

Figure 1.19: Molecular models of 1: 1 and 1:2 pyrene /β–cyclodextrin complexes

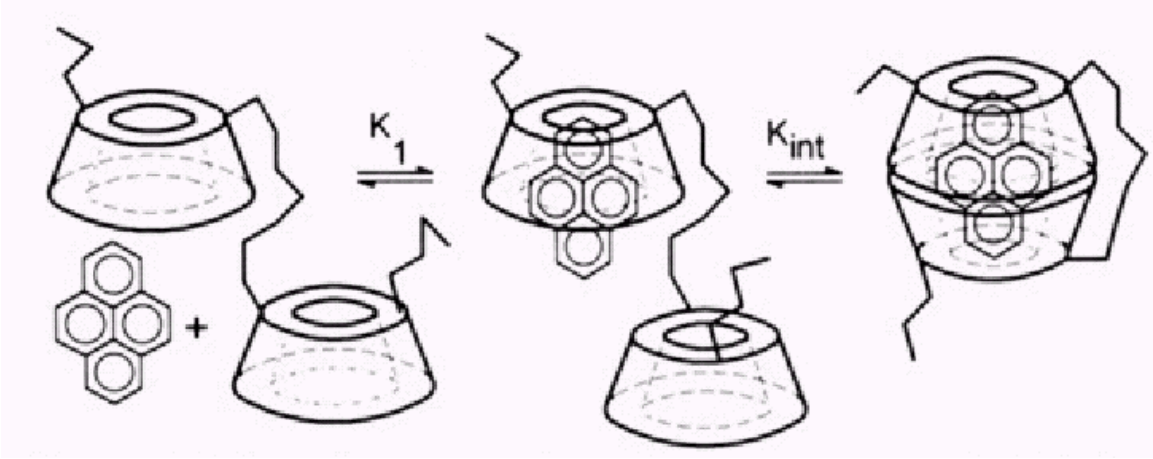
1:1 binding **2:1 binding**



encapsulate pyrene and protect it from any water contact. Based on above results clam shell model [Figure 1.20] was suggested for sequential formation of 1:1 and 1: 2 binding between β–cyclodextrin polymers and pyrene. Based on sequential formation of complexes, binding constant between ∝ or β–cyclodextrin and pyrene was estimated. For β–cyclodextrin system K_1 was 0.14 mM^{-1}, whereas for ∝-cyclodextrin it was 0.67 mM^{-1}. The overall binding constant for β–cyclodextrin complex K_1K_2 was 0.030 mM^{-2} which was lower than the value [0.8 mM^{-2}] reported by Pena et al., [1991b] but agreed well with value reported by Kusumoto et al., [1987] [0.01 mM^{-2}]. Edwards et al., [1978] considered that at low pyrene concentrations pyrene formed 1:1 complex [pyrene +

β–cyclodextrin → complex 1] with β–cyclodextrin characterized by the equilibrium constant K_1. At higher pyrene concentration the amount of 1:1 complex formed increases and leads to further association [2 complex 1 ↔ complex 2] characterized by the equilibrium constant K_2, wherein the complex 2 comprises more than one cyclodextrin and pyrene molecules. The authors estimated $K_1K_2 = 5.2\times 10^5\ M^{-2}$.

Figure 1.20: Clam shell model for β–cyclodextrin/pyrene complex



Steven et al., [1996] investigated kinetics of two stage excited process for the aqueous system of β–cyclodextrin and pyrene and showed that association of two molecules in the excited state was negligibly low. Excited state kinetics of pyrene was investigated while forming inclusion complex with β–cyclodextrin.

Nozakai et al., [1995] conjugated β–cyclodextrin on poly [N-isopropylacrylamide] [PNIPAM] and examined the effect of presence of PNIPAM chains in the inclusion phenomenon by β–cyclodextrin molecules. LCST of these polymers was found to be 33 ^{0}C for PNIPAM and 35 ^{0}C for β–cyclodextrin conjugated polymers. In the presence of free PNIPAM, fluorescence intensity at 25.5 ^{0}C was small, whereas at 41.5 ^{0}C the intensity increased due to the hydrophobic microenvironment around the ANS, which corresponds to aggregate globules of PNIPAM chains. In the presence of β–cyclodextrin polymers, fluorescence intensity was large at 19.5 ^{0}C as well as 40 ^{0}C. Equation 4 was used for the calculation of binding constant.

$1/\Delta F = 1/[\beta\text{–cyclodextrin}]\ K/[ANS] + 1/[ANS]$ [Equation 4]

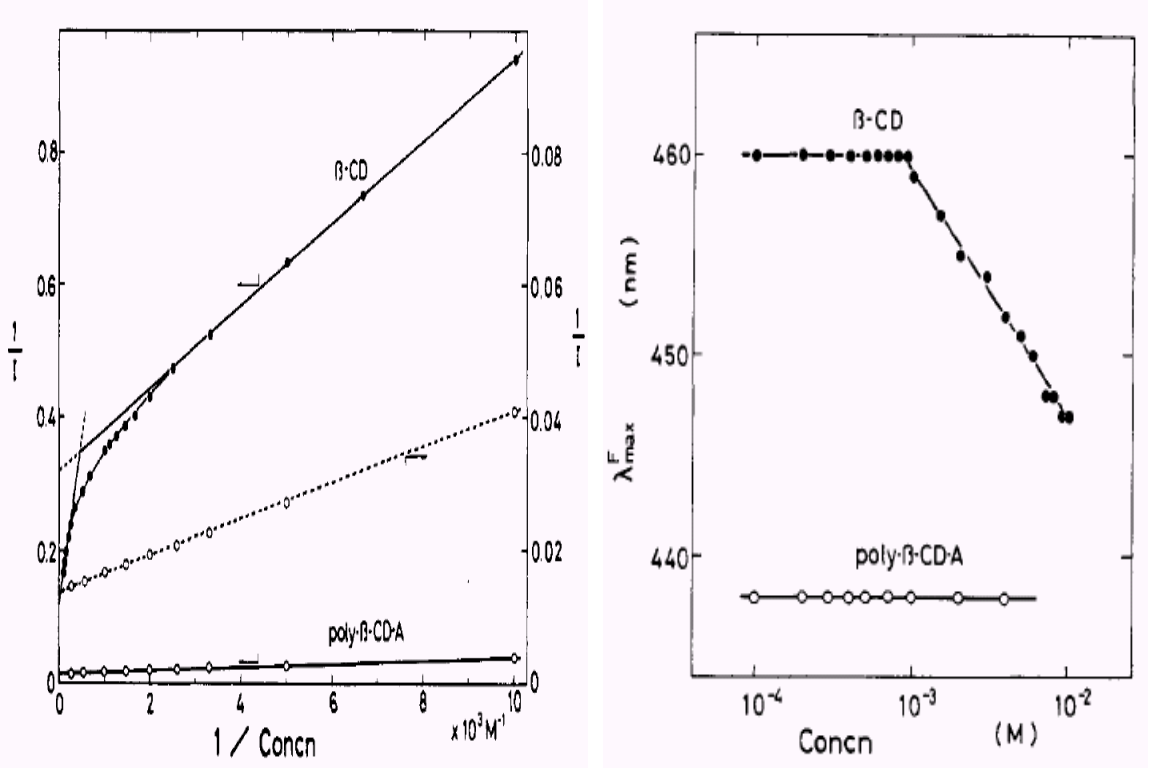
Where, ΔF = relative fluorescence intensity.

The results demonstrated that association constant for 1:1 complexation for β−cyclodextrin-ANS [84 M^{-1} at 11 ^0C] was much smaller than for free poly [NIPA]−ANS [1200 M^{-1} at 11 ^0C] and β−cyclodextrin-PNIPA [9000 M^{-1} at 11 ^0C] systems, because of the cooperativity of the β−cyclodextrin cavity and surrounding PNIPAM chains to bind ANS. In another approach, Nozaki et al., [1997] synthesized β−cyclodextrin gels crosslinked by epichlorohydrin and modified with PNIPA. The association constant of the ANS β−cyclodextrin complex was determined using equation 4 and shown to decrease from 84 to 55 M^{-1} with an increase in temperature from 10 to 60 ^0C. Similar results were obtained for β−cyclodextrin-PNIPA [9000 to 450 M^{-1}] and β−cyclodextrin-gel-PNIPA [5.6×10^5 to 1.0×10^5 M^{-1}]. The Van't Hoff plot for complexation showed that association phenomenon was affected by entropy below transition temperature of PNIPA chains and enthalpy above the transition temperature.

Harada et al., [1977] investigated cooperative effect of water-soluble dyes Rhodamine B, Auramine O, β-napthol, ANS, TNS and dimethylaminonapthalenesulfonyl phenylalanine [DNS-Phe] with polymers containing β−cyclodextrin. These compounds are virtually non-fluorescent but give fluorescence in presence of β−cyclodextrin. Fluorescence change was observed with napthylamine derivatives, which have been widely used as microenvironmental probes i.e. ANS, TNS and DNS-Phe. Especially interaction between β−cyclodextrin and polymer containing β−cyclodextrin with TNS was studied qualitatively by fluorescence titration. Titration plots of TNS with β−cyclodextrin and polymer-β−cyclodextrin were made [Figure 1.21a]. If the binding sites are homogeneous and independent, plot should give straight line [Figure 1.21a showed by dotted line]. The plot for β−cyclodextrin was linear at low concentration [$<10^{-3}$ Mole], but deviated from linearity at higher concentration [5×10^{-3} Mole] and yielded another straight line indicating the formation of 1:1 and 1: 2 complexes. Besides the enhancement of fluorescence, there was change in wavelength of the emission maximum on formation of TNS-β−cyclodextrin complexes as showed in Figure 1.21b. The emission maximum of the β−cyclodextrin-TNS system remained constant at 460 nm until the β−cyclodextrin concentration reached

Figure 1.21: Titration plots of TNS with β–cyclodextrin

a **b**



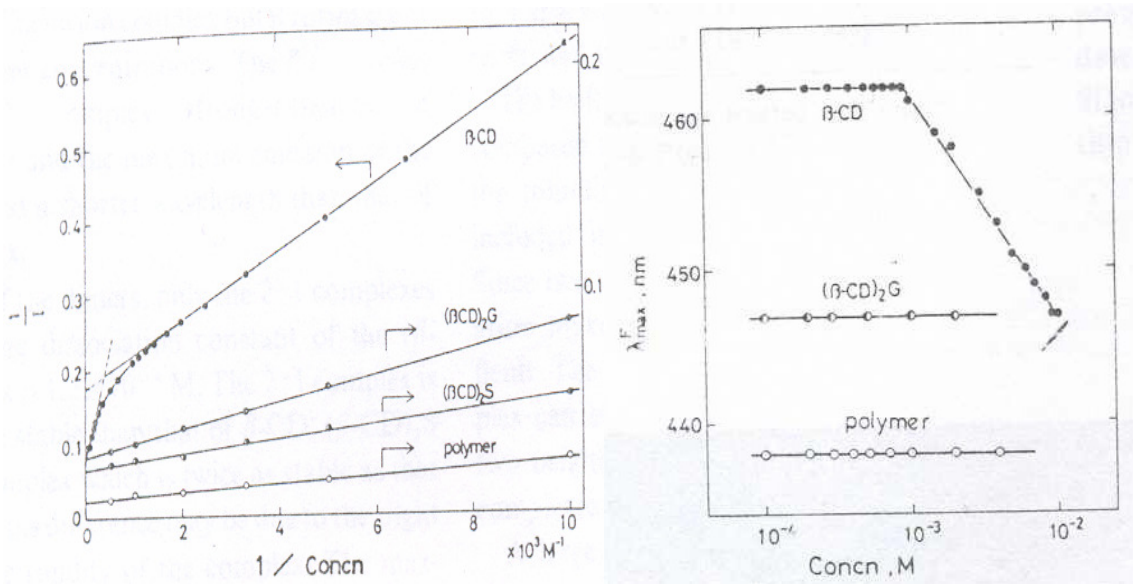
10^{-3} Mole and above this concentration it gradually shifted to shorter wavelength. The results indicated two-step binding for the β–cyclodextrin-TNS system, and 1: 2 binding for polymer-β–cyclodextrin-TNS system. These systems were also investigated by continuous variation method. The maximum molar fraction for β–cyclodextrin was 0.5 indicating 1:1 binding, whereas polymer-β–cyclodextrin showed at 0.66 indicating 1:2 stoichiometry.

Harada et al., [1980] investigated binding between dimer β–cyclodextrin and TNS. The interactions between dimer and TNS were studied by the fluorescence spectroscopy and compared with that of β–cyclodextrin and polymer-β–cyclodextrin conjugate. The fluorescence titration plot for dimer and polymer was found to be linear [Figure 1.22a], indicating 1:2 chelate binding, whereas plot for β–cyclodextrin showed two straight lines, indicating stagewise binding. The emission maximum of TNS with dimer as well as for polymer remained constant [Figure 1.22b] at 477 nm throughout the concentration range

studied, but for the β–cyclodextrin emission maximum was constant at 462 nm and then exhibited a gradual shift to a shorter

Figure 1.22: The fluorescence titration for TNS

a **b**



wavelength. These results indicated formation of chelate complexes for dimer and polymer. The stoichiometry was determined by the continuous variation method and showed maximum molar fraction 0.5 indicating 1:1 complex whereas dimer showed 0.66 indicating 1:2 stoichiometry. Dissociation constant for dimer β–cyclodextrin and polymer β–cyclodextrin and TNS ranged from 0.6 to 1.2 $\times 10^{-4}$ M. The values for 1:1 and 1:2 complex of β–cyclodextrin were 2.5×10^{-4} M and 5×10^{-2} M respectively. Thus 1:2 complexes for β–cyclodextrin are 400 times more stable than 1:1 complex.

Haskard et al., [1996], Fokke et al., [1994] and Russell et al., [1991] also investigated binding between TNS and various β–cyclodextrin dimers. These results were analogous to those reported by of Harada et al., [1980]. Fujita et al., [1984] also synthesized dimer β–cyclodextrin and estimated the association constant with TNS, ANS, methyl and ethyl orange by Klotz's method. The intensity and wavelength of TNS showed tenfold enhancement in intensity as showed by TNS in water, whereas dimer TNS demonstrated 50 fold enhancement. The wavelength of the fluorescence maximum of TNS complex was 436 nm. The authors concluded that dimer binds to TNS more strongly than

β–cyclodextrin. Martel et al., [1995] synthesized β–cyclodextrin conjugated poly [vinyl amine] polymer. DS of β–cyclodextrin was varied from 0.2 to 5%. These polymers were characterized by viscosity and indicated that even at low DS the polymer takes a folded conformation attributed to a dense hydrogen bonding network between amine groups and hydroxyl group of β–cyclodextrin. These complexes also exhibited 1:2 stoichiometry. Kondo et al., [1976] also investigated binding between α, β, γ-cyclodextrin and TNS. Results demonstrated that α-cyclodextrin forms 1:1 complex with TNS, whereas β and γ-cyclodextrin form 1:1 and 1:2 complexes with TNS. The dissociation constant of cyclodextrin and TNS complexes were 54.9 mM, 0.65 mM and 0.66 mM for α, β, γ-cyclodextrin respectively. The secondary dissociation constants for 1:2 complexes were 71.4 mM and 32.6 mM for β, γ-cyclodextrin respectively.

1.9.0 Concluding remarks

The review of literature on separation and recovery of cholesterol using technique such as molecular imprinting and affinity based separations, reveals that although the two can in principle be used for this purpose, there is a need to improve the performance. Molecular imprinting especially using non covalent interactions is being extensively investigated because of its versatality and flexibility *vis a vis* covalent binding approach. Major limitations of MIPs are a] low capacity because of poor active site utilization and b] lower selectivity because of non specific binding. The shrinkage caused during simultaneous polymerization / crosslinking, possibly distorts the cavities, which leads to poor active site utilization. To reduce the shrinkage, we have explored a two stage polymerization / crosslinking approach for the synthesis of MIPs. To increase the number of active sites, without lowering the degree of crosslinking, we have explored the use of cholesterol bearing crosslinkers. Finally to minimize non specific binding, we have synthesized crosslinkers containing hydrophilic groups. These strategies have been quite successful as will be seen later.

Affinity precipitation techniques are based on conjugation of ligands with smart polymers. The conjugation often leads to lowering of affinity between the ligand and the substrate. The use of cyclodextrin as an affinity ligand can overcome this as it forms very strong inclusion complex with cholesterol. Further, the deleterious effect of crowding phenomenon can be overcome by the introduction of spacers. Indeed, we show that with

increasing degree of conjugation of cyclodextrin the binding constant with cholesterol increases. The use of dimer cyclodextrin as an affinity ligand has been investigated for the first time and comparison in the behavior of the two ligands has been explained on the basis of the mechanisms of binding between cyclodextrin and pyrene by fluorescence spectroscopy.

Chapter 2

Objectives and Scope of the Work

The literature review on molecular imprinting and affinity based separation has revealed issues that need to be addressed to in this area. This work is directed towards the design, synthesis and evaluation of polymers that will address some of these issues. Thus present investigation has been undertaken with the following objectives.

1. To design and synthesize MIPs, which exhibit enhanced capacities as well as selectivities for cholesterol. Cholesterol bearing monomers will be synthesized to enhance cholesterol binding capacity. Efforts in the past have shown that the selectivity of MIPs is low. This could be attributed to non specific binding on the hydrophobic crosslinkers. To validate this, the role of hydrophilic crosslinker in minimizing nonspecific adsorption and enhancing capacity as well as selectivity will be studied. In order to increase the binding sites, the amount of functional monomer bearing cholesterol needs to be increased. However this leads to lower crosslink density. In order to overcome this constraint, cholesterol bearing crosslinkers will be synthesized to enhance binding sites population for cholesterol in the polymer, without lowering the degree of crosslinking.
2. To design and synthesize MIPs to minimize shrinkage and yield high percentage utilization of binding sites during BAS rebinding. Prior efforts showed that rebinding capacity of imprinted polymers was low due to the low active site utilization of imprinted cavity. This can be overcome by two stage polymerization / crosslinking approach developed in our laboratory. This approach may lead to high percentage utilization of imprinted active sites and hence to enhanced capacity and selectivity compared to conventional method. The approach involves selective polymerization of one vinyl group from divinyl monomer, with a functional monomer and subsequent crosslinking in the presence of the template.
3. To design and synthesize new affinity thermoprecipitating polymers containing β-cyclodextrin ligand for the separation / recovery of cholesterol from aqueous medium. Affinity precipitation techniques are based on conjugation of ligands with smart polymers. The conjugation often leads to lowering of affinity between the ligand and the substrate. The use of β-cyclodextrin as an affinity ligand can overcome this as it forms very strong inclusion complex with cholesterol. Further, the crowding effect can be overcome by the introduction of spacers. Binding

constant between β-cyclodextrin monomers, homopolymers, dimers and copolymers with cholesterol will be estimated by the solubility method. Study of binding constant between β-cyclodextrin or dimer β-cyclodextrin conjugated polymers and cholesterol as a function of DS of β-cyclodextrin to the polymer will be taken up.

4. The study of binding constant between β-cyclodextrin and cholesterol does not give any insights into the stoichiometry or mode of binding. This will be investigated by fluorescence spectroscopy. Pyrene will be selected as a model fluorescent probe and binding between β-cyclodextrin as well as dimer β-cyclodextrin conjugated polymers and pyrene will be investigated.

Chapter 3

Enhanced Capacities and Selectivities for Cholesterol in Aqueous Media by Molecular Imprinting: Role of Novel Crosslinkers

3.0.0 Introduction

The deleterious effect of cholesterol on human health is well documented [Huval et al., 2001, Holtmeier et al., 1996, Davidson et al., 2002]. Therefore attempts are being made to develop cholesterol or bile salt selective adsorbents that are biocompatible and clinically efficient [Huval et al., 2001, Holtmeier et al., 1996].

Molecularly imprinted polymers are being extensively investigated as selective adsorbents for cholesterol [Asanuma et al., 1997, 2000, Whitcombe et al., 1995, Hishiya et al., 1999, Sreenivasan 1998, 2001, 1998, 1997, 2001, Perez et al., 2001, Sellergren et al., 1998, Zhong et al., 2001, Natalia et al., 2000, Akimitsu et al., 2001, Hwang et al., 2002, Davidson et al., 2003]. The technique involves pre-organization of functional monomers around a template molecule, which resembles shape and size of the guest molecule, by either covalent, non-covalent or co-ordination interactions. Polymerization of the supramolecular assembly in the presence of an excess of crosslinker and subsequent removal of the template leads to polymers that retain the specific orientation of functional groups within the cavity created by the elution of the template molecule [Wulff et al., 1995, Shea et al., 1994, Mayes et al., 1997, Sellergren et al., 1997]. Approaches for the recovery of bile acids using molecularly imprinted polymers have also been reported [Huval et al., 2001]. However, the molecular interactions involved in the rebinding process are different than those involved in the case of cholesterol in view of the different functional groups involved in rebinding.

Approaches to devise cholesterol selective polymeric adsorbents using molecular imprinting methodology have been summarized in chapter 1. Broadly the researchers have exploited hydrogen bonding [Whitcombe et al., 1995] for rebinding from non aqueous media, and hydrophobic binding as well as inclusion complexes with cyclodextrin for rebinding from aqueous media [Asanuma et al., 1997]. Whitcombe and coworkers [1995] conjugated cholesterol with vinyl phenol through a readily hydrolysable carbonate ester linkage. After polymerization and removal of cholesterol by hydrolysis, rebinding was effected by hydrogen bonding between the hydroxyl group of cholesterol and the phenolic group on the polymer. The rebinding of cholesterol was evaluated in hexane and showed a fairly homogeneous population of binding sites. Sellergren and coworkers [1998] synthesized polymerizable derivatives of cholesterol and

bile acids to be used as amphiphilic monomers in the imprinting of highly crosslinked methacrylates with cholesterol. The polymers were prepared under conditions favoring apolar intermolecular interactions and cholesterol rebinding from intestinal mimicking fluids was evaluated. The capacity of molecularly imprinted polymer for cholesterol was 17 mg/g as against 13 mg/g exhibited by the non-imprinted polymer. Hwang and Lee [2002] adapted a similar approach wherein cholesteryl [4-vinyl] phenyl carbonate was used for covalent imprinting and 4-vinyl pyridine for non-covalent imprinting of cholesterol. As anticipated, covalent imprinting resulted in more selective adsorption of cholesterol as evaluated chromatographically. Asanuma and coworkers [1997] described the cholesterol recognition properties exhibited by polymers prepared by crosslinking of β-cyclodextrin, with diisocyanates in the presence of cholesterol. Zhong et al., [2001] prepared polymers comprising acryloyl derivatives of cyclodextrins which were imprinted using cholesteryl acrylate and N, N' diacryloyl piperazine as crosslinker. Since the high degree of crosslinker made rebinding from aqueous media difficult, hydrophilic monomers such as 2-hydroxy ethyl methacrylate were incorporated. The materials were capable of rebinding cholesterol also from aqueous media. One of the limitations of these polymers is their low capacity and low selectivity resulting from non-specific binding of cholesterol on to the hydrophobic crosslinking monomer used in polymerization [Zhong et al., 2001].

In this chapter we report alternative approaches for enhancing capacity and selectivity of molecularly imprinted polymers for cholesterol binding. In the first approach non-specific adsorption was minimized by incorporating hydrophilic crosslinkers. In the second approach crosslinkers containing covalently linked cholesterol rather than cholesterol containing monomers were incorporated so that the degree of crosslinking did not decrease when the loading of the cholesterol bearing moiety was increased. These approaches exploit the same mechanism for rebinding of cholesterol as envisaged by Sellergren et al., [1998]. In principle, cholesterol conjugate is prepared and brought in contact with cholesterol as a template and the assembly is polymerized in presence of excess crosslinker. The template cholesterol molecule is expected to bind to polymer cholesterol conjugate by hydrophobic binding. During the rebinding experiment cholesterol used as template is washed off and the imprinted polymer is brought in

contact with the guest cholesterol molecule in an aqueous medium where upon the latter is expected to bind to polymer cholesterol conjugate through hydrophobic binding. This approach is different than that reported by Whitcombe et al., [1995] discussed in the preceding paragraphs. These polymers were evaluated for rebinding of cholesterol from aqueous media. The results demonstrate that both higher cholesterol binding capacities as well as selectivities can be achieved.

3.1.0 Experimental

3.1.1 Materials

Ethylene glycol dimethacrylate [EGDMA], Methacrylic acid [MAA], Testosterone [Tes], Stigmasterol [Sti], Sodium cholate [NaC], N, N –dicyclohexylcarbodiimide [DCC], 4-[dimethylamino] pyridine [DMAP], Glycidyl methacrylate [GAM], Itaconic acid, were purchased from Aldrich. Sodium deoxycholate [NaDC], α,α'–azobis [isobutyronitrile] [AIBN], Potassium dihydrogen phosphate, Potassium carbonate [K_2CO_3], Sodium hydroxide, Cholesterol, Glycerol were supplied by S.D. Fine Chemicals, India, HPLC grade Methanol and Isopropanol were supplied by Qualigens Chemicals, India. All solvents supplied by local suppliers were purified as per standard procedure [Perrin et al., 1996].

3.1.2 Instrumentation

^{1}H NMR spectra were recorded on Bruker superconducting FT-NMR AC 300 operating at 300 MHz. IR spectra were recorded on Shimadzu 8300 FT-IR spectrometer. Electronic absorption measurements were done on Shimadzu UV 1601 spectrophotometer. The absorption wavelength for stigmasterol was 206 nm and for testosterone 241nm [Sreenivasan, 1997]. HPLC analysis was carried out using Waters HPLC system comprising 680 automated gradient controller, 510 solvent delivery pumps, 486 tunable absorbance detector and 746 dual channel integrator. A μ-Bondapak C_{18} column [Phenomenex] in conjunction with methanol-isopropanol [90:10 v/v] as mobile phase was used for estimation of cholesterol at 206 nm. Scanning electron micrographs [SEM] were recorded on Stereoscan 440, Leica. The pore surface area and pore volume of the porous copolymer samples were studied by mercury intrusion porosimetry in the pressure range of 0-4000 Kg/cm^2 using an Autoscan 60 mercury porosimeter from Quantachrome, USA. The mercury contact angle was 140^0.

3.1.3 Synthesis of polymerizable derivatives of cholesterol

3.1.3.1 Synthesis of monocholesteryl itaconate [M_1]

In a 250 ml capacity round bottom flask 7.73 g [0.02 Moles] cholesterol and 2.60 g [0.02 Moles] of itaconic acid were dissolved in 25 ml of THF. The flask was cooled in an ice bath and temperature was maintained between 0-5^0 C. 4.12 g [0.02 Moles] of DCC was dissolved in 5 ml THF and added to the above solution. 1% DMAP was added as a catalyst. Reaction mixture was stirred for 2-3 hrs in ice water bath and then at room temperature for 48 hrs. Dicyclohexyl urea [DCU] formed during the reaction was filtered off and filtrate was concentrated. The solid product was washed with water, 5% acetic acid, 0.5 N sodium bicarbonate solutions and brine and water [each 300 ml]. White solid was dried under vacuum and characterized.

Yield: 8.26 g [80%]

^{1}H NMR [300 MHz $CDCl_3$]: 0.67δ s [3H, 18-H_3], 0.85δ d [3H, 27-H_3], 0.87δ d [3H, 21-H_3], 0.92δ s [3H, 19-H_3], 1δ to 2.28 [steroid], 1.99δ s [3H, CH_3-CH_2], 3.18 δ s [-CH_2 of itaconate] 5.81δ and 6.42δ s [2H, CH_3=CH_2], 5.34δ s [1H, 6-H]

IR [KBr]: 3328.9 cm^{-1} -OH of COOH, 1710.7 cm^{-1} ester, 1650.5 cm^{-1} C=C

3.1.3.2 Synthesis of cholesteryl methacrylate [M_2]

Cholesteryl methacrylate [M_2] was synthesized as reported by Sellergren et al., [1998]

Yield: 5.56 g [94%]

^{1}H NMR [300 MHz $CDCl_3$]: 0.67δ s [3H, 18-H_3], 0.84δ d [3H, 27-H_3], 0.89δ d [3H, 21-H_3], 0.91δ s [3H, 19-H_3], 1δ to 2.28 [steroid], 1.98δ s [3H, CH_3-CH_2], 5.14δ and 5.20δ s [2H, CH_3-CH_2], 5.34δ s [1H, 6-H]

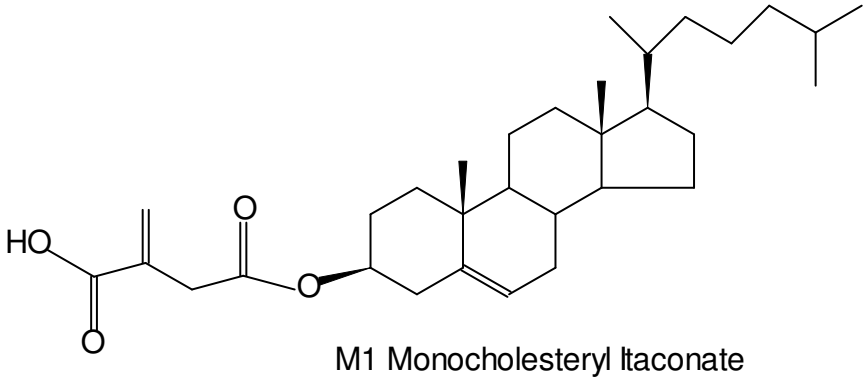
IR [KBr]: 1720.3 cm^{-1} ester, 1649 cm^{-1} C=C,

3.1.4 Synthesis of crosslinkers

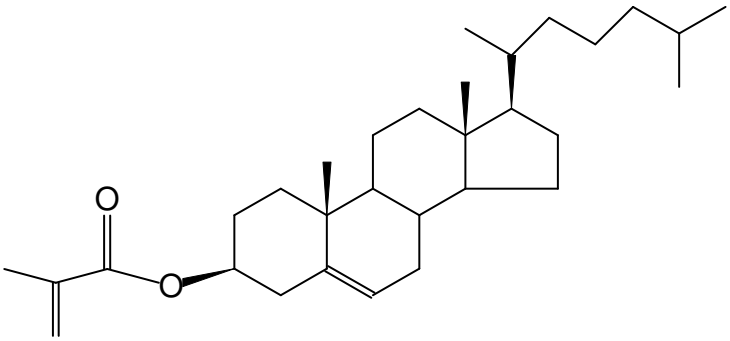
3.1.4.1 Synthesis of Glyceroldimethacrylate [C_2]

In a 500 ml round bottom flask 110 g [0.8 Moles] anhydrous K_2CO_3 was placed in 500 ml of dry acetone. To this solution 50.90 ml [0.6 Moles] of MAA was added in a drop wise manner over 30 min under vigorous stirring at room temperature. The reaction was continued for 2 hrs to complete the formation of potassium methacrylate. Further 26.4 ml [0.2 Moles] GMA was added over 2 hrs in a dropwise manner. The reaction was continued at room temperature for 12 hrs and then at reflux for another four hours. The

Figure 3.1: Polymerizable derivatives of cholesterol



M1 Monocholesteryl Itaconate



M2 Cholesteryl Methacrylate

reaction mixture was cooled to room temperature and filtered to remove unreacted potassium methacrylate and potassium carbonate. Acetone was evaporated under vacuum at 35 ^{0}C. The crude product was dissolved in diethyl ether, washed repeatedly with water to remove traces of K_2CO_3 and potassium methacrylate. Ether layer was dried over Na_2SO_4. Ether was removed under vacuum to yield an oily liquid.

Yield: 77.3 g [60%]

^{1}H NMR [300 MHz $CDCl_3$]: 2.00δ s [6H, -CH_2], 3.36δ m [1H, CH-OH], 3.99δ to 4.6δ 2dd [4H -CH_2-O- and O-CH_2-], 5.8 δ, 6.2 δ 2s [4H, CH_2=C-].

IR [Neat]: 3425.3 cm^{-1} –OH, 1720.4 cm^{-1} ester, 1639.4 cm^{-1} of C=C

3.1.4.2 Synthesis of Glyceryldicholesteryl itaconate [C_3]

In a 250 ml capacity round bottom flask 1.5 g [0.003 Moles] of monocholesteryl itaconate and 0.21 ml [0.003 Moles] of glycerol were dissolved in 25 ml of THF. The

flask was cooled in an ice bath and temperature was maintained between 0-5^0 C. 0.62 g [0.003 Moles] of DCC was dissolved in 5 ml THF and added to the above solution. 1% DMAP was added as a catalyst. It was stirred for 2-3 hrs in ice water bath and then at room temperature for 48 hrs. DCU formed during reaction was filtered off and workup was followed by same method reported for M_1.

In the next step 2.0 g [0.003 Moles] of glycerylmonocholesteryl itaconate was coupled with 1.5 g [0.003 Moles] of monocholesteryl itaconate by DCC coupling by the same procedure as reported earlier

Yield: 2.2 g [65%]

^{1}H NMR [300 MHz $CDCl_3$]: 0.67δ s [6H, 18-H_3], 0.84δ d [6H, 27-H_3], 0.87δ d [6H, 21-H_3], 0.92δ s [6H, 19-H_3], 1δ to 2.28 [steroid], 1.82δ s [6H, CH_3-CH_2], 3.7δ m [1H CH-OH], 3.50δ to 4.16δ 2dd [4H –CH_2-O- and O-CH_2-], 3.02 δ s [-CH_2 of itaconate] 6.53δ and 6.98δ s [2H, CH_3-CH_2], 5.34δ s [1H, 6-H].

IR [KBr]: 1697.2 cm^{-1} Ester, 3327 cm^{-1} OH, 1625 cm^{-1} C=C.

3.1.4.3 Synthesis of monocholesteryl itaconate glycerol methacrylate [C_4]

Synthesis of glyceryl itaconate ester

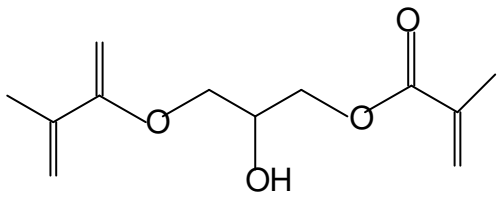
In a 1000 ml round bottom flask 10 ml [0.076 Moles] of GA and 10 g [0.076 Moles] of itaconic acid were added along with 2 g of hydroquinone and 500 ml of benzene. To this solution 1.5 ml of pyridine was added as catalyst and the solution was refluxed for 5 hr. Benzene was recovered and the residue washed first with 1% sodium bicarbonate solution and then with water to remove itaconic acid. The filtrate was dried on anhydrous sodium sulfate and concentrated under vacuum. The product obtained was characterized by IR and NMR.

Yield: 15 g [75%]

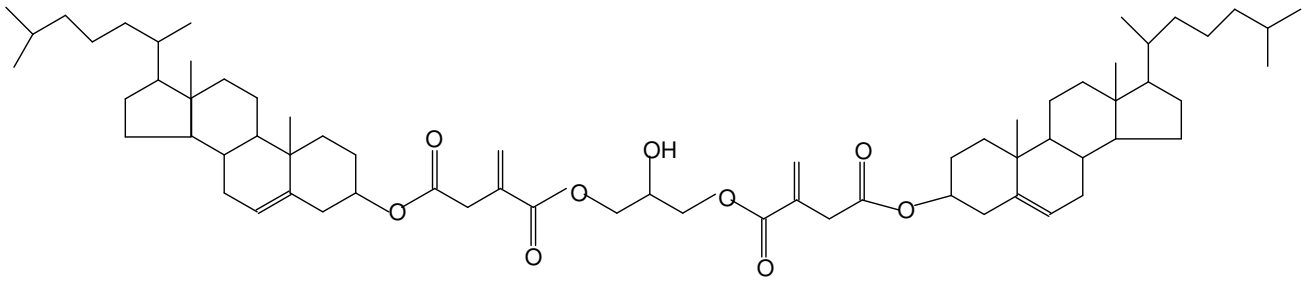
^{1}H NMR [300 MHz $CDCl_3$]: 2.00δ s [6H, 2CH_3], 3.3δ m [-CH-OH], 4.01δ to 4.07δ dd [2H, -CH_2-O-], 4.5δ to 4.56δ dd [2H, -CH_2-O-], 5.66δ and 6.2δ s [4H, =CH_2].

IR [Nujol]: 1720.3 cm^{-1} Ester, 1695 cm^{-1} –COOH, 1645 cm^{-1} C=C.

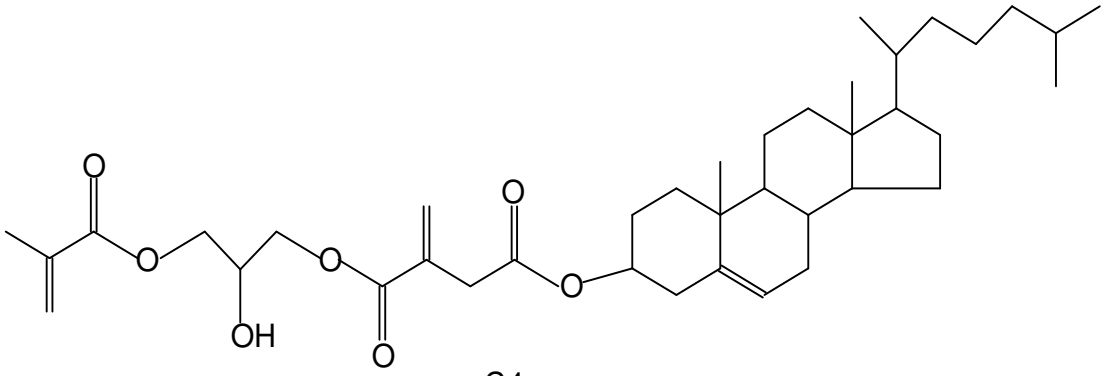
Figure 3.2: Hydrophobic, hydrophilic and cholesterol bearing crosslinkers used for imprinting



C2



C3



C4

Condensation of glyceryl itaconate and cholesterol

In a 250 ml capacity round bottom flask 14.98 g [0.039 Moles] of cholesterol and 10 ml [0.039 Moles] of glycerylitaconate ester were dissolved in 25 ml of THF. The flask was cooled in an ice bath and temperature was maintained between 0-5^0 C. 7.99 g [0.039 Moles] of DCC was dissolved in 5 ml THF and added to the above solution. 1% DMAP was added as a catalyst. Solution was stirred for 2-3 hrs in ice water bath and then at room temperature for 48 hrs. DCU formed during reaction was removed by filtration and workup was performed by the same method reported for M_1.

Yield: 16 g [65%]

^{1}H NMR [300 MHz $CDCl_3$]: 0.67δ s [3H, 18-H3], 0.82δ d [3H, 27-H3], 0.86δ d [3H, 21-H3], 0.93δ s [3H, 19-H3], 1δ to 2.28δ [steroid], 1.89δ s [3H, CH_3-CH_2].

IR [Nujol]: 1720.3 cm^{-1} Ester, 1645 cm^{-1} C=C.

3.1.5 Synthesis of imprinted polymers

In 20 ml test tube, predetermined quantities of monomer, crosslinker and cholesterol were dissolved in ethanol. [For details of precise quantities used in each experiment, please refer to Table 3.1]. For the synthesis of non-imprinted polymers no cholesterol was used to serve as a template during polymerization. The test tubes were purged with nitrogen for 20 min and 1% by weight of AIBN was added. Tubes were maintained in a hot water bath at 60^0 C for 16 hrs. The template cholesterol was extracted from the imprinted polymers by Soxhlet extraction for 48 hrs in methanol. Complete extraction was confirmed by verifying that further extraction did not yield any cholesterol. The polymer was crushed and sieved through a mesh to 37 micron size.

3.1.6 Cholesterol binding studies

Preparation of intestinal mimicking solution [A] [Sellergren et al., 1998]

200 ml water was added to 125 ml of a 0.2 Molar potassium dihydrogen phosphate solution and 95 ml of 0.2 Molar sodium hydroxide solution. Then 24.5 g of sodium deoxycholate [NaDC] and 16.5 g sodium cholate [NaC] were added. The pH was adjusted to 7.5 ± 0.1 with 0.2 Molar sodium hydroxide solution and final volume was made upto of 500 ml using water. After purging with nitrogen for 30 min, the solution was stored in dark at room temperature.

Preparation of cholesterol standard solution [B]

To 500 ml of [A] above, 900 mg of cholesterol was added and the solution was sonicated for 3 hrs at 50^0 C. The solution was then purged with nitrogen for 30 min and stored in dark at room temperature.

Adsorption of cholesterol from intestinal mimicking solution

10 mg of dry polymer was suspended into 5 ml of intestinal mimicking solution [IMS]. The samples were then stirred in a circulatory shaking bath at room temperature for 24 hrs. The solution was centrifuged to separate the polymer and the supernatant solution was estimated for cholesterol by HPLC.

3.1.7 Selectivity studies

The selectivity studies were performed in water/THF mixture [5/6 v/v] since the steroids testosterone and stigmasterol were insoluble in IMS. In a 50-ml conical flask, 10 mg of polymer was weighed and 4 ml of above steroid solution was added. Flask was stirred in a circulatory shaking bath at room temperature for 24 hrs. The polymer suspension was centrifuged [1000 rpm for 30 min] and concentration of ligand in the supernatant was determined by UV spectroscopy monitoring for stigmasterol at 206 nm and testosterone at 214 nm. The amount of steroid bound to the polymer was calculated by difference.

3.1.8 Swelling studies

Equilibrium swelling studies were carried out for all polymers in water at 25^0 C as per standard procedure [Sellergren et al., 1998].

3.2.0 Results and discussion

The importance of lowering cholesterol is well established [Huval et al., 2001, Holtmeier et al., 1996, Davidson et al., 2002]. In view of the problems associated with the administration of cholesterol lowering agents such as statins, there is an increasing emphasis on use of polymeric adsorbents as sequestrants for bile acids as well as cholesterol. Since the functionalities available on the two are not the same, the choice of functional groups for sequestering cholesterol and bile acids differs. Polymers, which bind to bile acids through ionic interactions, have been synthesized and binding capacities of polymeric adsorbents have been reported [Huval et al., 2001].

Table 3.1: Preparation of adsorbents for the cholesterol binding experiments [a]

Polymer	Mole ratio	Weights g	Monomer	Crosslinker
P_1	20:4:1	2.5:1.146:0.243	M2	C1
P_2	5:1:1	2.5:0.286:0.243	M2	C1
P_3	20:4:1	0.4:0.162:0.033	M2	C2
P_4	20:4:1	2.5:1.257:0.243	M1	C1
P_5	9:1:1	0.4:0.125:0.075	-----	C2, C4
P_6	7:3:3	0.4:0.483:0.290	------	C2, C4
P_7	5:5:5	0.4:1.127:0.678	------	C2, C4
P_8	2:8:8	0.9:4.50:2.713	-------	C2, C4
P_9	9:1:1	2.5:0.900:0.541	-----	C1, C4
P_{10}	7:3:3	1.25:1.736:1.044	------	C1, C4
P_{11}	5:5:5	1.25:4.052:2.438	------	C1, C4
P_{12}	2:8:8	0.416:5.40:3.251	--------	C1, C4
P_{13}	2:1:1	1.25:3.321:1.210	------	C1, C3

[a] The polymers were prepared as described in the experimental section

Where, C_1 = EGDMA

C_2 = Glyceroldimethacrylate

C_3 = Glyceryldicholesteryl itaconate

C_4 = Monocholesteryl itaconate glycerol methacrylate

M_1 = Monocholesteryl itaconate

M_2 Cholesteryl methacrylate

Since cholesterol contains no ionizable groups, the only interactions for binding to cholesterol are either hydrogen bonding or hydrophobic interactions. Further, for binding in aqueous media, only the later can be exploited. Imprinting using a covalent approach is reported to be more efficient than that using a non-covalent approach [Davidson et al., 2002, Asanuma et al., 1997, 2000, Whitcombe et al., 1995]. Non-covalent imprinting methodology has also been extensively investigated for the imprinting of cholesterol and subsequent rebinding from non aqueous media [Sreenivasan 1998].

In particular Sellergren et al., [1998] synthesized a large number of imprinted polymers containing cholesterol, bile acid derivatives and EGDMA as the crosslinker. These were then evaluated for the selective adsorption of cholesterol from simulated intestinal fluids. Typically the ratio of the crosslinker, methacrylic acid and the steroid monomer was [10:2:1] and the ratio of the steroid monomer to cholesterol used as template was [2:1]. Methacrylic acid was incorporated as to bind to cholesterol through hydrogen bonding and also to repel bile acids in the intestine.

In view of the large excess of hydrophobic crosslinker, viz. EGDMA used in the synthesis of the crosslinked polymer, we believed that at least part of non specific binding of cholesterol would be due to hydrophobic binding with the crosslinker. In order to explore if this were indeed so, we synthesized a crosslinker which would have hydrophilic group on the crosslinker backbone. Apart from the selectivity in recognition, the capacity of the adsorbent is equally critical. An increase in the content of the steroid monomer, which would present the rebinding sites for higher capacity, leads to a lower degree of crosslinking and consequently a loss in selectivity. In order to circumvent this problem, we synthesized crosslinkers containing cholesterol. Copolymerization of these along with conventional crosslinkers was expected to lead to enhanced cholesterol binding capacity without loss in selectivity, since the crosslink density remained the same. Apart from these two variables which govern the chemical composition of the polymer, the polymerization conditions as well as the monomer composition influence the morphological structure of the cross linked polymer as reflected in pore volume, pore size, surface area etc, which also influence the adsorption capacity. In the following sections we explain the effect of these variables on the binding capacity for cholesterol as well as selectivity *vis a vis* other steroids.

3.2.1 Polymer synthesis and characterization

Sellergren et al., [1998] incorporated methacrylic acid in the crosslinked polymer structure to provide a hydrogen bonding site for cholesterol and also repel any bile acids. The crosslinker used in this work *viz* EGDMA was highly hydrophobic. It may be noted here that methacrylic acid is ionized at pH beyond 4 and renders polymers containing methacrylic acid hydrophilic. Since we wished to investigate the effect of hydrophilicity of crosslinker on suppressing non specific binding, we did not want this to be

complicated by the swelling caused by ionization of methacrylic acid. In the absences of methacrylic acid, swelling of network would be a good measure of the hydrophilicity of the matrix. Hydrophilicity was enhanced by incorporating either ionizable groups in the monomer itself, as in the case of monocholesteryl itaconate [Figure 3.1] or by incorporating hydroxyl groups in the crosslinker, as in the case of glycerol dimethacrylate. In order to increase the concentration of cholesterol bearing conjugates [Figure 3.2] without lowering the degree of crosslinking, we synthesized hydroxyl bearing crosslinkers monocholesteryl itaconate glycerol methacrylate and glyceryl dicholesteryl itaconate. The latter allowed us to double the loading of the binding sites compared to the former. The ratio of cholesterol conjugate to template cholesterol as well as the cholesterol bearing crosslinker to conventional crosslinker was varied [Table 3.1]. All polymerizations were carried out in presence of ethanol as the porogen as this is

Table 3.2: Adsorbents for cholesterol binding: Swelling ratio and surface area

Polymer	Swelling ratio*	Pore volume of MIP cm^3/g	Pore surface area of MIP m^2/g
P_1	1.00	2.1892	32.47
P_2	3.00	2.3743	38.24
P_3	3.00	1.3101	20.81
P_4	3.00	2.0704	46.59
P_5	7.00	2.4929	53.25
P_6	4.00	1.2165	52.40
P_7	3.00	1.2527	19.58
P_8	-	1.5378	18.00
P_9	4.00	2.4457	48.62
P_{10}	3.00	1.9149	50.84
P_{11}	1.00	1.3729	37.35
P_{12}	2.00	-	-

* Weight of swollen polymer/weight of dry polymer

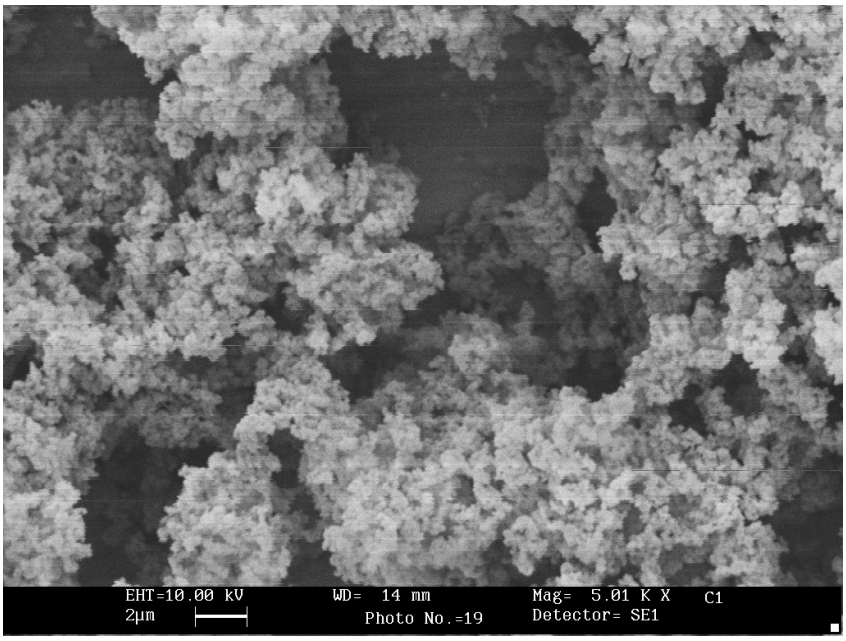
supposed to lead to macroporous resins with low swelling [Sellergren et al., 1998]. Those prepared in the presence of dichloromethane are gel type and exhibit high degree of swelling [Sellergren et al., 1998]. Since the crosslinked polymers are to be used as cholesterol sequestrants in 'intestinal mimicking' media, the swelling measurements were made in water rather than other solvents. Polymers were characterized for their surface area and pore volume [Table 3.2]. The scanning electron microscope imaging indicated that the cholesterol imprinted polymers were more porous than the corresponding non imprinted polymers [Figure 3.3].

3.2.2 Evaluation of polymers prepared using cholesterol bearing monomers

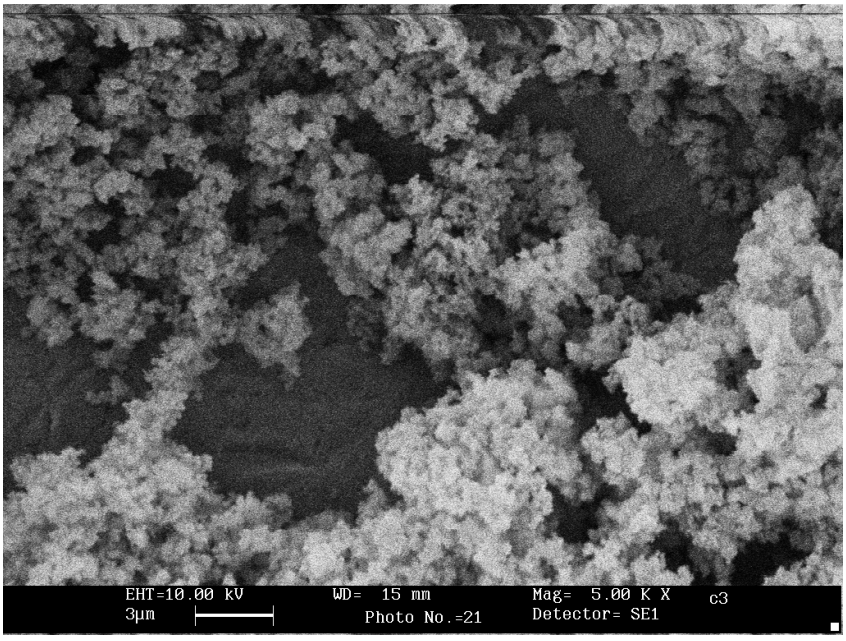
In this series of experiments, ethylene glycol dimethacrylate was copolymerized with cholesteryl methacrylate in the mole ratio 5:1. Polymerization was carried out using the functional monomer: template mole ratio of 4:1 [P_1] and 1:1 [P_2] respectively. It was observed that imprinting of cholesterol in stoichiometric proportion [1:1] led to a higher binding capacity for cholesterol for P_2 [23 mg/g] as compared to the case wherein excess of functional monomer [4:1] was used for P_1 [16.7 mg/g] [Table 3.3]. This can be attributed to the preferential self-association involving the cholesterol bearing monomers rather than the intermolecular association between the cholesterol bearing monomer and cholesterol. In contrast, when the functional monomer and the template are incorporated in the stoichiometric proportions, the intermolecular association between the cholesterol bearing monomer and the template molecule *viz* cholesterol will predominate, leading to higher imprinting efficiency. The formation of highly porous structure with higher surface area leads to higher swelling ratio [*viz* 3] as compared to the polymer synthesized using a high monomer template ratio, which exhibits a low swelling ratio [*viz* 1]. The exposure of the binding sites during rebinding leads to higher cholesterol binding capacity. In contrast, when ethylene glycol dimethacrylate was replaced by glycerol dimethacrylate, the porosity as well as surface area both decreased and yet swelling ratio was high [*viz* 3], since the crosslinker is now more hydrophilic as compared to EGDMA. As a result, the binding capacity of the imprinted polymer P_3 decreased [8 mg/g]. Yet the nonspecific hydrophobic binding was suppressed as indicated by very low cholesterol binding capacity of the corresponding non-imprinted polymer [3 mg/g]. Substitution by

Figure 3.3: Scanning electron microscope [SEM] of imprinted vs non imprinted polymers [P_9]

Imprinted polymer



Non imprinted polymer



glycerol dimethacrylate leads to higher selectivity for cholesterol *vis a vis* a non imprinted polymer [2.6] as compared to the hydrophobic crosslinker EGDMA [1.45].

Thus while we have succeeded in improving the selectivity by using a more hydrophilic crosslinker, we still need to enhance the binding capacity of the imprinted polymer for cholesterol. In order to further explore the effect of hydrophilicity of the matrix on cholesterol binding capacity, cholesteryl methacrylate was replaced by cholesteryl mono itaconate. While the swelling ratio of this polymer [P_4] was the same [*viz* 3] as that in case of P_2 and P_3, the porosity was comparable and the surface area was much higher than in case of both P_2 and P_3 which leads to increased exposure of the cholesterol binding sites within the porous matrix. Accordingly the cholesterol imprinted copolymer comprising mono cholesteryl itaconate and ethylene glycol dimethacrylate P_4 exhibits the highest binding capacity [32.5 mg/g] in this series of adsorbents without loss of imprinting efficiency [1.4]. It may be noted that the ratio of the cholesterol bearing monomer and template in this case is 4:1, and bringing this down to 1:1 may lead to further enhancement in capacity.

3.2.3 Evaluation of polymers prepared using cholesterol conjugated crosslinkers

In the previous section it was shown that the substitution of EGDMA by glycerol dimethacrylate resulted in enhanced selectivity of the imprinted polymer to cholesterol *vis a vis* non imprinted one, but the cholesterol binding capacity was low. This could perhaps be increased by increasing the concentration of cholesteryl methacrylate. However, this would lead to a decrease in the crosslinker concentration and loss in selectivity. To over come this problem, we synthesized cholesterol bearing crosslinker monocholesteryl itaconate glycerol methacrylate [C_4]. This crosslinker can be looked upon as glycerol methacrylate containing an additional methylene group conjugated with cholesterol. The incorporation of this crosslinker would help in increasing cholesterol binding without sacrificing the crosslink density. Although increasing cholesterol loading is expected to enhance capacity without sacrificing the cross link density and hence the selectivity, it must be borne in mind that this will also depend on the porosity and surface area of the resulting polymer and that these morphological features can not be independently controlled. The results for the polymer samples P_5 to P_8 [Table 3.3]

Table 3.3: Rebinding of cholesterol

Polymer	Rebinding by MIP mg/g	Rebinding by Non-MIP mg/g	Imprinting Efficiency α*
P_1	16.7	11.5	1.4
P_2	23.0	18.2	1.3
P_3	8	3	2.6
P_4	32.5	23.8	1.4
P_5	28.6	21.5	1.3
P_6	23.6	15.7	1.5
P_7	18.2	15.2	1.2
P_8	33.4	19.0	1.7
P_9	43.7	11.7	3.7
P_{10}	20.0	10.3	1.9
P_{11}	31.2	13.6	2.2
P_{12}	32.0	22.9	1.4
P_{13}	19.9	10.7	1.9

α = MIP/non-MIP

demonstrate, that at constant ratio of cholesterol bearing crosslinker to cholesterol [1:1], the selectivity of the imprinted polymers to cholesterol *vis a vis* non imprinted polymers remains practically unchanged, at least the values show no systematic decline as one would anticipate if the crosslinker concentration were to decrease. This is because, although the ratio of the crosslinker to cholesterol bearing crosslinker is varied, there is no change in crosslink density. In contrast, the cholesterol binding capacity for both imprinted as well as non-imprinted polymer decreases, when the concentration of the cholesterol bearing crosslinker is increased. This could be attributed to decrease in the pore volume as well as surface area, which implies that although as indicated by the chemical composition the amount of cholesterol available for the hydrophobic binding has increased in principle, the fraction available on the pore surface has decreased as a

result of decrease in porosity and surface area. In the case of polymer P_8, the ratio of crosslinker to cholesterol bearing crosslinker has increased four folds [1:4] as compared to that in case of polymer P_7. Thus while the porosity has marginally increased and surface area decreased, a four fold increase in cholesterol concentration results in a larger available population of cholesterol binding sites on the surface which leads to higher cholesterol binding capacity in case of both imprinted and non imprinted polymers. Yet another feature to be noted is that these polymers are highly hydrophilic as indicated by their swelling ratios [*viz* 7-3]. It is possible that very high hydrophilicity suppresses the binding of cholesterol.

To test this hypothesis, we replaced glycerol dimethacrylate by EGDMA. The results for the polymer P_9 to P_{12} indicate that for identical composition, the swelling ratio decreases when glycerol dimethacrylate is replaced by EGDMA. Thus polymers P_5 and P_9 are identical in monocholesteryl itaconate glycerol methacrylate content as well as porosity and to a certain extent the surface area, which implies that parts of the sites are not available on the surface for binding with cholesterol. Yet the cholesterol binding capacity of the polymer P_9 is 43.7 mg/g, which is, 50% higher than polymer P_5 [28.6 mg/g]. It is also note worthy that the cholesterol imprinted polymer P_9 exhibits higher selectivity [3.7] with respect to cholesterol over the corresponding non imprinted polymer. In the case of polymer P_5 this value is [1.3]. Although the proportion of cholesterol bearing crosslinker is increased in case of polymer P_{10}, decreased porosity accompanied by a modest increase in surface area leads to lower cholesterol binding capacity as well as selectivity *vis a vis* non imprinted polymer. A further increase in cholesterol content as in case of polymer P_{11} more than compensates for this lowering and leads to a higher cholesterol binding capacity. Further increase in the cholesterol bearing crosslinker has no effect. Thus although the effect of polymer composition and morphology cannot be independently controlled, we have achieved higher cholesterol binding capacity as well as selectivity compared to the non imprinted polymer, by judicious choice of a cholesterol bearing hydrophilic crosslinker and EGDMA.

The results of cholesterol binding capacity of polymers P_9-P_{12} also highlight the role of molecular imprinting *vis a vis* self stacking of cholesterol in the rebinding experiment. In this series of polymers, the degree of cross linking remains constant irrespective of the

amount of cholesteryl ligand incorporated, since it has been conjugated with a crosslinker. In a typical rebinding experiment 10 mg of the polymer is brought in contact with 5 ml of intestinal mimicking fluid, which contains 4.6 mMoles/L cholesterol. In the case of polymer P_9, 10 mg polymer, which exhibits a swelling ratio of 4, the concentration of cholesteryl ligand is 0.1 Mole/L. Since the ligand is a part of crosslinked structure, self-association between cholesteryl ligand will not be favored. Similarly since free cholesterol concentration is much lower than, the cholesteryl ligand concentration, the self-association between free cholesterol will not be favored over intermolecular association between cholesterol ligand and free cholesterol. The latter is favored due to large apolar contact. It may be further noted that as the cholesteryl ligand concentration increases from P_9 to P_{11} the swelling ratio decreases. As a result of these two effects, the cholesteryl ligand concentration within the swollen particle increases 20 folds, yet the cholesterol rebinding capacity in the case of non-imprinted polymers remains practically unaltered. This indicates that in the case of non-imprinted polymers the guest cholesterol molecule does not stack over the cholesteryl conjugate. In contrast, the cholesterol binding capacity of cholesterol imprinted polymers is always higher than the corresponding non imprinted polymers which indicates that apolar association between the guest cholesterol molecule and the cholesteryl ligand is effective only in the case of cholesterol imprinted polymers. The increase in the binding capacity of non-imprinted polymer P_{12} can be attributed to very large excess in cholesterol ligand concentration.

Under the conditions of synthesis of the cholesterol imprinted polymer, the concentration of cholesterol in alcohol was 0.073 Moles/L the solubility of cholesterol is eight times higher. Also the mole ratio of cholesterol to cholesterol bearing ligand increases from 1:1 to 1:4. We therefore expect no preferential self-association of cholesterol over intermolecular association with cholesteryl ligand.

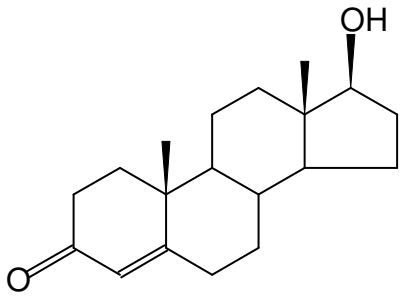
In order to further explore if the binding capacity of the polymer could be increased by increasing the amount of cholesterol in the cholesterol bearing crosslinker, we replaced the crosslinker monocholesteryl itaconate glycerol methacrylate [C_4] by glycerol dicholesteryl itaconate [C_3]. A comparison of the molecular structure of the two reveals that for the same molar composition, the crosslinker C_3 offers twice the number of cholesterol binding sites. The structure of this crosslinker is analogous to the

multifunctional vinyl monomer derived from 3,5 dibromobenzoic acid, propargyl alcohol and cholesterol, which contained two cholesterol receptor sites [Davidson et al., 2003]. The molecularly imprinted polymer based on this tweezer monomer exhibited very high selectivity [5.4]. We would thus expect the polymer [P_{13}] to offer higher cholesterol binding capacity. However the results do not validate the same. This can be attributed to the following factors. For the same molar composition, the polymer P_{13} is more hydrophobic than the polymer comprising crosslinker C_4. Also the presence of two cholesterol molecules in the vicinity of one another is likely to result in hydrophobic association between the two. This would result in lower availability of cholesterol as template binding site. The crosslinker C_3 can also be looked upon as two moles of cholesteryl methacrylate [M_2] conjugated through a hydrophilic spacer. A comparison of the results for polymers P_2 and P_{13} reveals that even when amount of cholesterol binding sites available for imprinting is increased two folds, the cholesterol binding capacity has actually marginally decreased. Yet the selectivity has increased which could be attributed to the presences of hydrophilic hydroxyl group in the spacer.

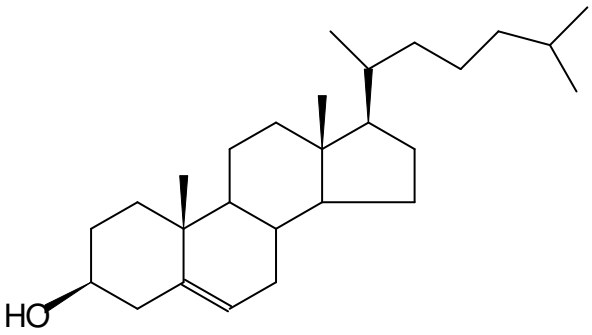
3.2.4 Selectivity measurements: Role of sorbate structure

The selectivity of molecularly imprinted polymer was evaluated in two ways. 1] We evaluated the binding capacity of cholesterol imprinted polymers for binding of cholesterol as well as two related molecules stigmasterol and testosterone. Stigmasterol has the same structure as cholesterol, but for the unsaturation in the side link and incorporation of ethyl linkage at C_{24} position [Figure 3.4]. Testosterone on the other hand has no side chain, and is over all more hydrophilic than cholesterol or stigmasterol. If the rebinding involved hydrophobic binding, we would expect testosterone binding capacity to be lower than stigmasterol binding capacity. 2] We also evaluated the capacities of corresponding non-imprinted polymers towards all three sorbents. The results summarized in Table 3.4 indicate that in all cases cholesterol imprinted polymers have higher binding capacities for cholesterol than for either stigmasterol or testosterone. This is only to be expected since imprinting is expected to create shape selective cavities within the polymer structure. As a result $\alpha_{Cho/Sti}$ and $\alpha_{Cho/test}$ are always higher than unity. Further it is also note worthy that binding capacities for testosterone are always lower than those for stigmasterol. An analysis of three-dimensional structures of the three

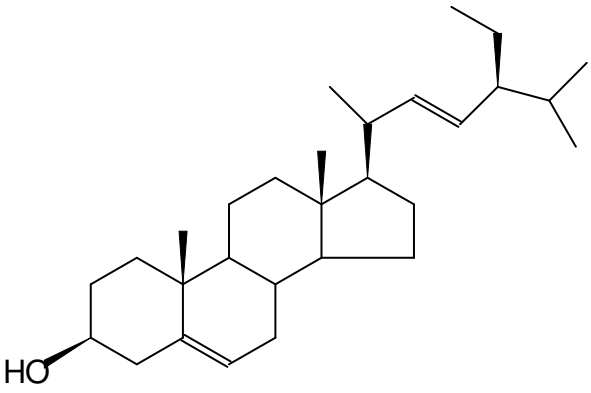
Figure 3.4: Steroids used for selectivity studies



testosterone



Cholesterol



Stigmasterol

steroids reveals that that the space fitting requirements of cholesterol versus stigmasterol are only marginally different. Testosterone in contrast can fit into the same cavity as cholesterol and yet the binding capacity for testosterone is lower. This is because testosterone is more hydrophilic than either cholesterol or stigmasterol. Hence $\alpha_{Cho/Test}$ values for all imprinted polymers are greater than $\alpha_{Cho/Sti.}$ Table 3.4 also summarizes the binding capacities of non-imprinted polymers for cholesterol, stigmasterol and testosterone. In the absence of imprinting, rebinding is driven by hydrophobic binding alone. In the case of non-imprinted polymers, therefore the value $\alpha_{Sti/Test}$ could be either greater or lower than unity.

Table 3.4: Selectivity studies

Polymer	Cholesterol mg/g	Stigmasterol mg/g	Testosterone mg/g	α_{sti} mg/g	α_{test} mg/g	$\alpha_{Sti/Test}$ mg/g
P_1	13.5 [9.1]	7.8 [4.2]	6.5 [3.2]	1.73[2.16]	2.0[2.84]	1.2[1.3]
P_3	13.2 [5.9]	9.7 [3.9]	8.1 [4.3]	1.36[1.51]	1.6[1.37]	1.1[0.9]
P_4	22.7 [17.2]	8.7 [4.5]	7.1 [5.1]	2.60[3.82]	3.1[3.37]	1.2[0.8]
P_5	25.0 [17.2]	12.2 [7.8]	7.8 [5.4]	2.04[2.20]	3.2[3.18]	1.5[1.4]
P_6	12.7 [5.2]	8.9 [4.2]	6.2 [3.3]	1.42[1.24]	2.0[1.58]	1.4[1.2]
P_7	14.2 [10.1]	7.5 [3.8]	6.2 [3.5]	1.89[2.66]	2.3[2.88]	1.2[1.0]
P_9	38.5 [14.0]	17.0 [15.0]	8.1 [6.4]	2.30[0.93]	4.7[2.19]	2.0[2.3]
P_{10}	13.3 [5.3]	7.8 [3.8]	6.7 [4.1]	1.70[1.40]	1.9[1.3]	1.1[0.9]
P_{11}	26.5 [14.5]	8.6 [4.8]	7.2 [3.4]	3.08[3.02]	3.7[4.26]	1.2[1.4]
P_{13}	21.2[11.8]	11.2[5.2]	7.5[3.2]	1.90[2.26]	2.8[3.6]	1.5[1.6]

α_{sti} = MIP cholesterol / MIP stigmasterol

α_{test} = MIP cholesterol / MIP testosterone,

$\alpha_{Sti/Test}$ = MIP stigmasterol/MIP testosterone

The numbers in the parentheses show the selectivity of the non-imprinted polymer

In summary the use of stoichiometric proportion of functional monomer especially cholesterol monoitaconate [M_1] and template has led to polymers having higher binding capacity for cholesterol. The choice of the functional crosslinker has led to polymers with enhanced capacities. A more effective method of increasing the binding capacity without affecting the selectivity was to use crosslinkers containing cholesterol. The selectivities of these MIPs for cholesterol *vis a vis* stigmasterol and testosterone further demonstrate the merit of these crosslinkers. Though the polymer composition and morphology could not be independently controlled, this approach has led to MIPs having higher recognition ability for cholesterol in the aqueous medium. The rebinding studies using stigmasterol and testosterone have further highlighted the role of hydrophobic interactions during rebinding.

3.3.0 Concluding remarks

Polymers containing cholesterol bearing monomers and crosslinker imprinted with cholesterol exhibit enhanced affinity and capacity for cholesterol from intestinal mimicking media. It was shown that the non-specific binding on to imprinted and non-imprinted polymers could be reduced by hydrophilic modification. Cholesterol functionalized crosslinkers were synthesized to improve the recognition ability of MIPs. This approach led to polymers having stronger affinity for cholesterol than reported earlier. The polymers also had very good selectivity for cholesterol as compared to other steroids. The adsorptive capacity seems to arise from binding sites induced by the hydrophobic association between cholesterol units in the polymer backbone and the presence of cholesterol during polymerization. The adsorbents exploiting this approach may find applications for selective binding of steroids from aqueous medium.

Chapter 4

Enhancing Active Site Utilization in Molecularly Imprinted Polymers: A New Synthetic Approach

4.0.0 Introduction

Polymers bind bile acids preventing their reabsorption in small intestine. To maintain the balance, liver produces bile acids by enzymatically cleaving cholesterol, which results in the net reduction of plasma cholesterol [Parkinson et al., 1970, Shepherd J 1989, LaRosa, 1989, Fears et al., 1990, Polli et al., 1995, Nau et al., 1973]. A wide range of bile acid sequestrants [BAS] has been exploited for the treatment of hypercholesterolemia [Stedronsky, 1994, Mandeville et al., 1997, The Dow Chemical Company, 1958, Terinent et al., 1960, Thale et al., 1978, Tkahashi et al., 1980, Class S.D, 1991, Raghavan et al., 1997, Schreiber et al., 2001, Benson et al., 1997, Homma et al., 1996, Gore et al., 2004]. Anion-exchange polymers, based on copolymers of styrene [Cook et al., 1978, Wagner A.F, 1978, Jaxa-Chamiec et al., 1990, Pierre et al., 1992] and acrylic monomers have been investigated for this purpose [Borzatta et al., 1980, Grier et al., 1980, Pierre et al., 1986, Zhu et al., 1992, Kobayashi et al., 1995a, 1995b]. Although the bile acid binding capacities of these sequestrants under *in-vitro* conditions are of the order of 500 mg/g, under *in-vivo* conditions only about 2% of the binding capacity is realized. Hence the dose requirements are as high as 16-24 g/day [Huval et al., 2004]. Yet this is a better approach than the removal of cholesterol, by adsorbents as these adsorbents exhibit very low capacities and selectivities [Gore et al., 2004]. Huval et al., [2001] reported the use of molecular imprinting technique for the synthesis of BAS. Imprinted polymers were synthesized by partially neutralizing poly [allylamine hydrochloride] and crosslinking with epichlorohydrin in the presence of the template sodium cholate [NaC]. The imprinted polymers exhibited enhanced capacity and cooperativity during rebinding as compared to non imprinted polymers.

For the synthesis of molecularly imprinted polymers [MIPs], conventionally a molecular assembly comprising a functional monomer, template and a crosslinker is polymerized / crosslinked. The template is then leached out to create a size / shape selective cavity. In this chapter we report a new two stage approach for the synthesis of MIPs. We prepared a soluble copolymer of allylamine hydrochloride and methylene bis acrylamide [MBAM], crosslinked it in the presence of the template, NaC and then leached out the template to create a cavity selective to NaC. Our approach exploits the same mechanism for

rebinding of NaC as envisaged by Huval et al., [2001]. The results demonstrate that the two stage approach leads to better capacity utilization of rebinding sites and selectivity.

4.1.0 Experimental

4.1.1 Materials

Sodium cholate [NaC], Allylamine, Methylene bisacrylamide [MBAM], 2,6 Dimethyl β-cyclodextrin [DMCD], Sodium taurocholate [NaT] were purchased from Aldrich [Sigma-Aldrich, Bangalore, India] Potassium persulfate [$K_2S_2O_8$], Potassium dihydrogen phosphate, Sodium hydroxide were supplied by S.D. Fine Chemicals, India, HPLC grade Methanol was supplied by Qualigens Chemicals, India. All solvents supplied by local suppliers were purified as per standard procedure [Perrin et al., 1996].

4.1.2 Instrumentation

^{1}H NMR spectra were recorded on Bruker [Bruker, Karlsruhe, Germany] superconducting FT-NMR AC 300 operating at 300 MHz. Electronic absorption measurements were carried out on Shimadzu UV 1601 spectrophotometer [Shimadzu, Kyoto, Japan]. High Pressure Liquid Chromatography [HPLC] analysis was carried out using Waters HPLC [Waters Milford, MA] system comprising 680 automated gradient controller, 510 solvent delivery pumps, 486 tunable absorbance detector and 746 dual channel integrator. A μ - Bondapak C_{18} column [Phenomenex, Torrance, USA] was used. The mobile phase was methanol-deionized water [80:20 v/v]. Flow rate 1 ml/min was used for estimation of cholic acid at 210 nm [retention time 9.2 min] as well as for taurocholic acid [retention time 2.7 min]. Deionized water was obtained using Millipore Direct-Q water purification system [Millipore, Quentin Yvelines Cedex, France], The pore surface area and pore volume of the porous copolymer samples were estimated by mercury intrusion porosimetry in the pressure range of 0-4000 Kg/cm^2 using an Autoscan 60 mercury porosimeter from Quantachrome, USA. The mercury contact angle was 140^0.

4.1.3 Synthesis of methylene bis acrylamide [MBAM] and dimethyl β-cyclodextrin [DMCD] complex

In a 250 ml conical flask 13.31 g [1.00×10^{-2} Mole] of DMCD and 1.54 g [1.00×10^{-2} Mole] of MBAM were dissolved in 196 ml of distilled water. The solution was stirred for 24 hrs at room temperature. Water was evaporated and the complex was characterized by ^{1}H NMR.

Yield: 14.40 g [97%]

^{1}H NMR [300 MHz D_2O]: 6.19 δ d [2H, **CH_2**-CH], 5.62 δ s [1H, -**CH**-CO-], 4.99 δ m [1H, C_1-**H** of DMCD], 4.54 δ s [2H, -NH-**CH_2**-NH-], 3.31 δ s [3H, C_6-O-**CH_3**], 3.40 δ s [3H, C_2-O-**CH_3**], 3.71-3.64 δ m [5H, C_2, C_3, C_4, C_5, C_6 protons of DMCD]. Peak at 5.07 δ indicates the formation of inclusion complex [Schneider et al., 1998].

4.1.4 Synthesis of allylamine hydrochloride monomer

Allylamine was converted to the hydrochloride salt by the method reported earlier [Bergthaller, 1982]. To a 200 ml water-jacketed reaction kettle equipped with a mechanical stirrer, 9 g [2.46×10^{-1} Mole] concentrated hydrochloric acid was added. The acid was cooled to 4 ^{0}C using circulating water bath. Allylamine 8 g [1.40×10^{-1} Mole] was added dropwise with stirring, maintaining the temperature between 5-10 ^{0}C. After the addition was complete, 4.94 g of the liquid was removed by vacuum distillation at 60 ^{0}C and washed with acetone. A white solid compound was obtained and characterized.

Yield: 10.4 g [80%]

^{1}H NMR [300 MHz D_2O]: 5.82 δ m [2H, **CH_2**-], 5.31 δ m [1H, -**CH**-], 3.52 δ d [2H, CH-**CH_2**-]

4.2.0 Synthesis of imprinted polymers

4.2.1.1 Synthesis of MIPs by simultaneous polymerization / crosslinking

In a 100 ml round bottom flask, predetermined quantities of crosslinker, monomer and NaC were dissolved in water [Table 4.1]. In particular for the synthesis of polymer P_1, 2.72 g [2.91×10^{-2} Mole] of allylamine hydrochloride, 1.25 g [2.91×10^{-3} Mole] of NaC and 1.50 g [9.70×10^{-3} Mole] of MBAM were dissolved in 40 ml of distilled water. Control polymers were synthesized in identical manner excluding NaC from the recipe. 1 % by weight of potassium persulfate was added as an initiator and the flask was purged with nitrogen for 30 min. Flask was maintained in a hot water bath at 65 ^{0}C for 18 hrs. The template NaC was extracted from the imprinted polymer by Soxhlet extraction for 48 hrs in methanol. Complete extraction was confirmed by verifying that further extraction did not yield any NaC. The polymer was crushed and sieved through a mesh to obtain particles less than 37 micron size.

4.2.1.2 Synthesis of MIPs by sequential polymerization / crosslinking

Stage1: Synthesis of copolymer of MBAM and allylamine hydrochloride

Predetermined quantities of complex and monomer were dissolved in water [Table 4.2]. In particular, for the synthesis of polymer P_8, 4 g [2.69×10^{-3} Mole] of complex and 0.754 g [8.07×10^{-3} Mole] of allylamine hydrochloride were dissolved in 120 ml of distilled water in a round bottom flask. 1 % by weight of potassium persulfate was added as an initiator and nitrogen was purged for 30 min. Flask was maintained in a hot water bath at 65^0 C for 18 hrs. Copolymer was precipitated into methanol and separated by filtration. Polymer was dried under vacuum and characterized.

Yield: 0.58 g [50%]

^{1}H NMR [300MHz D_2O]: 5.44 δ m [2H, **CH_2**- of MBAM], 5.26 δ m [2H, -**CH**-CO-], 4.54 δ m [2H, -NH-**CH_2**-NH-], 3.05 δ s [1H, -**CH**- of allylamine], 2.27 δ m [1H, -CH-**CH**-], 1.77 δ d [2H, -CH-**CH_2**-]

Stage 2: Crosslinking of MBAM / allylamine hydrochloride copolymer in the presence of template NaC

Predetermined quantities of copolymer of MBAM and allylamine hydrochloride and template were dissolved in water [Table 4.3]. In particular for the synthesis of polymer P_8, 0.768 g of the MBAM / allylamine hydrochloride copolymer [containing 1.15×10^{-3} Mole of allylamine hydrochloride] and 0.050 g [1.15×10^{-4} Mole] of NaC as template were dissolved in 2.5 ml of distilled water. The protocols for the synthesis of control polymers and crosslinking in the presence of template were the same as in section 2.5.1.

4.2.2 Acid value estimation

Incorporation of functional monomer allylamine hydrochloride into the polymers was calculated by estimating acid value [Lele et al., 1999].

4.2.3 Sodium cholate binding studies

The NaC solutions were prepared in an aqueous phosphate buffer [pH 7.4] [Baille et al., 2000]. The concentration of stock solution was 2.6 mg/ml.

In a 50 ml conical flask, 10 mg of polymer was weighed and 4 ml of stock bile solution was added. Flask was stirred in a circulatory shaking water bath at 37 ^{0}C for 3 hrs. The polymer suspension was centrifuged [1000 rpm for 30 min]. In 2 ml of supernatant 200 µl acetic acid was added and the solution was diluted to 10 ml with methanol.

Table 4.1: MIPs prepared by simultaneous polymerization / crosslinking

Polymer	Mole ratio C:M:T	In feed			In product M/g $\times 10^{-3}$		In product % by wt	
		C	M	T	C	M#	C	M
P_1	1:3:0.3	1.5 g 9.7×10^{-3} M	2.72 g 2.9×10^{-2} M	1.25 g 2.9×10^{-3} M	5.0	2.0	72	28
P_2	1:3:0.6	1.5 g 9.7×10^{-3} M	2.72 g 2.9×10^{-2} M	2.50 g 5.8×10^{-3} M	5.2	2.1	71	29
P_3	1:3:3	1.5 g 9.7×10^{-3} M	2.72 g 2.9×10^{-2} M	12.56 g 2.9×10^{-2} M	4.9	2.5	77	23
P_4	1:3:6	1.5 g 9.7×10^{-3} M	2.72 g 2.9×10^{-2} M	25.00 g 5.8×10^{-2} M	5.0	2.0	72	28
P_5	1:9:9	1.5 g 9.7×10^{-3} M	8.40 g 8.9×10^{-2} M	38.66 g 8.9×10^{-2} M	4.6	3.0	61	39
P_6	1:13:13	0.75 g 4.8×10^{-3} M	5.91 g 6.3×10^{-2} M	27.21 g 6.3×10^{-2} M	4.6	2.9	62	38
P_7	1:18:18	0.75 g 4.8×10^{-3} M	8.18 g 8.7×10^{-2} M	37.67 g 8.7×10^{-2} M	4.3	3.4	56	44

C = Crosslinker, M = Monomer, T = Template NaC, M# = Calculated by acid value

Table 4.2: Synthesis of copolymers of MBAM and allylamine hydrochloride

Polymer	Mole ratio C: M	In Feed		In product M/g × 10^{-3}		In product % by wt	
		C	M	C	$M^{\#}$	C	M
P_8	1:3	4 g 2.6×10^{-3} M	0.75 g 8.0×10^{-3} M	5.5	1.5	79	21
P_9	1:3	4 g 2.6×10^{-3} M	0.75 g 8.0×10^{-3} M	5.5	1.5	79	21
P_{10}	1:3	4 g 2.6×10^{-3} M	0.75 g 8.0×10^{-3} M	5.5	1.5	79	21
P_{11}	1:3	4 g 2.6×10^{-3} M	0.75 g 8.0×10^{-3} M	5.5	1.5	79	21
P_{12}	1:9	4 g 2.6×10^{-3} M	2.32 g 2.4×10^{-2} M	5.3	1.9	74	26
P_{13}	1:13	4 g 2.6×10^{-3} M	3.20 g 3.5×10^{-2} M	5.4	1.6	77	23
P_{14}	1:18	4 g 2.6×10^{-3} M	4.50 g 4.8×10^{-2} M	5.3	1.9	73	27

C = Complex, M = Monomer, $M^{\#}$ = Calculated by acid value.

Table 4.3: MIPs prepared by sequential polymerization / crosslinking

Polymer	**Mole ratio M: T**	In feed	
		Copolymer g	**T**
P_8	3:0.3	0.768	0.05 g 1.15×10^{-4} M
P_9	3:0.6	0.768	0.099 g 2.30×10^{-4} M
P_{10}	3:3	0.768	0.61 g 1.15×10^{-3} M
P_{11}	3:6	0.768	0.99 g 2.30×10^{-3} M
P_{12}	9:9	0.744	0.63 g 1.42×10^{-3} 5M
P_{13}	13:13	0.844	0.71 g 1.35×10^{-3} M
P_{14}	18:18	0.875	0.88 g 1.66×10^{-3} M

T = Template NaC .

Concentration of cholic acid was determined by HPLC, monitoring absorbance at 210 nm [retention time 9.2 min]. The amount of NaC bound to the polymer was calculated by difference.

4.2.4 Selectivity studies

Sodium taurocholate [NaT] solutions were prepared in an aqueous phosphate buffer [pH 7.4]. The concentration of stock solution was 2.6 mg/ml.

In a 50 ml conical flask, 10 mg of polymer was weighed and 4 ml of stock NaT solution was added. Flask was stirred in a circulatory shaking bath at 37^0 C for 3 hrs. The polymer suspension was centrifuged [1000 rpm for 30 min]. In 2 ml of supernatant, 200 µl acetic acid was added and the solution was diluted to 10 ml using methanol. Concentration of taurocholic acid was determined by HPLC, monitoring absorbance at 210 nm [retention time 2.7 min]. The amount of sodium taurocholate [NaT] bound to the polymer was calculated by difference.

4.2.5 Swelling studies

Equilibrium swelling studies were carried out for all polymers in water at 25^0 C as per standard procedure [Gore et al., 2004]. The results are summarized in Table 4.4.

4.2.6 Estimation of binding constant

10 mg of polymer was shaken with solutions of NaC in phosphate buffer at pH 7.4. Total nine concentrations of NaC were selected in the range between 1.2 to 6 mM. The solution was stirred at 37^0 C for 3 hrs. After sedimentation of the polymer, the supernatant was analyzed by HPLC for the rebinding of NaC. The amount of NaC adsorbed was calculated by subtraction using a calibration curve obtained from the same experiment without the polymer. The binding constant was obtained from Hill's equation [Huval et al., 2001].

$$LS = S_{max} \quad K^n [L]^n / 1 + K^n [L]^n$$

In this equation, K is the intrinsic binding constant in mM^{-1}, LS is the density of bound sites [mmol per g of polymer], S_{max} is the total density of sites [mmol per g of polymer], L is the free bile acid concentration in mM, and n is the cooperativity of binding.

Table 4.4: Adsorbents for NaC binding: Swelling ratio and surface area

Polymer	Swelling ratio [a]	Pore volume of MIP cm^3/g	Pore surface area of MIP m^2/g
P_1	1.07 [1.22]	0.1241	151.36
P_2	1.08 [1.19]	0.2532	162.64
P_3	1.06 [1.20]	0.2814	168.55
P_4	1.05 [1.24]	0.3697	178.86
P_5	1.04 [1.18]	0.3186	171.66
P_6	1.08 [1.21]	0.3284	161.53
P_7	1.10 [1.20]	0.3476	164.24
P_8	1.22 [1.00]	0.3984	87.21
P_9	1.24 [1.03]	0.3475	78.22
P_{10}	1.21 [1.04]	0.4561	51.45
P_{11}	1.20 [1.02]	0.3875	67.15
P_{12}	1.24 [1.07]	0.5881	27.24
P_{13}	1.26 [1.09]	0.5295	26.57
P_{14}	1.28 [1.12]	0.4987	57.82

a = Weight of swollen polymer/weight of dry polymer, The numbers in parentheses indicate the swelling ratio of the non-imprinted polymers, whereas numbers preceding the parentheses indicate the swelling ratio of the imprinted polymers

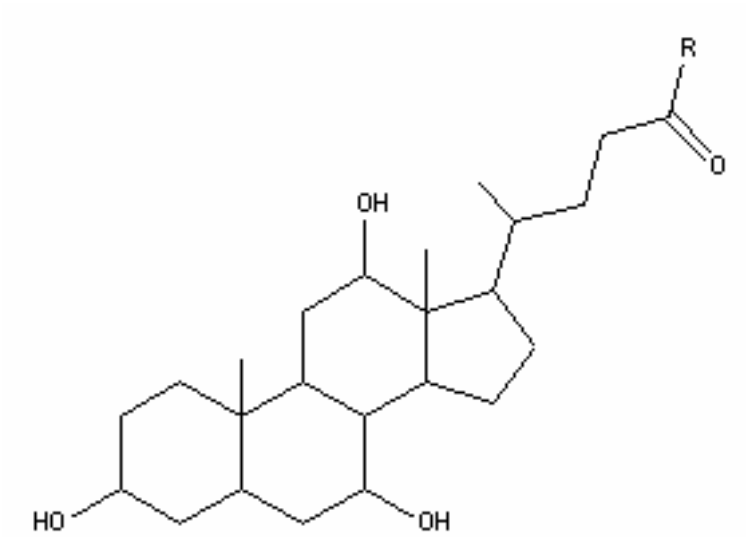
4.3.0 Results and discussion

Cholic acid and chenodeoxycholic acid are the primary bile acids, which undergo deconjugation and dehydroxylation to deoxycholic acid and lithocholic acid. Cholic acid also undergoes conjugation with glycine and taurine to form glycocholic acid and taurocholic acid respectively. Johns and Bates [1970] reported that the affinity constants for bile salts with cholestyramine increase with decrease in the number of hydroxyl substituents. It would, therefore, follow that NaC would have the lowest affinity constant. Nichifor et al., [2001] emphasized the need to develop new sorbents, which would exhibit enhanced affinity towards hydrophilic bile acids. Cholic acid is a carboxylic acid whereas taurocholic acid has a sulfonic acid group and would be expected to bind strongly if ion exchange were the dominating binding mechanism. We therefore selected NaC as the template for imprinting and NaT as the competing bile acid to check the efficiency of imprinting [Figure 4.1].

In their studies, Wu et al., [1996] argued that protonated resins generally are more effective sorbents than the quaternized ones. This was attributed to the swelling characteristics as well as the possibility that the protonated pendent groups developed hydrogen bond interactions with the bile acid anions. We therefore chose allylamine hydrochloride as the functional monomer for our investigations. Rebinding study was carried out at 37 ^{0}C in phosphate buffer at pH 7.4, to mimic the *in-vivo* conditions in the small intestine [Benson et al., 1997, Fordtran et al., 1966]. Incubation time for the polymer was 3 hrs, which is the transit time in the small intestine [Christensen et al., 1985]

In the development of adsorbents based on MIPs the issues that need to be addressed to are 1] capacity, which is reflected in terms of the number of active sites and the efficiency of utilization of the active sites 2] imprinting effect, which is reflected in terms of the enhanced binding capacity achieved over the corresponding non imprinted polymer as well as the binding capacity for the imprinted molecule *vis a vis* a competing molecule and finally 3] strength of binding which is reflected in the association constant as well as the cooperativity of binding .

Figure 4.1: Bile acids used for selectivity study



Where,
Sodium cholate [NaC], R = ONa
Sodium taurocholate [NaT], R = -NH-CH_2-CH_2-SO_3H

In the subsequent sections we first show that the simultaneous polymerization / crosslinking of allylamine hydrochloride and MBAM in the presence of the template NaC, yields polymers which exhibit enhanced binding capacity for NaC. We then demonstrate that the synthesis of the same copolymer in the first step and crosslinking in the presence of the template in the subsequent step results in higher binding capacity for NaC indicating enhanced functional group utilization and selectivity over NaT.

4.3.1 Rebinding of NaC from MIPs prepared by simultaneous polymerization / crosslinking

In this series of experiments, allylamine hydrochloride was polymerized with MBAM in the presence of the template NaC. Mole ratio of crosslinker to functional monomer was varied [Table 4.1]. Crosslinking had to be carried out in the concentration range; 1.04 to 4.49 Moles/lit for polymers P_1 to P_7 because of limited solubility of MBAM in water. Crosslinking under these conditions results in poor binding between allylamine hydrochloride and NaC, leading to lower capacity as well as selectivity. Enhancement in binding capacity primarily depends on the functional groups available for rebinding of

the sorbate molecule [Gore et al., 2004, Wu et al., 1996]. In order to evaluate this effect, we increased the mole ratio of template NaC to the functional monomer, allylamine hydrochloride, from 0.1 [P_1] to 2 [P_4]. The rebinding capacity increased from 290 to 357 mg/g. Thus, with increasing ratio of template to functional monomer, binding with the functional monomer improved. However the ratio of template to functional monomer could not be raised beyond 2 because of the limited solubility of the template. For the same reason the ratio was maintained 1 in subsequent experiments when amount of functional monomer was increased further.

In order to enhance the incorporation of functional monomer into the crosslinked polymer, we increased the mole ratio of functional monomer to crosslinker from 3 to 18, [P_4 to P_7]. But only a relatively modest increase in allylamine hydrochloride content [28 to 44%] was observed. This is because of the low reactivity of allylamine hydrochloride [Harada et al., 1985]. When these polymers were evaluated for NaC rebinding from the phosphate buffer at pH 7.4, the bile acid rebinding capacity was increased from 357 mg/g [P_4] to 475 mg/g [P_7]. In view of variation in the polymer composition resulting during polymerization, the extent of utilization of functional groups was used as a measure of the effect of templating on rebinding. The utilization of the functional groups remained practically constant at 33%. The rebinding capacity for the non-imprinted polymers was always lower.

These results are consistent with those reported by Huval et al., [2001] wherein, the maximum binding capacity for NaC increased from 460 mg/g to 847 mg/g, when the amount of NaC used in imprinting was increased from 2.31 to 11.53 mmol. Higher capacities of the imprinted polymers can therefore be attributed to the presence of the template selective high affinity binding sites created during the template mediated polymerization / crosslinking reaction. The authors further concluded that the imprinting imparts additional binding properties to the polymers, which results in reduced desorption of bile acids as the polymer particle moved along gastrointestinal [GI] tract.

The fact that for the polymers $P_1 \rightarrow P_4$ the utilization of the binding sites increases with the ratio of template to functional monomer, highlights the role played by the template molecule in increasing the population of the active sites which can rebind the bile acid as a result of both the electrostatic as well as the imprinting effect. In the case of polymers

$P_4 \rightarrow P_7$ increase in the functional monomer content leads to increase in the rebinding capacity for NaC although the extent of utilization of the sites is not increased.

It may be noted that the rebinding capacity of 33 - 40% is very high compared to the values of around 5% normally reported in the literature. It is even more remarkable since the polymer contains 56 - 77% crosslinker as against 95% conventionally employed. This is probably because the rebinding primarily takes place through electrostatic interaction.

In the case of polymers $P_1 \rightarrow P_4$ wherein the ratio of template to functional monomer was increased from 0.1 to 2, the ratio of binding capacity of imprinted polymer *vis a vis* non imprinted polymer increased consistently from 1.01 to 1.23, which is not surprising. During the synthesis of polymers P_3 and $P_5 \rightarrow P_7$, the ratio of template to functional monomer was maintained constant [1]. Crosslinker content of the polymer decreased from 77 to 56%. Yet the ratio of binding capacity of imprinted polymer *vis a vis* non imprinted polymer increased consistently from 1.21 to 1.35.

For the imprinted polymers $P_1 \rightarrow P_4$ the selectivity for the template molecule NaC versus the competing molecule NaT, was fairly high [1.4 ± 0.15] although there was no systematic variation in relation to the ratio of template to functional monomer [Table 4.5]. In contrast, for polymers P_3 and $P_5 \rightarrow P_7$ the selectivity was rather low [1.1 ± 0.1] which can be attributed to a certain extent to lower crosslinker content and the fact that the dominating binding mechanism is electrostatic interaction.

In summary, molecular imprinting by simultaneous polymerization / crosslinking results in enhanced binding capacities for the template molecule with increasing ratio of template to functional monomer. The extent of utilization of functional groups remains practically constant with increasing functional monomer content. The binding capacities of the imprinted polymers for the template molecule are higher as compared to the corresponding non imprinted polymers. However the selectivities *vis a vis* competing taurocholic acid are low and do no show any systematic trend. This is probably because the dominant rebinding mechanism is electrostatic interaction. During simultaneous polymerization / crosslinking in the presence of the template there is considerable shrinkage which results in the distortion of the cavity created during imprinting which could also lead to poor selectivity.

Table 4.5: Rebinding and selectivity study

Polymer	NaC[#] mg/g	α *	U% [+]	NaT mg/g	$\alpha_{NaC / NaT}$
P_1	290 [285]	1.01	33	186 [182]	1.55
P_2	310 [287]	1.08	34	220 [187]	1.40
P_3	345 [285]	1.21	36	281 [185]	1.22
P_4	357 [290]	1.23	40	247 [192]	1.44
P_5	422 [335]	1.25	33	387 [237]	1.09
P_6	435 [340]	1.27	34	395 [239]	1.10
P_7	475 [351]	1.35	33	416 [210]	1.14
P_8	325 [279]	1.16	51 [43]	197 [95]	1.64
P_9	385 [286]	1.34	60 [45]	215 [97]	1.79
P_{10}	435 [290]	1.50	68 [45]	185 [91]	2.35
P_{11}	475 [282]	1.68	74 [44]	175 [99]	2.71
P_{12}	571 [310]	1.84	70[38]	196 [66]	2.91
P_{13}	579 [325]	1.78	84 [47]	187 [61]	3.09
P_{14}	587 [341]	1.72	71 [41]	195 [55]	3.01

α * = Binding capacity of NaC imprinted polymer for NaC / Binding capacity of non imprinted for NaC

$\alpha_{NaC / NaT}$ = Binding capacity of NaC imprinted polymer for NaC / Binding capacity of NaC imprinted polymer for NaT

= The numbers in parentheses indicate the rebinding capacity of the non-imprinted polymers, whereas numbers preceding the parentheses indicate the rebinding capacity of the imprinted polymers,

U%$^+$ = The numbers in parentheses indicate the % utilization of functional monomer for the non-imprinted polymers, whereas numbers preceding the parentheses indicate the % utilization of functional monomer for the imprinted polymers.

4.3.2 Synthesis of MIPs by sequential polymerization / crosslinking

Nichifor et al., [2001] argued that the binding of bile acid to a cationic hydrogel involves a cooperative mechanism. The first interaction between the ligand and the bile acid anion is electrostatic. The binding of the subsequent bile acid anion to the cation in juxta position to the one already occupied by electrostatic interaction is aided by the hydrophobic binding between the bile acid molecules already bound. Since the binding of subsequent bile acid molecule to the polymer is enhanced by the binding between the polymer and the bile acid bound in the preceding step, the binding is cooperative in nature and is not multipoint binding. The polymer polyallylamine and the sorbate, cholic acid is the same in the present case and that reported by Huval et al., [2001]. The rebinding is therefore enhanced by the cooperative effect in both cases.

We therefore explored sequential polymerization / crosslinking approach to check if it could lead to enhanced rebinding capacity and selectivity. This involves the synthesis of copolymer of allylamine hydrochloride and the crosslinker MBAM so that the polymer is still soluble in aqueous solution. The functional group can then bind to the template and the polymer crosslinked in the presence of template. However, such synthetic methodologies are not reported in the literature. Very recently Li et al., [2005] reported an analogous approach in which a random copolymer of t-butyl methacrylate and 2 (trimethyl silyloxy) ethyl methacrylate was prepared using atom transfer radical polymerization. An unsaturated group was then introduced in the polymer structure by reaction with methacryloyl chloride and the carboxylate group generated by the hydrolysis of t-butyl methacrylate. This polymer was then templated with theophylline, crosslinked and used for rebinding of theophylline and its analogues. Matsui et al., [2002, 2004] synthesized a linear pre-polymer of poly(methacrylic acid) and 4-vinylbenzyl, in which unsaturated group was introduced in the polymer structure by reaction between carboxylic group of poly(methacrylic acid) and 4-vinylbenzyl chloride. This polymer was then templated with cinchonidine in the presence of ethylene glycol dimethacrylate. In addition, MIPs were also synthesized from the mixture of methacrylic acid, styrene and 4-vinylbenzyl methacrylate in the presence of template cinchonidine. The formation of pre-polymer method showed higher imprinting efficiency as compared to the conventional method. Rather than incorporating the unsaturation in an additional post

polymerization step, we exploited a novel approach developed in our group to synthesize polymers containing pendant unsaturation, for sequential polymerization, imprinting and crosslinking.

Cyclodextrins [CD] are well known to form inclusion complexes with various compounds [Szejli et al., 1996, 1998, Harada, 1998, Wenz G, 1994]. This inclusion leads to significant changes in the solution properties of guest molecules. Born et al., [1995, 1994, 1991] and Jeromin et al., [1998] reported solubilization and polymerization of water insoluble monomers such as long chain acrylates in presence of cyclodextrin in aqueous media. Kulkarni et al., [2004a, 2004b] recently reported that divinyl monomers form inclusion complex with cyclodextrin. The vinyl group included in the cavity does not take part in polymerization. Thus homo and copolymers of divinyl monomers, which are soluble in solvents, can be formed. The unreacted vinyl group can subsequently be polymerized by free radical polymerization.

This methodology was used for the synthesis of MIPs in aqueous solution for the first time. We prepared the complex comprising DMCD and MBAM in aqueous solution as discussed in the experimental section [Figure 4.2]. During the complex formation, the ratio of crosslinker to DMCD in the feed was 1:1. The composition of the complex was established by ^{1}H NMR. The peak at 4.99 δ m [C_1-**H**] corresponds to seven protons of DMCD and at 4.54 δ s [-NH-**CH_2**-NH-] corresponds to two protons of MBAM. Integration of these peaks confirmed the formation of 1:1 complex. As a result of complexation, the solubility of MBAM in aqueous medium is enhanced. Since one of the two vinyl groups is protected, MBAM behaves like a vinyl monomer and leads to water soluble copolymer.

In the first step, a water soluble copolymer of MBAM and functional monomer allylamine hydrochloride was synthesized [Figure 4.3]. Copolymer was precipitated in methanol and separated by filtration. DMCD was washed out with methanol. Complete removal of DMCD was confirmed by ^{1}H NMR analysis of the copolymer. The analysis showed peaks for vinyl groups, which were protected by DMCD during polymerization. In the next step, polymerization of this free vinyl group was carried out in the presence of template in aqueous medium for the synthesis of MIPs.

Figure 4.2: Complexation of MBAM and DMCD

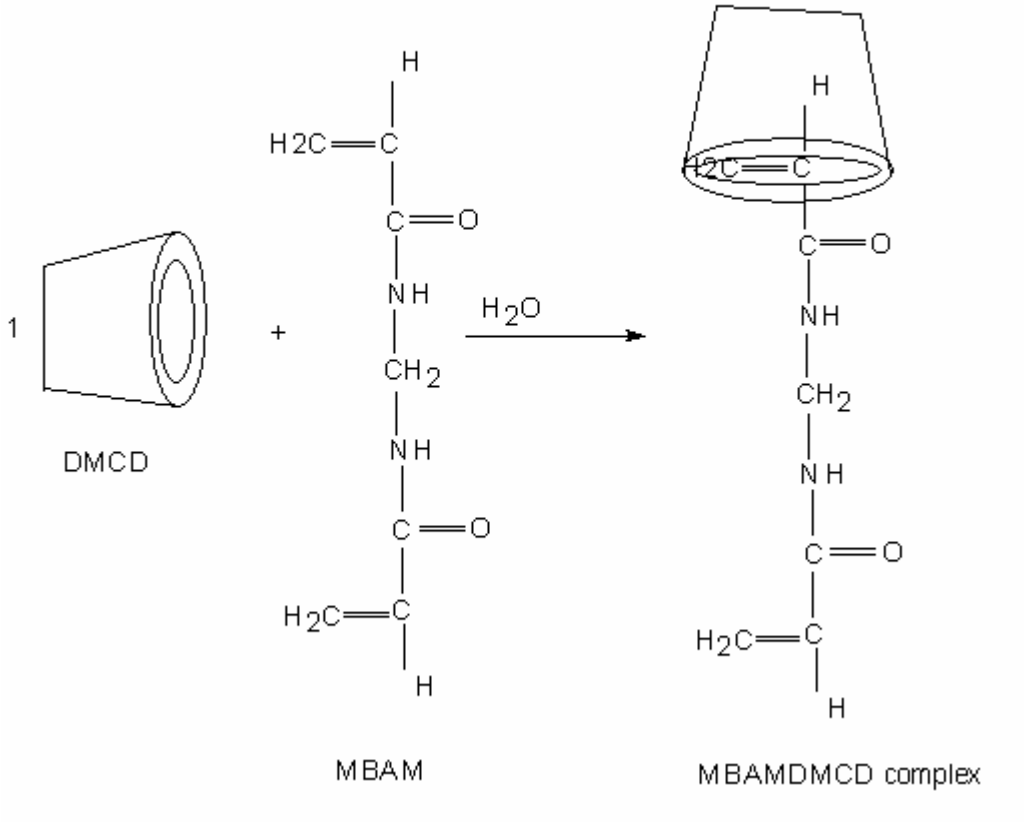
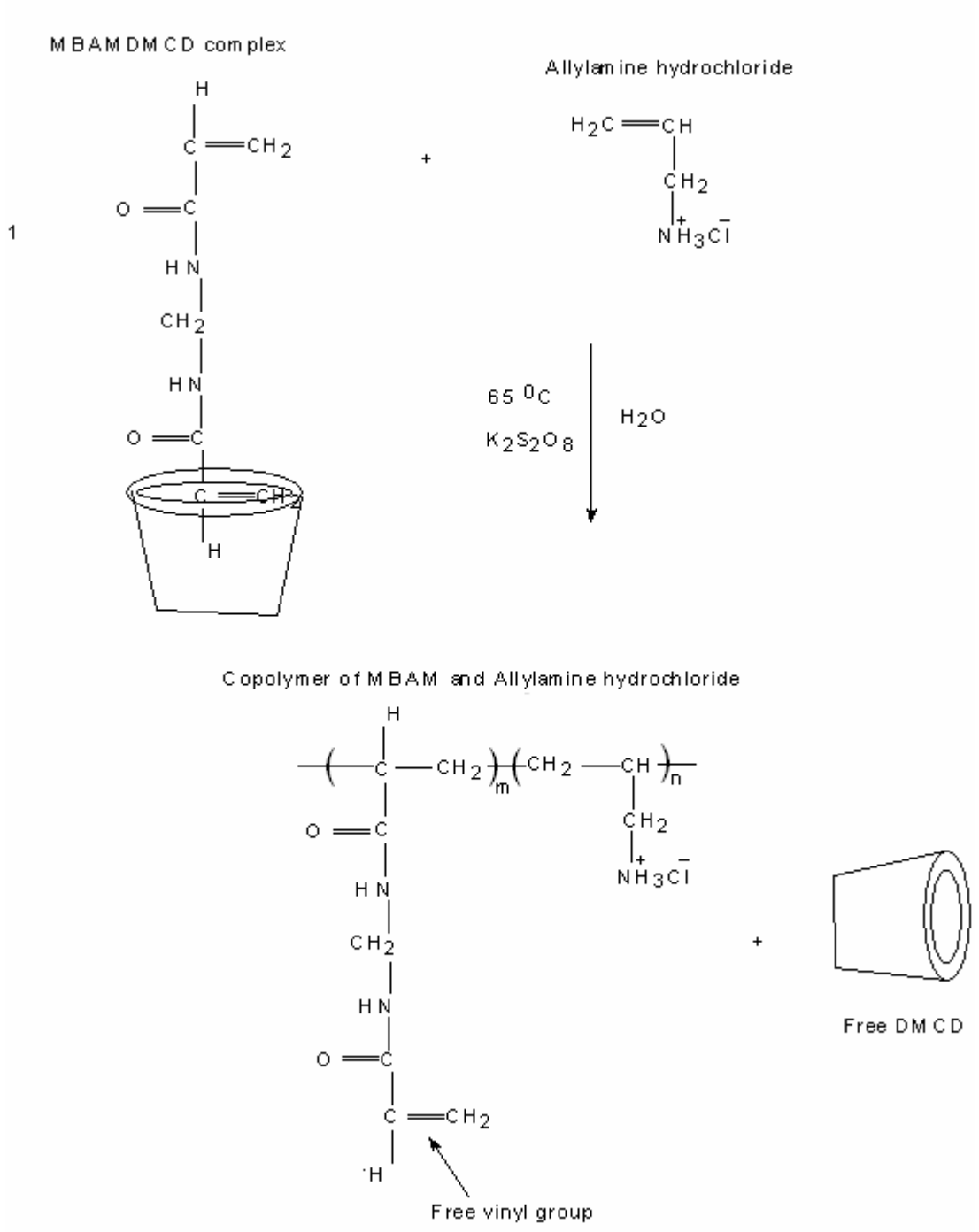


Figure 4.3: Synthesis of copolymer of MBAM and allylamine hydrochloride



In the case of the MIPs prepared by simultaneous polymerization / crosslinking technique the supramolecular assembly comprising the functional monomer, template molecule and the crosslinker is polymerized / crosslinked to form a MIP. The binding in this case is between the functional monomer allylamine hydrochloride and the template NaC. In contrast, in case of the MIPs prepared by the sequential polymerization / crosslinking technique, the supramolecular assembly comprising the polymer containing the functional monomer as well as the crosslinker and the template is crosslinked to form a MIP. The binding in this case is between the functional polymer polyallylamine hydrochloride and the template NaC. During this process initial binding between NaC and allylamine hydrochloride repeat unit is primarily electrostatic in nature. However the binding of subsequent NaC molecules to the allylamine hydrochloride molecule repeat unit is enhanced by the hydrophobic binding between NaC molecules. This interaction is cooperative in nature as described by Huval et al., [2001], Wu et al., [1996] and Regen et al., [1998]. Secondly, the shrinkage during crosslinking of a preformed polymer would be much lower than that during simultaneous polymerization / crosslinking of monomers, leading to more effective rebinding. In order to enhance the binding capacity for NaC, the incorporation of allylamine hydrochloride in the polymer needs to be enhanced. However this could not be achieved by increasing the proportion of allylamine hydrochloride in the feed, because of its low reactivity [Harada et al., 1985].

4.3.3 Rebinding of NaC from MIPs prepared by sequential polymerization / crosslinking

As in the previous case we prepared a copolymer containing 79% MBAM and 21% allylamine hydrochloride. This was crosslinked in the presence of increasing amounts of NaC so that the ratio of template to functional monomer increased from 0.1 to 2 [$P_8 \rightarrow P_{11}$]. The template was then leached from the polymer by first precipitating the polymer in methanol and subsequently by washing with water and methanol respectively. The polymers were then dried under vacuum and sieved to particle size less than 37 micron.

The rebinding experiment showed that for the polymers $P_8 \rightarrow P_{11}$ the capacity for NaC increased systematically from 325 mg/g to 475 mg/g with increasing mole ratio of template to functional monomer from 0.1 to 2. This binding capacity is significantly higher than the values obtained for the MIPs of comparable composition obtained by

simultaneous polymerization / crosslinking route [$P_1 \rightarrow P_4$]. Accordingly in this series of polymers the utilization of functional monomer is significantly higher [51 – 74%].

Increase in the ratio of functional monomer to crosslinker, from 3 to 18 [$P_{11} \rightarrow P_{14}$] at a constant ratio of template to functional monomer [1] resulted in an initial increase in the rebinding capacity, which then plateaued at 580 mg/g. It may be noted here that in the post polymerization, imprinting and crosslinking approach used by Li et al., [2005], the binding capacity of the imprinted polymer was 180 μ mol/g against an anticipated capacity of 900 μ mol/g. Thus the active site utilization was about 20%. In contrast the approach used by us led to the highest capacity utilization of 84% [P_{13}]. The efficiency of imprinting as measured by $\alpha_{\text{MIP / non-MIP}}$ was also higher [~ 1.7] as compared to that reported in the previous section [~ 1.3] but lower than that [2.0] reported by Huval et al., [2001]. Indeed, it would have been instructive to compare our results with those reported by Huval et al., [2001]. The authors reported amine equivalent and amount of crosslinker used in the preparation of the MIPs, but the degree of neutralization was not reported. Similarly the porosity and surface area of the MIPs have not been reported. The rebinding experiments were carried out in BES buffer at pH 6.8, while we have used phosphate buffer of pH 7.4. It is therefore not possible to compare our results with those reported by Huval et al., [2001].

Simultaneous polymerization / crosslinking in the presence of template molecule leads to the formation of template selective cavities. But shrinkage during polymerization leads to a certain degree of distortion of the cavities. The crosslinking of the polymer template assembly results in comparatively lower shrinkage, minimizing the distortion of the cavities. Further the imprinted polymers prepared by sequential polymerization / crosslinking technique exhibit greater swelling and pore volume than the polymers prepared by simultaneous polymerization / crosslinking method [Table 4.4]. This further enhances the accessibility of the binding sites on the polymer during rebinding, leading to both higher capacity as well as selectivity *vis a vis* non imprinted polymer.

The major advantage of sequential polymerization / crosslinking methodology is seen in high selectivities achieved in competitive binding experiments. For the polymers $P_8 \rightarrow P_{11}$ with increasing ratio of template to functional monomer from 0.1 to 2, the ratio of binding capacity for NaC over NaT increased systematically from 1.64 to 2.71 [Table

4.5]. Also for the polymers P_{10} and $P_{12} \rightarrow P_{14}$ the ratio increased from 2.35 to 3.55. Taurine conjugated bile acids are the major constituents of the bile acid pool in the gut and the binding occurs via electrostatic interaction. The fact that NaC is selectively bound over NaT, highlights that molecular imprinting induced hydrophobic interaction plays dominant role over the electrostatic interaction.

4.3.4 Enhancing bile acid binding

Stedronsky [1994] attributed low *in-vivo* efficiency of cholestyramine resins to rapid dissociation of the bound bile acid and transport through the wall of the ileum. In a subsequent investigation Regen et al., [1998] demonstrated that the exchange of the taurocholate by chloride ion from the anion exchange resin was controlled by film diffusion rather than particle diffusion. The ion exchange process would be particle diffusion controlled and delay the loss of the sorbed bile acid, if the crosslink density is increased. However one of the difficulties with this approach is that the sorption of the bile acid would also be limited by particle diffusion. Hydrophobic interactions would slow down the rate of dissociation, without limiting the rate of sorption.

Benson et al., [1997] reported a novel bile acid sequestrant, which exhibited higher affinity and slower dissociation than cholestyramine under *in-vitro* conditions. The sequestrant SKF 97426 was a copolymer of 11-trimethylammonioundecylmethacrylate chloride and ethylene glycol bismethacrylate. The enhanced binding of the bile acid to the polymer was attributed to the hydrophobic interaction between the β surface of cyclopentanophenanthrene ring of the bile acid and the alkyl pendant chain of 11-trimethylammonioundcylmethacrylates.

Zhang et al., [2000] demonstrated that conjugation of cholic acid with the anion exchange resin and subsequent cloistering with taurocholic acid enhanced the affinity of the resins toward taurocholate and delayed its rate of release, which was now to a certain extent controlled by particle diffusion.

The binding constant for NaC was estimated by generating the adsorption isotherm and fitting the data into the Hill's equation. Similar results were obtained for imprinted polymers synthesized by simultaneous polymerization and crosslinking of allylamine hydrochloride and MBAM in the presence of template NaC. In both cases, control experiments were carried out in the absence of the template. The results are summarized

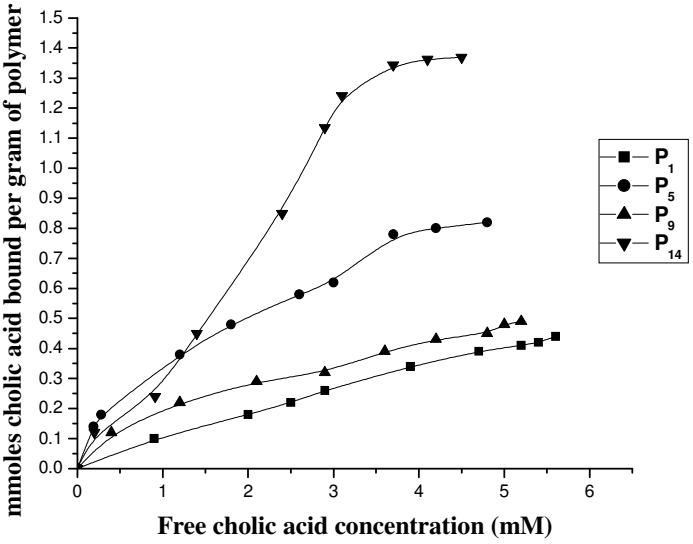
Table 4.6: Study of association constants and cooperativity

Polymer	Cooperative parameter [n]	α_n	Association constant [K] mM^{-1}	α_K
P_1	1.21	1.04	0.21	1.00
P_2	1.20	1.03	0.22	1.04
P_3	1.17	1.02	0.23	1.15
P_4	1.22	1.06	0.25	1.19
P_5	1.20	1.04	0.29	1.20
P_6	1.24	1.10	0.33	1.43
P_7	1.34	1.13	0.27	1.58
P_8	1.33	1.18	0.28	2.33
P_9	1.36	1.21	0.26	2.16
P_{10}	1.35	1.22	0.27	2.70
P_{11}	1.37	1.22	0.28	2.33
P_{12}	1.25	1.30	0.22	2.22
P_{13}	1.20	1.23	0.36	4.50
P_{14}	1.17	1.23	0.17	4.35

α_n = Cooperativity of MIP/Non-MIP

α_K = Association constant of MIP/Non-MIP

Figure 4.4: Binding isotherm



in Table 4.6. A typical isotherm plot for polymers P_1, P_5, P_9 and P_{14} is given in Figure 4.4 In the case of polymers synthesized by simultaneous polymerization / crosslinking technique, no systematic variation in cooperativity parameter was noted either with increasing ratio of functional monomer to template or the ratio of crosslinker to functional monomer. Also, the enhancement in the cooperativity parameter over the non imprinted polymers was insignificant. Most of the non imprinted polymer also exhibited cooperative effect [n >1]

In the case of polymers synthesized by sequential polymerization, imprinting, crosslinking technique, the cooperativity parameter showed a marginal enhancement over the polymer prepared by simultaneous polymerization / imprinting crosslinking technique. But the relative enhancement over the non imprinted polymers was of the order of 22 - 30%. This was because many of the non imprinted polymers did not show cooperativity effect [n < 1]. This clearly illustrates the cooperative effect operating during the binding of NaC to poly [allylamine hydrochloride-MBAM] polymer during the crosslinking step. An examination of the association constants shows that for the imprinted polymers the values varied between 0.20 to 0.35 but did not show any systematic variation with either the ratio of functional monomer to template or the ratio

of crosslinker to functional monomer. A comparison with the non imprinted polymer on the other hand shows that in both cases the ratio increases systematically. The relative enhancement in the case of polymers synthesized by sequential polymerization, imprinting, crosslinking technique was much higher than in the case of polymers synthesized by simultaneous polymerization, imprinting crosslinking technique because of the low values resulting for the non imprinted polymers. It would be interesting to see if this results in enhanced bile acid binding under *in- vivo* conditions. These investigations are in progress.

4.4.0 Concluding remarks

Polymeric adsorbents for removal of bile acids from the GI tract provide a viable alternative for the treatment of hypercholesterolemia. The major limitation of currently used adsorbents is their low *in-vivo* binding capacity especially for trihydroxy bile acids. This can be overcome if the binding between polymer and the bile acid could be enhanced. This paper presents a new synthetic approach viz. the sequential polymerization, imprinting and crosslinking for the synthesis of bile acid imprinted polymers, which results in enhanced capacity utilization of active sites. It also leads to enhanced association with the bile acid. A number of possibilities for enhancing binding capacities and selectivities of bile acids which open up based on this approach.

Chapter 5

Enhancing Cholesterol - β-Cyclodextrin Binding: Role of Polymer Architecture

5.0.0 Introduction

Cyclodextrin is cyclic oligomer of glucose, which forms inclusion complexes with hydrophobic guests [Siegel et al., 1975]. This property has been exploited for the recovery of many hydrophobic biomolecules such as cholesterol from egg yolk, creams, milk etc [Risch et al., 1995, Oakenfull et al., 1991, Ogura 1995,Cao et al., 1996]. Asanuma and coworkers [1998,1997,1999] described the recognition properties exhibited by β-cyclodextrins, crosslinked with diisocynates in the presence of cholesterol. The materials were capable of rebinding cholesterol also in aqueous media. However, their utility is limited, as they cannot be readily recovered. This can be overcome by conjugating β-cyclodextrins with thermosensitive polymers. Strong and selective rebinding in water depends on the hydrophobic effect and requires a large Van der Waal contact area between cholesterol and host, which is provided by cyclodextrins [Wallimann et al., 1997 and Fersht 1985].

The association constants and stoichiometry of complexation between β-cyclodextrin and cholesterol have been studied by many researchers as mentioned in chapter 1. In this chapter we report the synthesis of monomers and dimers containing β-cyclodextrin as well as homopolymers and copolymers containing β-cyclodextrin as well as its dimer. The association constants with cholesterol were determined by the solubility method. It was observed that in polymers containing β-cyclodextrin, the association constant increased with degree of substitution. This suggests that the mechanism of binding shifts from stagewise to chelate type. On the other hand in polymers containing β-cyclodextrin dimers, the association constants were independent of the degree of substitution since the binding was always chelate type.

5.1.0 Experimental

5.1.1 Materials

β-Cyclodextrin [CD], Sodium cholate, Acrylic acid, [1-ethyl-3-[3-dimethylamino] propyl] carbodiimide hydrochloride [EDC], Dicyclohexyl carbodiimide [DCC], N-isopropylacrylamide [NIPA], N, N, N', N' tetra methyl ethylene diamine [TEMED], Ammonium Persulfate were purchased from Aldrich Chemical Company Inc., Milwaukee, WI, USA. Cholesterol, Methanol, Sodium deoxycholate, m-nitrophenol, P-toluene sulphonyl chloride [PTSA], Sodium iodide, Sodium sulphide, Tris buffer, 2-

Aminoethanol, Ferric chloride, Phenol, Ethylene diamine, Pyridine [dried on KOH], Sodium hydroxide [NaOH], Hydrochloric acid [HCl], Sulphuric acid [H_2SO_4] analytical grade were purchased from local suppliers. All solvents were purified as per standard procedures [Perrin et al., 1996].

5.1.2 Instrumentation

^{1}H NMR spectra were recorded on Bruker superconducting FT-NMR AC 300 operating at 300 MHz. ^{13}C NMR was recorded on DRX500 MHz spectrometer under proton-band decoupling conditions using deuterated chloroform and dimethyl sulfoxide in volume ratio 1: 0.5. The spectra for pure cholesterol and dimer were obtained under identical conditions to serve as reference. The number of scans needed to achieve a good signal to noise ratio was between 20000 and 50000. Differential scanning calorimetry [DSC] was performed using Perkin Elmer DSC equipped with a Thermal Analysis Data Station [TADS] at a heating rate of 5^0C/min in nitrogen atmosphere. The temperature and energy scales were calibrated using standard procedure. Temperature range used was 50^0-210^0 C. The sample size was 2-4 mg. Duplicate determinations were carried out for each sample. IR spectra were recorded on Shimadzu 8300 FT-IR spectrometer. Electronic absorption measurements were done on Shimadzu UV 1601 spectrophotometer.

5.1.3 Monomer Synthesis

5.1.3.1 Synthesis of N-Acryloylethyl-6-amino-β-cyclodextrin [M_1]

β-Cyclodextrin was converted to primary monotosyl cyclodextrin [Leckchiri et al., 1995] and to [aminoethyl] amino-β-cyclodextrin, [Nozaki et al., 1995] according to the procedure reported. The free amino group was coupled with acrylic acid by condensation in the presence of EDC.

In a 250 ml round bottom flask 10 g [0.0087 Moles] of [aminoethyl] amino-β-cyclodextrin and 0.6 ml [0.0087 Moles] of acrylic acid were dissolved in sodium acetate buffer solution of pH 5.5. 2 g [0.0087 Moles] of EDC was added in above solution as a coupling agent and the contents were stirred at room temperature for 12 hrs. Reaction mixture was concentrated and precipitated into cold acetone. The residue was filtered and dried at room temperature

Yield: 25%

^{1}H NMR N-Acryloylethyl-6-amino-βcyclodextrin [M_1] [300 MHz DMSO d6]: 3.06δ d [-NH-CH_2-CH_2-], 2.3δ d [-NH-CH_2-CH_2-], 4.8δ m [7H1], 3.38δ m [7H2], 2.79δ to 3.38δ m [7H2 to 7H6], 5.40δ and 5.59δ d [CH2=], 5.52δ m [=CH]

IR [KBr]: 1639.4 cm^{-1}, -C=C-, -OH [broad]: 3425.3 cm^{-1}, -COO Ester: 1720.4 cm^{-1}

5.1.3.2 Synthesis of Acryloyl-β-cyclodextrin [M_2]

Acryloyl-β-cyclodextrin [M_2] was synthesized according to the procedure reported by Harada et al., [1976].

In a 250 ml round bottom flask 11.76 g (0.01 Moles) β-cyclodextrin and 2.0 g (0.01 Moles) of acetonitrile solution of m-nitrophenyl acrylate was dissolved in 120 ml of carbonate buffer [pH 11]. The reaction mixture was shaken vigorously for five minutes and neutralized with dilute HCl [pH 3] and cooled in ice water bath. The unreacted acrylate crystallized out and was filtered off. The solution was poured in to cold acetone and product was precipitated and separate by filtration.

Yield: 30%

^{1}H NMR Acryloyl-β-cyclodextrin [M_2] [300 MHz DMSO d6]: 4.82δ m [7H1], 3.31δ to 3.62δ m [7H2 to 7H6], 5.19δ and 5.06δ d [CH_2=], 6.19δ m [=CH].

IR [KBr]: 1640.4 cm^{-1} -C=C-, 3450.3 cm^{-1} –OH [broad], 1721 cm^{-1} –COO ester.

5.1.3.3 Synthesis of N-Acryloyl-6-aminocaproyl-β-cyclodextrin [M_3]

N-Acryloyl-6-aminocaproyl-β-cyclodextrin [M_3] was synthesized according to the procedure reported by Harada et al., [1976].

In a 250 ml round bottom flask 3.46 g (0.0118 Moles) of acryloyl 6-ACA-m-nitrophenyl ester and 13.5 g (0.0118 Moles) of β-cyclodextrin were dissolved in a mixture of 100 ml of carbonate buffer [pH 11] and 50 ml of DMF. Reaction mixture was stirred for 30 min at room temperature and neutralized with HCl [pH 3] and kept in fridge. Unreacted β-cyclodextrin was precipitated and filtered off. Filtrate was concentrate and precipitated in cold acetone.

Yield: 25%

^{1}H NMR N-acryloyl-6-aminocaproyl-β-cyclodextrin [M_3] [300 MHz DMSO d6]: 4.8δ m [7H1], 3.27δ m [7H2], 3.6δ to 3.87δ m [7H3 to 7H6], 2.4δ and 2.73δ m [[CH_2]$_5$], 5.76δ and 5.78δ d [CH_2=], 6.2δ m [=CH].

IR [KBr]: 1642.4 cm^{-1} -C=C-, 3550.3 cm^{-1} –OH [broad], 1720 cm^{-1} –COO ester.

5.1.3.4 N-Acryloylethyl-amino-βcyclodextrin [M_4]

β-Cyclodextrin was converted to secondary monotosyl-β-cyclodextrin according to the procedure reported [Ueno et al., 1982]. Synthesis of [aminoethyl] amino-β-cyclodextrin derivative and condensation with acrylic acid was carried out as reported earlier.

Yield: 30%

^{1}H NMR N-Acryloylethyl-amino-βcyclodextrin [M_4] [300 MHz DMSO d6]: 4.79δ m [7H1], 3.59δ to 3.8δ m [7H2 to 7H6], 3.1δ d [-NH-CH_2-CH_2-], 2.2δ d [-NH-CH_2-CH_2-], 5.4δ and 5.63δ d [CH_2=], 6.1δ m [=CH].

IR [KBr]: 1642.4 cm^{-1} -C=C-, 3550.3 cm^{-1} –OH [broad], 1720 cm^{-1} –COO ester.

5.1.4 Synthesis of β-cyclodextrin dimers

β-Cyclodextrin-C_6-sulphide [D_1] and β-cyclodextrin-C_6-disulphide [D_2] were synthesized as reported by Breslow et al., [1996] and Fujita et al., [1984]. β-Cyclodextrin-C_6-sulphide was converted to acryloyl-β-Cyclodextrin-C_6-sulphide [M_5] derivative using the same procedure reported in the previous paragraphs.

^{1}H NMR β-Cyclodextrin-C_6-sulphide [D_1] [300 MHz D_2O]: 3.93 to 4.05δ d [4H6], 4.71 to 5.03δ m [7H1], 3.2 to 3.6δ m [7H2 to 7H3], 3.7 to 3.9δ m [7H4 to 7H5].

^{13}C NMR [500 MHz DMSO d6]: 102.24 δ [C-1], 72.15 δ [C-2], 73.14 δ [C-3], 81.91 δ [C-4], 72.66 δ [C-5], 60.20 δ [C-6]

Yield: 25%

^{1}H NMR Acryloyl-β-Cyclodextrin-C_6-sulphide [M_5] [300 MHz D_20]: 4.80δ to 3.61δ m [for cyclodextrin], 6.05δ and 6.28δ d [CH_2=], 6.45δ m [=CH].

IR [KBr]: 1644.4 cm^{-1} -C=C-, 3550.3 cm^{-1} –OH [broad], 1722 cm^{-1} –COO ester.

Yield: 30%

^{1}H NMR Acryloyl-β-cyclodextrin-C_6-disulphide [D_2] [D_2O]: 3.93 to 4.05δ d [4H6], 4.71 to 5.03δ m [7H1], 3.2 to 3.6δ m [7H2 to 7H3], 3.7 to 3.9δ m [7H4 to 7H5].

^{13}C NMR [500 MHz DMSO d6]: 102.27 δ [C-1], 72.15 δ [C-2], 73.13 δ [C-3], 81.94 δ [C-4], 72.69 δ [C-5], 60.19 δ [C-6]

Yield: 27%

5.1.4.1 Synthesis of β-Cyclodextrin-C_3-sulphide [D_3]

In a 250 ml round bottom flask 1.3 g [0.001Moles] of secondary monotosyl-β-cyclodextrin was refluxed with 50 ml of 0.25% aqueous K_2CO_3 for 5 hrs and neutralized with dilute HCl [pH 3] to yield β-cyclodextrin 2,3-alloepoxide [Fujita et al., 1986]. 1.11 g [0.001 Moles] of β-cyclodextrin 2,3-alloepoxide and 0.152 g [0.002 Moles] of sodium sulfide [Na_2S] were dissolved in 30 ml of DMF and solution was refluxed at 70 ^{0}C for 7 hrs [Nesanas et al., 2000]. The solution was concentrated and the dimer was precipitated into acetone. The residue was filtered and dried at room temperature.

Yield: 25%

^{1}H NMR β-cyclodextrin-C_3-sulphide [D_3] [300 MHz D_2O]: 4.4δ broad [7H3], 4.8 to 5.2δ m [7H1], 3.2 to 3.8δ m [7H2, 7H4, 7H6].

^{13}C NMR [500 MHz DMSO d6]: 102.28 δ [C-1], 72.15 δ [C-2], 73.13 δ [C-3], 81.95 δ [C-4], 72.69 δ [C-5], 60.22 δ [C-6]

5.1.5 Synthesis of 2-aminoethyl acrylate hydrochloride [2-AEA] [M_6]

Synthesis of 2-aminoethanol hydrochloride

20 ml of ethanolamine was dissolved in 40 ml of concentrated HCl and this solution was warmed for 1 hour. After cooling, water was removed under vacuum [Lele et al., 1999]. The residue was washed with acetone and dried under vacuum.

Yield: 95%

Condensation of 2- aminoethanol hydrochloride with acrylic acid

In a 250-ml capacity round bottom flask 8 ml [0.10 Moles] of acrylic acid and 10 g [0.10 Moles] of 2-amino ethanol hydrochloride were dissolved in 30 ml of DMF. To this solution 21 g [0.10 Moles] of DCC was added by dissolving in 5 ml DMF. Solution was stirred for 2-3 hrs on ice water bath, then at room temperature for 48 hrs. Dicyclohexyl urea [DCU] salt formed during reaction was filtered off and the filtrate was concentrated. The monomer was precipitated in to diethyl ether.

Yield: 70%

^{1}H NMR 2-AEA [M_6] [300 MHz D_20]: 5.76δ and 5.72δ d [CH_2=], 6.26δ m [=CH], 3.54δ d [-O-CH_2-], 2.81δ d [-CH_2-NH-]

IR [KBr]: 1644.4 cm^{-1} -C=C-, 3550.3 cm^{-1} –NH [broad], 1722 cm^{-1} –COO ester.

5.1.6 Polymers synthesis

5.1.6.1 Synthesis of co-polymers of NIPA with cyclodextrin monomers

Copolymers of NIPA with various β-cyclodextrin bearing monomers were synthesized, since we intended to use these polymers for recovery of cholesterol by affinity precipitation technique. In a 20-ml test tube NIPA and the comonomer were weighed in mole ratio [9:1] and 2 ml of dimethyl sulfoxide [DMSO] was added as a solvent. AIBN, 1% by weight of the total monomer was added and the tube was purged with nitrogen for 15 min. Polymerization was carried out at 60^0C in water bath for 16 hrs. Polymers were recovered by precipitation in diethyl ether. The exact amounts of monomers and initiator used are summarized in Table 5.1.

5.1.6.2 Synthesis of Poly [2- amino ethyl acrylate]

In a 20 ml test tube 1 g of 2-amino ethyl acrylate hydrochloride was dissolved in 2 ml of water. Test tube was purged with nitrogen for 20 minutes. 1% of ammonium per sulfate and 20 µl TEMED were added as initiator and test tube was maintained at 40^0 C in water bath for 16 hrs. The solution was cooled and precipitated in cold acetone. The salt of homopolymer of 2-AEA was obtained. To deprotect the salt, methanol containing KOH equivalent to the hydrochloride was added to the polymer solution. The solution was refrigerated for two hours and the salt was filtered off. After concentrating the filtrate, homopolymer was precipitated into cold acetone.

5.1.6.3 Synthesis of co-polymers of NIPA and 2-AEA

In a 20-ml test tube 2-AEA and NIPA in predetermined mole ratios were added along with 3 ml of distilled water [see Table 5.1]. Tube was purged with nitrogen for 15 min and ammonium per sulfate, 1% by weight of the total monomer and 20 µl TEMED were added as initiator. The test tube was maintained at 40^0 C for 16 hrs and the polymer was precipitated into diethyl ether. The hydrochloride was removed as described earlier. β-Cyclodextrin or the dimer was conjugated on to the polymer as reported by Seo et al., [1987].

5.1.7 Cholesterol binding studies

The association constant between cholesterol and β-cyclodextrin was determined by the solubility method [Breslow et al., 1996]. The solutions containing β-cyclodextrin in

water were stirred with an excess of cholesterol for 24 hrs at 25 ^{0}C and the filtrate was analyzed

Table 5.1: Synthesis of copolymers of NIPA, β-cyclodextrin monomers and 2-AEA

Monomer	Mole ratio NIPA: Monomer	Weight NIPA: Monomer g	Initiator g
M_1	9:1	0.5:0.6	0.01
M_2	9:1	0.5:0.5	0.01
M_3	9:1	0.5:0.6	0.01
M_4	9:1	0.5:0.6	0.01
NIPA: 2AEA	1:1	0.5:0.6	0.01
	3:2	0.5:0.4	0.001
	2:8	0.5:2.6	0.031

for cholesterol by a colorimetric method [Rajendraprabhu, et al., 1980]. The plot of β-cyclodextrin concentration versus concentration of cholesterol in aqueous medium was plotted. The plot was linear and from the slope and intercept [S_0], the association constant for the complexation between cyclodextrin and cholesterol was calculated according to the following equation [Breslow et al., 1996].

$$K_a = \text{Slope}/S_0\,[1-\text{Slope}]$$

5.1.8 Preparation of β-cyclodextrin dimer and cholesterol complexes

The complex comprising cholesterol and β-cyclodextrin dimer was prepared according to the procedure reported by Claudy et al., [1991]. 1 g of cholesterol was dissolved in 25 ml of n-hexane. The solution was stirred and warmed up to 55 ^{0}C. A 10% by weight solution of β-cyclodextrin dimer in water was prepared. Varying amounts of cholesterol were then added so as to adjust the mole ratios of the two. The solution was then allowed to stand. A white microcrystalline powder was formed. After 24 hrs the powder was filtered and washed with water and hexane and dried at 40 ^{0}C for 18 hrs. After grinding the product was stored at room temperature.

5.2.0 Results and discussion

5.2.1 Polymer synthesis and characterization

Polymers containing β-cyclodextrin were synthesized either by the polymerization of the appropriate cyclodextrin bearing monomer or by substitution of β-cyclodextrin on a preformed polymer. In the first approach, different monomers containing β-cyclodextrin were synthesized. To evaluate the effect of choice of binding site on β-cyclodextrin and spacer length between vinyl group and β-cyclodextrin on the association constant with cholesterol, we synthesized different vinyl monomers [Figure 5.1] containing pendent β-cyclodextrin. These monomers were copolymerized with N-isopropylacrylamide [NIPA] by free radical polymerization, since the intended use of polymers for the recovery of cholesterol from aqueous medium by affinity thermoprecipitation method. Incorporation of β-cyclodextrin monomers into the copolymers was quantified colorimetrically using phenol sulphuric acid method [Dubois et al., 1956]. It was observed that increasing the content of β-cyclodextrin monomer in the feed did not enhance incorporation of β-cyclodextrin in to the polymer and LCST estimated by the turbidimetry method [Boutris et al., 1997], remained practically constant. Polymers showed only 2-3 % incorporation of β-cyclodextrin. This could be due to poor reactivity of monomers containing β-cyclodextrin monomers. Since our aim was to study the association constant with cholesterol as a function of β-cyclodextrin content, polymers having higher β-cyclodextrin content were synthesized by the substitution route. 2AEA.HCl [M_6] was synthesized as reported in experimental section and copolymerized with NIPA by free radical polymerization. Protection of free amino group by the hydrochloride protects the O→ N acyl migration [Notes 1970] and leads predominantly to the pendent amino end group. The hydrochloride group was deprotected using potassium hydroxide in methanol and the free amino groups were estimated colorimetrically by ninhydrin method [Jayaraman, et al., 1985]. β-Cyclodextrin was covalently linked to the polymer by the reaction between free amino group and β-cyclodextrin tosylate [Seo et al., 1987]. Copolymers in which upto 67% of amino groups were substituted by β-cyclodextrin could be synthesized by this method.

Figure 5.1: β-Cyclodextrin monomers M_1 to M_6

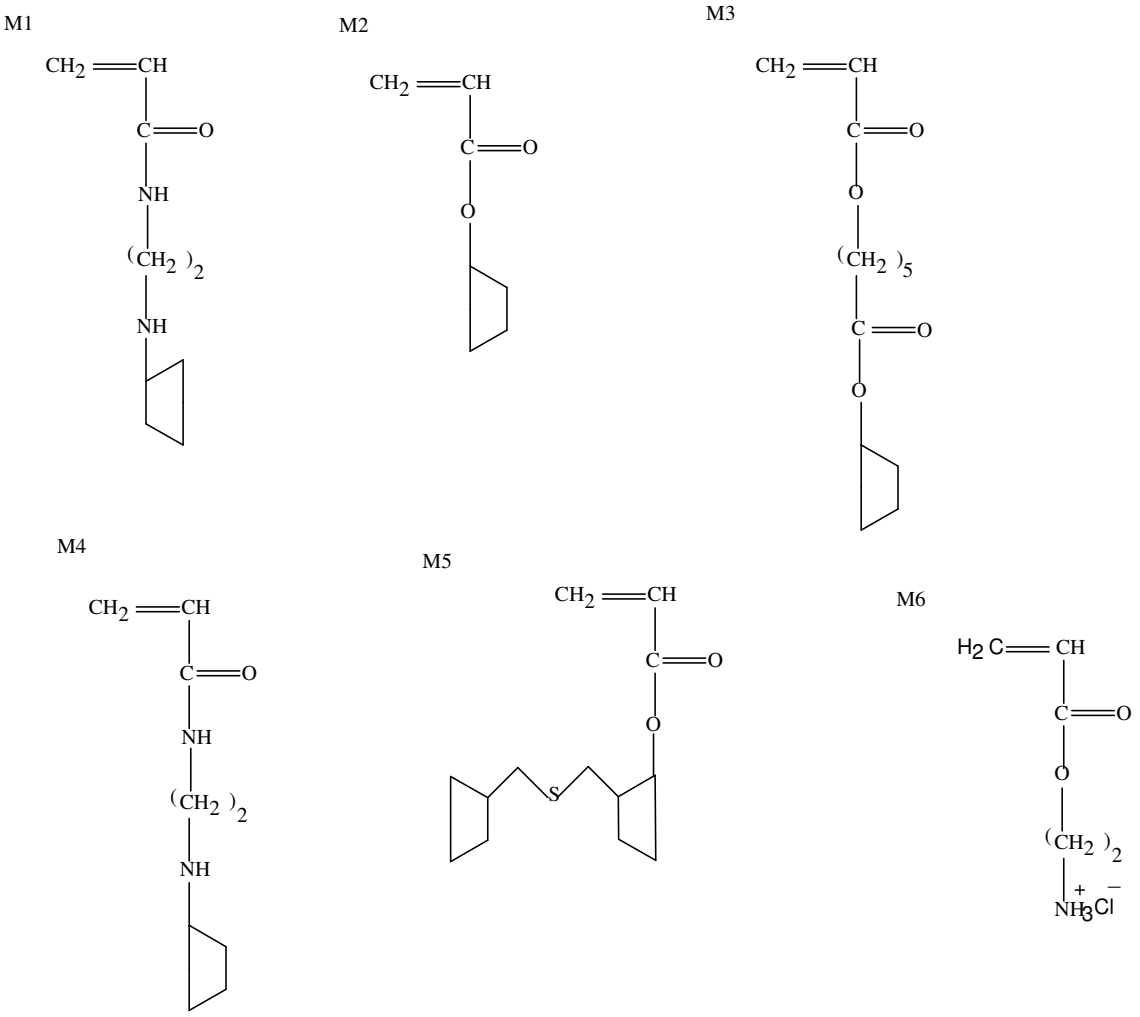
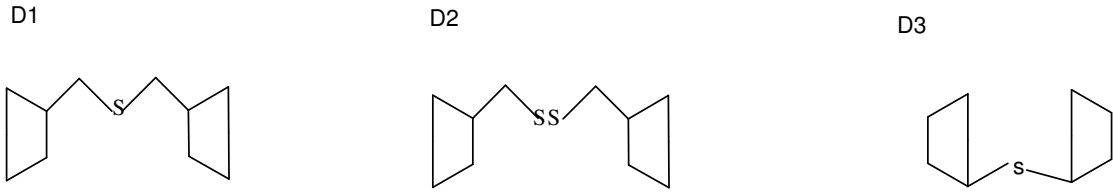


Figure 5.2: β-Cyclodextrin dimers D_1 to D_3



Breslow et al., [1996] reported that cholesterol binds more strongly to β-cyclodextrin dimers than β-cyclodextrin. But the effect of binding site and spacer length was not studied. We synthesized three types of dimers D_1, D_2, and D_3 [Figure 5.2]. In dimer D_1, two β-cyclodextrin rings are linked through a sulphur bridge on the primary site of β-cyclodextrin. In dimer D_2, two β-cyclodextrin rings are linked through a bridge containing two sulphur atoms conjugated on the primary site of β-cyclodextrin, whereas in dimer D_3 two β-cyclodextrin rings are linked through a single sulphur bridge on the secondary site of β-cyclodextrin. Dimer D_1 was converted in to the vinyl monomer [M_5] so that it could be polymerized as well as copolymerized with NIPA. These dimers were characterized by ^{1}H NMR and ^{13}C NMR. Dimer D_1 was converted to the secondary tosylate and covalently linked to the polymer containing 2-amino ethyl acrylate using the procedure reported earlier. Incorporation of β-cyclodextrin dimer and LCST were estimated by the same procedures as reported earlier. Stoichiometry of the complex between dimer and cholesterol was estimated by DSC and ^{13}C NMR analysis [Claudy et al., 1991].

5.2.2 Cholesterol binding with monomers and polymers containing β-cyclodextrin

Complexes comprising cholesterol and different vinyl monomers of β-cyclodextrin were prepared and their association constants were determined by the solubility method [Breslow et al., 1996]. The value for β-cyclodextrin was 0.92×10^4 M^{-1}, which was little lower than that reported by Frijlink et al., [1991] [1.7×10^4 M^{-1}]. Association constants for the various monomers were comparable to that for β-cyclodextrin [Table 5.2]. Nesanas et al., [2000] reported that modification of primary site leads to higher association constant than binding at secondary site, because binding of large molecule like cocaine takes places from secondary site. Cholesterol is much larger in size as compared to cocaine and hence would be expected to enter the cavity from the secondary site of β-cyclodextrin. In monomer M_1 [Figure 5.1] β-cyclodextrin is conjugated through primary hydroxyl site to the vinyl backbone. Since secondary site is available for binding with cholesterol, this monomer exhibits higher association constant than monomers M_2 - M_4.

Homopolymers of monomers M_1 to M_4 were synthesized and their binding with cholesterol was estimated. Homopolymer of M_3 showed enhanced association constant

Table 5.2: Association constants [K_b M^{-1}] between β-cyclodextrin monomers, homopolymers and copolymers with cholesterol

Monomer	Monomer	Homopolymer	Copolymer	LCST of copolymer ^0C
β-Cyclodextrin	0.92×10^4	-	-	-
M_1	0.87×10^4	0.90×10^4	5.4×10^4	38
M_2	0.71×10^4	1.16×10^4	1.4×10^4	31
M_3	0.43×10^4	1.93×10^4	1.7×10^5	34
M_4	0.63×10^4	0.70×10^4	1.6×10^4	31

than others, which could be due to the spacer effect. However no substantial increase in the association constant was observed for other polymers in relation to the corresponding monomers. This result is analogous to that reported by Breslow et al., [1996] for soluble epichlorohydrin crosslinked β-cyclodextrin.

Host-guest complexation between cholesterol and copolymers containing β-cyclodextrin and NIPA having low β-cyclodextrin content was investigated. These copolymers exhibited enhanced association constant for cholesterol as compared to the monomers and homopolymers [Table 5.2]. This could be attributed to the segmental mobility of the polymer chain, which would bring at least two β-cyclodextrin units in close proximity as to include cholesterol. Hollas et al., [1998] argued that at low degrees of substitution of β-cyclodextrin in the polymer, the two cyclodextrin units would belong to different polymer chains, i.e. complexation would be intermolecular. At higher degrees of substitution, the two cyclodextrin units from the same chain would be involved, i.e. complexation would be intramolecular and also chelate type. Similar cooperative binding has been reported earlier for the interaction between fluorescent compounds and

polymers containing β-cyclodextrin [Hollas et al., 1998, Wallimann et al., 1997]. In this work it was not possible to distinguish between inter or intramolecular complex formation between cholesterol and the polymer directly, but using techniques like fluorescence spectroscopy and choice of a hydrophobic florescent probe such as pyrene probable mode of binding between β-cyclodextrin and cholesterol could be established [Chapter 6]. Copolymer of M_3 [N-acryloyl-6-aminocaproyl-β-cyclodextrin] exhibits higher association constant [1.7×10^5 M^{-1}] than the copolymer of M_1 [N-Acryloylethyl-6-amino-β-cyclodextrin] 5.4×10^4 M^{-1}, M_2 [Acryloyl-β-cyclodextrin] 1.4×10^4 M^{-1} and M_4 [N-Acryloylethyl-amino-β-cyclodextrin] 1.6×10^4 M^{-1} respectively. This could be due to the increased spacer length in the case of M_3. Although the incorporation of β-cyclodextrin monomer was only 2-3%, LCST increased from 31 to 38^0 C, which could be attributed to the hydrophilicity of the β-cyclodextrin monomer [Feil et al., 1993].

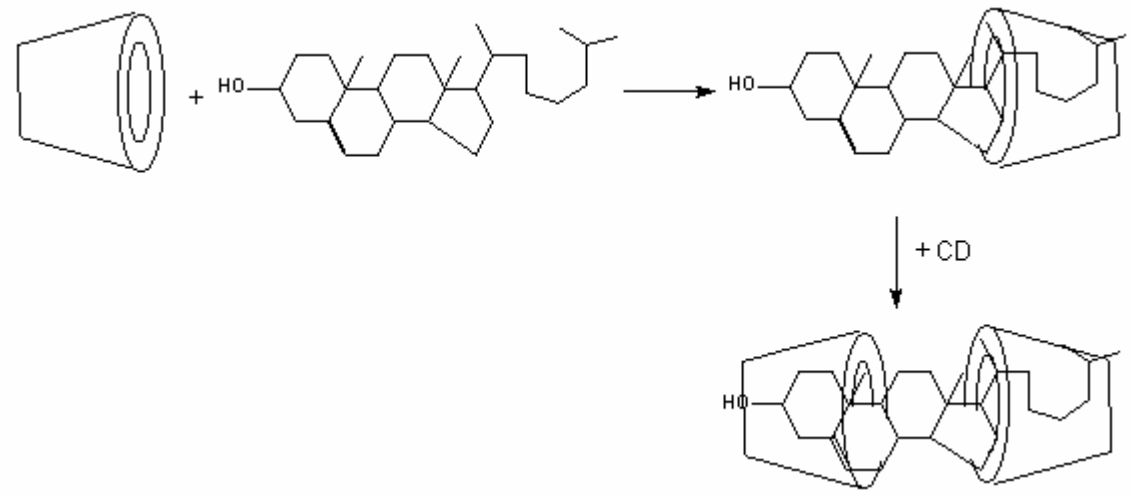
5.2.3 Cholesterol binding with copolymers containing higher loadings of β-cyclodextrin

Breslow et al., [1996] evaluated the association constant between epichlorohydrin crosslinked β-cyclodextrin polymer and cholesterol. The polymer prepared using epichlorohydrin, β-cyclodextrin ratio 8:1 was still water soluble. The value [5.07×10^4 M^{-1}] was only three times higher compared to the β-cyclodextrin [1.7×10^4 M^{-1}] although more significant enhancement was anticipated. This was attributed to hindered approach of cholesterol in the β-cyclodextrin cavities, as the latter were linearly crosslinked. To overcome this limitation we synthesized polymers containing increasing degree of substitution of β-cyclodextrin in the side chain. Cholesterol is 15 A^0 long, while β-cyclodextrin cavity is 7.8 A^0 deep. Complete inclusion of cholesterol, would therefore need two or more β-cyclodextrin rings [Breslow et al., 1996].

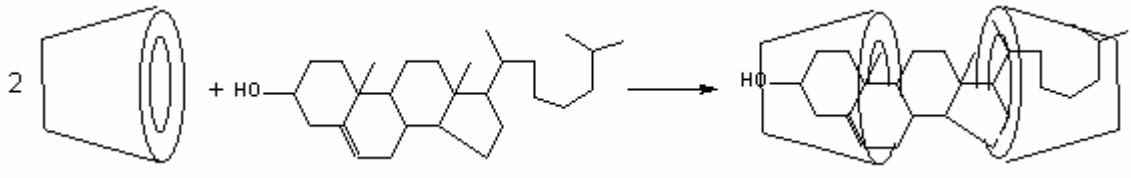
Binding between β- cyclodextrin and cholesterol can take place either by a stagewise mechanism at low β-cyclodextrin content or chelate type at higher β-cyclodextrin content [Figure 5.3] It has been reported that at high β-cyclodextrin content, chelate like complexes between β-cyclodextrin and guest are formed due to high local β-cyclodextrin concentration and result in very high association constant [Hollas et al., 1998]. Therefore

Figure 5.3: Stagewise and chelate binding between β-cyclodextrin and cholesterol

Stagewise binding between β-cyclodextrin and cholesterol



Chelate binding between β-cyclodextrin and cholesterol



we expected the association constant between cholesterol and the polymer β-cyclodextrin conjugate to increase with β-cyclodextrin content.

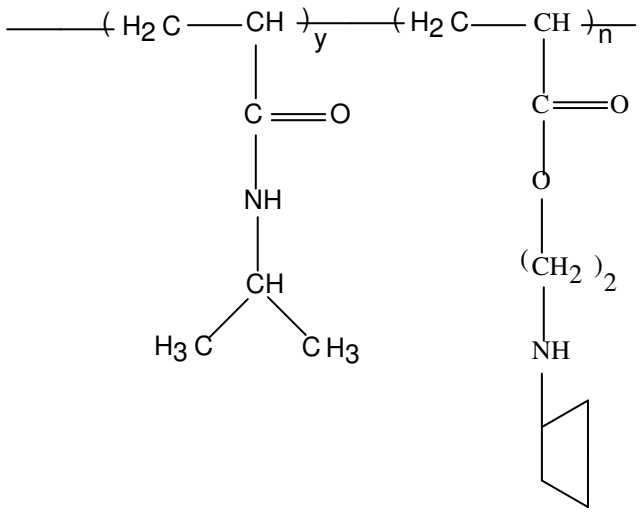
In view of the difficulties in preparing copolymers of high β-cyclodextrin content from the respective monomers, we substituted β-cyclodextrin on copolymers of 2-AEA and NIPA to increase the β-cyclodextrin substitution, to upto 67 % of total amino groups in 2-AEA [Figure 5.4]. As the degree of substitution increased from 7.4 to 67 % [P_1 to P_6], the association constants increased as expected [Table 5.3]. This enhancement was three orders of magnitude as compared to three folds increase reported by Breslow et al., [1996]. Such a strong dependence of association constant on the β-cyclodextrin content can only be explained by the chelate type binding. If this were the only effect to operate, one would expect the association constant to approach the value for the dimer D_1 i.e. 2.4×10^6 M^{-1}. The fact that the highest value for the polymer β-cyclodextrin conjugate *viz* 3.8×10^8 M^{-1} exceeds this by two orders of magnitude, implies an additional contribution. This is discussed below.

Nozaki et al., [1995] conjugated β-cyclodextrin with poly N-isopropylacrylamide [P [NIPA]] and estimated the association constants for 8-anilino-1-napthalenesulfonic acid ammonium salt [ANS]. In this case more than one P [NIPA] chain was conjugated with β-cyclodextrin unit but there were no β-cyclodextrin repeat units along the chain. Below the lower critical solution temperature of the polymer, the association constant for the system ANS-β-cyclodextrin-EDA-P [NIPA] was two orders of magnitude higher than that for ANS-β-cyclodextrin. P [NIPA] attached to β-cyclodextrin had more hydrophobic microenvironment than free P [NIPA] chains because of the enhanced concentration of P [NIPA] chains in the vicinity of β-cyclodextrin. The enhanced association constants resulted from the cooperativity between β-cyclodextrin and the P [NIPA] chains to bind ANS.

In the present case not only the formation of intramolecular complexes between cholesterol and β-cyclodextrin units from the same chain but also the contribution from P [NIPA] segments attached to the β-cyclodextrin units, leads to enhanced binding with cholesterol. As a result the association constant exceeds that for the dimer. Similar considerations explain why the association constants for dimer β-cyclodextrin conjugated

Figure 5.4: Polymers conjugated by β-cyclodextrin and β-cyclodextrin dimer

P1 to P6



P7 to P9

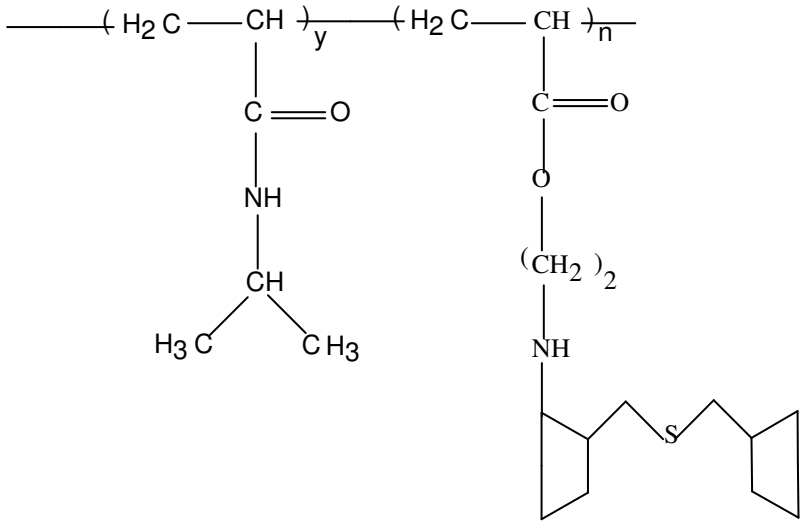


Table 5.3: Association constants of 2-AEA homopolymers and copolymers substituted with β-cyclodextrin and β-cyclodextrin dimer

Polymer	LCST ^{0}C	Mole % β-Cyclodextrin	NH_2 Mole %	$[K_b]$ M^{-1}
P_1	37	7.4	42.1	7.3×10^4
P_2	39	8.2	40.9	9.0×10^4
P_3	34	22.21	12.28	3.1×10^5
P_4	34	40.69	38.3	1.6×10^7
P_5	35	51.16	27.8	3.0×10^8
P_6	> 50	67.4	11.4	2.2×10^7
P_7	35	15.78	17.78	4.0×10^6
P_8	34	34	45	3.4×10^6
P_9	35	41	38	1.4×10^6

to P [NIPA] and 2-AEA copolymers, exceeds that for the dimer [see later]. The highest value 3.8×10^8 M^{-1} obtained by us is of the same order of magnitude quoted [Breslow et al., 1989] for medium affinity antibodies. Increasing β-cyclodextrin content reduces the distance between cyclodextrin moieties along the chain and leads to an increase in local β-cyclodextrin concentration. However, polymer P_6 containing 67% β-cyclodextrin substitution exhibited lower association constant [2.2×10^7 M^{-1}] than P_5 [3.0×10^8 M^{-1}]. This could be due to the crowding effect of β-cyclodextrin units.

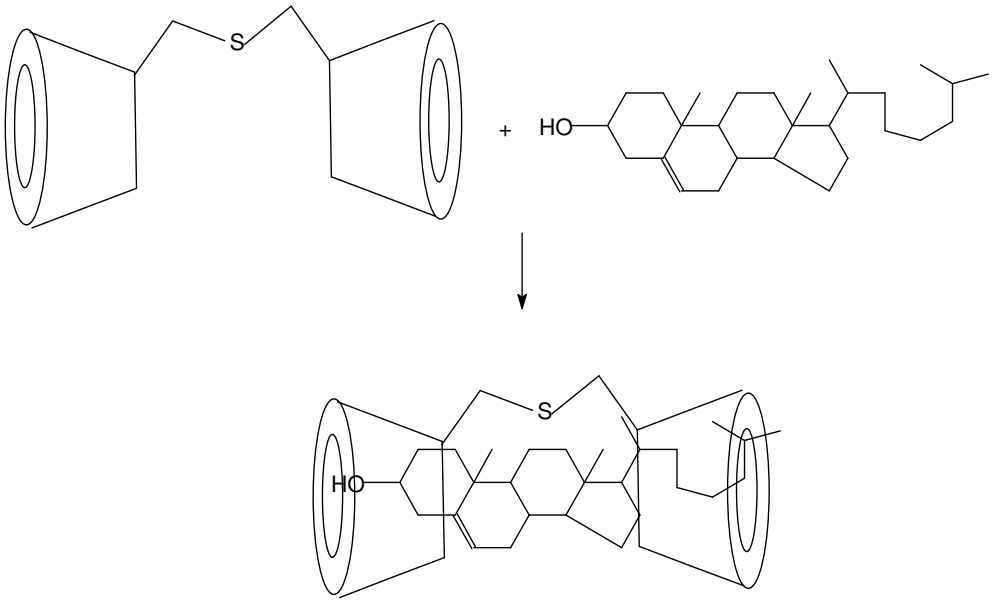
5.2.4 Cholesterol binding with dimers

Polymers containing high degree of β-cyclodextrin substitution showed enhanced association constant as compared to β-cyclodextrin. This could result from two β-cyclodextrin units coming together and forming an inclusion complex with cholesterol in a stagewise manner or a chelate. This led us to study the binding between cholesterol, β-cyclodextrin dimer and polymer containing β-cyclodextrin dimer as a function of degree of substitution. We synthesized β-cyclodextrin dimers D_1, D_2 and D_3 as explained earlier.

For dimer D_1 the association constant was 2.4×10^6 M^{-1}, which compared fairly well with the value [3.3×10^6 M^{-1}] reported earlier by Breslow et al., [1996]. The value for dimer D_1 reported by us is two orders of magnitude higher as compared to β-cyclodextrin [0.92×10^4 M^{-1}]. This is an indication that cholesterol forms a complex with the dimer D_1 which involves cooperative binding of cholesterol by the two β-cyclodextrin units. The stoichiometry of complex was further confirmed by ^{13}C NMR spectroscopy and is discussed later. It may be noted that although this conclusion appears trivial, this is not so. Breslow et al., concluded that " not all dimers automatically bind strongly and those that do are still selective for their guests". For instance Nesanas et al., [2000] reported that cocaine binds to β-cyclodextrin forming a 1:1 complex with a binding constant 570 M^{-1}. The complexation with dimer D_1 involves two cocaine molecules with identical binding constant of 800 M^{-1}, which is only marginally higher than that for β-cyclodextrin. Dimer D_2 exhibited slightly lower association constant [1.63×10^6 M^{-1}] than D_1, while in the case of D_3 the association constant [2.66×10^3 M^{-1}] was lower even than that for β-cyclodextrin. Cholesterol being a large molecule would prefer entry into the cavity from the secondary face of β-cyclodextrin and the same is hindered when the site is blocked. This observation is consistent with the findings of Nesanas et al., [2000] who reported very low association constant [310 M^{-1}] for association between cocaine and D_3 [β-cyclodextrin-C_3-sulphide] *vis a vis* cocaine and D_1 [β-cyclodextrin-C_6-sulphide] [570 M^{-1}]. Molecular modeling studies by Choi et al., [2001] and Bonnet et al., [2002] demonstrated that tail to tail orientation in dimer of β-cyclodextrin helps produce a more stable β-cyclodextrin cholesterol complex [Figure 5.5]. On the other hand, when the two β-cyclodextrin rings are linked head to head, inclusion complex formed with cholesterol was less stable and therefore exhibited lower association constant as compared to D_1, D_2 or β-cyclodextrin.

To evaluate binding between cholesterol and polymer β-cyclodextrin dimer conjugates, we first conjugated the dimer D_1 to form the vinyl monomer [M_5]. The association constant for the monomer M_5 was 1.04×10^6 M^{-1}, whereas for the copolymer with NIPA containing 3 mole % of M_5, the value was four times higher [5.4×10^6 M^{-1}].

Figure 5.5: Chelate binding between β-cyclodextrin dimer and cholesterol



5.2.5 Binding between polymers containing β-cyclodextrin dimer and cholesterol

As described earlier, conjugation of β-cyclodextrin with the polymer backbone leads to enhancement in association constant as a result of formation of 2:1 complex involving two β-cyclodextrin units. In the case of dimers, the association constant was enhanced since the two β-cyclodextrin units were always in juxta position. It was therefore interesting to investigate effect of substitution of dimer β-cyclodextrin on the association constant. Dimer D_1 was conjugated with the copolymer comprising NIPA and 2-AEA. The degree of substitution was varied between 15.7 to 41% [P_7-P_9]. The enhancement in the association constant was at best three times that of the dimer [Table 5.3]. As mentioned earlier, the enhancement over the value for the dimer is due to the contribution from the NIPA segments.

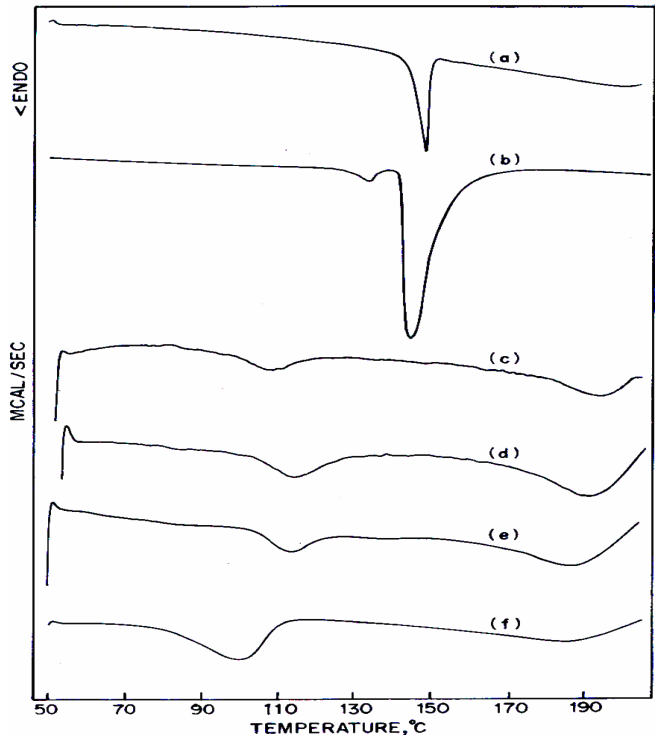
5.2.6 Structural characterization of cholesterol complexes

Having studied association between cholesterol and β-cyclodextrin dimer and its polymer conjugates in solution, we isolated complexes prepared using β-cyclodextrin dimer to cholesterol in ratios 1:1, 1:2 and 1:3 in the feed. The complexes were then characterized

by DSC and ^{13}C NMR to establish the stoichiometry of the inclusion complex. The DSC scan for cholesterol presented in Figure 5.6a exhibits an endothermic peak at 148.99 ^0C, which corresponds to the melting point of cholesterol 148.5 ^0C [Sigma Library of FT-IR Spectrum, 1986] and is well within the range 148 ^0C to 150 ^0C.

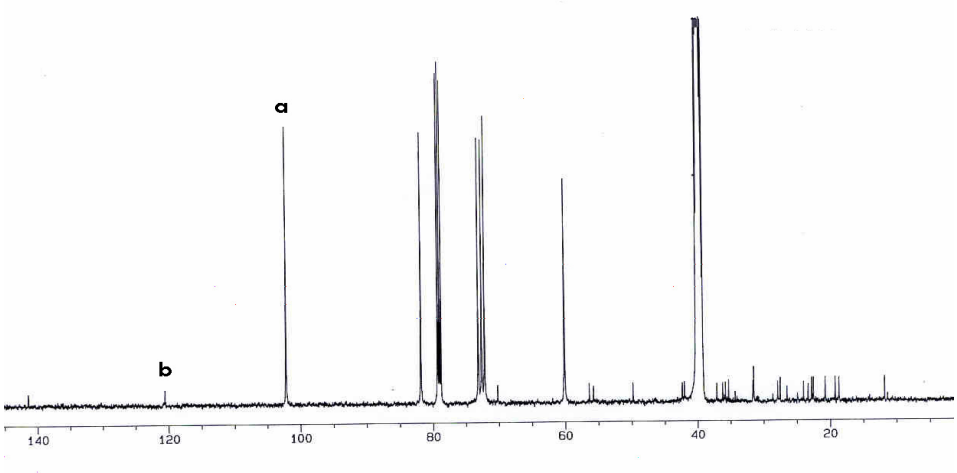
However the scans for the inclusion complexes prepared using dimer: cholesterol ratio 1:1, 1:2 and 1:3 in the feed [Figure 5.6f] did not exhibit any endothermic peak in the vicinity of 148 ^0C. This indicates the amorphous character of cholesterol in the inclusion complex. Fromming et al., [1993] too commented that molecular cholesterol in an inclusion complex does not show a melting peak. In fact Figure 5.6b shows endothermic peak corresponding to melting of cholesterol since cholesterol, which was not included in the complex, was not completely washed off. The endothermic peak disappeared when the complex was repeatedly washed with hexane [Figure 5.6 c].

Figure 5.6: Differential Scanning Calorimetry [DSC] for inclusion complexes between β-cyclodextrin dimer and cholesterol (a to f explained in the text)



These complexes were further subjected to ^{13}C NMR analysis to ascertain the stoichiometry of the complex [Figure 5.7]. The characteristic signals at 102.2 ppm corresponding to seven carbons [C_1] of β-cyclodextrin and at 120.1 ppm corresponding to one carbon [C_6] of cholesterol were used. These signals were specially chosen, as they are well isolated from the other signals of the spectrum. The calculations were performed using electronic integration of peak areas. Ratios of integration were in close agreement [Table 5.4] with proposed stoichiometry. Thus although the mole ratio of dimer to cholesterol in feed was varied, the inclusion complex always comprised dimer β-cyclodextrin and cholesterol in mole ratio 1:1.

Figure 5.7: ^{13}C NMR for inclusion complex between β-cyclodextrin dimer and cholesterol



a = Corresponds to fourteen carbon of cyclodextrin

b = Corresponds to one carbon of cholesterol

Table 5.4: β-Cyclodextrin dimer and cholesterol stoichiometry by ^{13}C NMR

Dimer	Mole ratio in feed β-Cyclodextrin Dimer: Cholesterol	Stoichiometry Found by ^{13}C NMR
D1	1:1	1.2
	1:2	1.1
	1:3	0.86
D2	1:1	0.84
	1:2	0.87
	1:3	1.2

5.3.0 Concluding remarks

β-Cyclodextrin was modified on primary as well as secondary site to obtain acryloyl derivatives. The length of the spacer segment was varied. Homopolymers and copolymers containing these monomers were synthesized and association constants with cholesterol were determined by the solubility method. The association constants increased with β-cyclodextrin content of the copolymers and the mechanism of complexation shifted from stagewise to chelate type. In the case of dimers D_1 and D_2, which were conjugated on the primary side, the association constant was enhanced by orders of magnitude. In the case of dimer D_3, which was conjugated on the secondary side, the association constant was lower than that for β-cyclodextrin. Since the dimers of β-cyclodextrin always formed chelate type complexes, the association constant between cholesterol and polymers containing β-cyclodextrin dimers, was independent of the degree of substitution. The stoichiometry of complexes was further confirmed by ^{13}C NMR.

Chapter 6

Complexation between Pyrene and Cyclodextrin Polymers: Fluorescence Study

6.0.0 Introduction

Cyclodextrins are increasingly being used for the encapsulation of drugs, preparation of supramolecular complexes and recovery of biomolecules. [Szjtli et al., 1982, 1996]. The ability of β-cyclodextrin to form inclusion complexes with suitable molecules has been exploited to develop tools in analytical chemistry and biochemistry [Li et al., 1992]. The mode of binding and stoichiometry between cyclodextrin and guest plays an important role in designing new polymers for effective complexation in these applications. Binding between cyclodextrin and guest molecule has been investigated by various method such as, X-ray analysis [Udachin et al., 1998], molecular modeling [Choi et al., 2001, Bonnet et al., 2001, 2002], fluorescence spectroscopy [Szjtli et al., 1996, Fromming et al., 1994, Karpovich et al., 1995, Xu et al., 1993, Hollas et al., 1998, 1998a, Kusumoto et al., 1987, Munoz et al., 1991, Jorg et al., 1999] etc.

Complexation between pyrene and β-cyclodextrin conjugated polymers has been extensively studied [Xu et al., 1993, Hollas et al., 1998, Kusumoto et al., 1987, Munoz et al., 1991]. β-Cyclodextrin conjugated polymers formed 2:1 complexes either by stagewise or chelate type mechanism depending upon the degree of substitution [DS]. It was concluded that the formation of 1:1 complex provides the driving force for the formation of 2:1 complexes between β-cyclodextrin and pyrene when the latter is formed by a stagewise mechanism.

In this chapter, we report binding between pyrene and β-cyclodextrin as well as dimer β-cyclodextrin conjugated polymers as a function of degree of substitution (DS) using fluorescence spectroscopy. A stagewise formation of intermolecular 1:1 [β-cyclodextrin: pyrene] and 2:1 complexes at low DS [below 5%] and intramolecular 2:1 chelate type complex at higher DS [above 5%] was confirmed. In contrast, dimer β-cyclodextrin and dimer β-cyclodextrin conjugated polymers always formed intramolecular 2:1 chelate complexes independent of the DS. We further show that in β-cyclodextrin conjugated polymers the association constant increases with DS while in the latter case it is independent of DS.

6.1.0 Experimental

6.1.1 Materials

β-Cyclodextrin, Pyrene, Acrylic acid, Dicyclohexyl carbodiimide [DCC], N-isopropylacrylamide [NIPA], N, N, N', N' tetra methyl ethylene diamine [TEMED] and Ammonium persulfate were purchased from Aldrich Chemical Company Inc., Milwaukee, WI, USA, P-toluene sulphonyl chloride [PTSC], Sodium iodide, Sodium sulphide, Tris buffer, 2-Aminoethanol, Phenol, N, N dimethyl acetamide [DMAc], Pyridine, Potassium hydroxide [KOH], Sodium chloride [NaCl], Hydrochloric acid, Sulphuric acid analytical grade were purchased from local suppliers. All solvents were purified as per standard procedures [Perrin et al 1966].

6.1.2 Instrumentation

^{1}H NMR spectra were recorded on Bruker superconducting FT-NMR AC 300 operating at 300 MHz. ^{13}C NMR was recorded on DRX500 MHz spectrometer. IR spectra were recorded on Shimadzu 8300 FT-IR spectrometer. Electronic absorption measurements were done on Shimadzu UV 1601 spectrophotometer. Fluorescence spectra were recorded at room temperature [~ 25^{0} C] on a Spex Fluorolog 2, unit using photon-counting technique. The excitation slit was set to 5.4 nm and the emission slit to 0.9 nm. The excitation wavelength was 335 nm, and the emission spectra were recorded from 350 to 450 nm [Hollas et al., 1998]. 2×10^{-7} Mole/Lit aqueous pyrene stock solution was used for fluorescence measurements [Munoz et al., 1991]. Deionized water used was from Millipore Direct – Q water purification system. Stock solutions of β-cyclodextrin and polymer conjugates of β-cyclodextrin or its dimer were prepared in deionized water. Viscosity measurements were carried out at 25^{0} C in 0.1 Molar NaCl solutions on Ubbelohde Viscometer [Schott AVS 350] using capillary number 0a and capillary diameter [ϕi] 0.53 mm.

6.2.0 Monomer synthesis

6.2.1 Synthesis of 2-aminoethyl acrylate hydrochloride [2-AEA]

Synthesis of 2-amino ethanol hydrochloride

In a 100 ml capacity round bottom flask 20 ml of 2- amino ethanol was dissolved in 40 ml of concentrated hydrochloride and this solution was warmed for 1 hour. After cooling,

water was removed under vacuum [Lele et al., 1999]. The residue was washed with acetone and dried under vacuum.

Condensation of 2- amino ethanol hydrochloride with acrylic acid

In a 250 ml capacity round bottom flask 8 ml [1×10^{-1} Mole] acrylic acid and 10 g [1×10^{-1} Mole] of 2-amino ethanol hydrochloride were dissolved in 30 ml DMF. To this, a solution containing 21 g [1×10^{-1} Mole] of DCC in 10 ml DMF was added. Solution was stirred for 2-3 hrs on ice water bath, then at room temperature for 48 hrs. Dicyclohexyl urea formed during reaction was filtered off and the filtrate was concentrated. The monomer was precipitated in to diethyl ether and characterized further.

^{1}H NMR 2-AEA [300 MHz D_20]: 5.76δ and 5.72δ d [CH_2=], 6.26δ m [=CH], 3.54δ d [-O-CH_2-], 2.81δ d [-CH_2-NH-]

IR [KBr]: 1644.4 cm^{-1} -C=C-, 3550.3 cm^{-1} –NH [broad], 1722 cm^{-1} –COO ester.

Yield: 70%

6.3.0 Synthesis of β-cyclodextrin dimer

β-Cyclodextrin-C_6-sulphide was synthesized as reported by Breslow et al., [1996] and Fujita et al., [1984].

^{1}H NMR β-Cyclodextrin-C_6-sulphide [300 MHz D_2O]: 3.93 to 4.05δ d [4H6], 4.71 to 5.03δ m [7H1], 3.2 to 3.6δ m [7H2 to 7H3], 3.7 to 3.9δ m [7H4 to 7H5].

^{13}C NMR [500 MHz DMSO d6]: 102.24 δ [C-1], 72.15 δ [C-2], 73.14 δ [C-3], 81.91 δ [C-4], 72.66 δ [C-5], 60.20 δ [C-6]

Yield: 25%

6.4.0 Polymer synthesis

6.4.1 Synthesis of NIPA and 2-AEA copolymer

In a 250 ml round bottom flask 0.71 g [6.3×10^{-3} Mole] NIPA and 4 g [2.5×10^{-1} Mole] of 2-AEA were added to 25 ml of distilled water. 20 µl TEMED and 1% by weight of ammonium per sulfate were added and flask was purged with nitrogen for 30 min. Flask was maintained in a hot water bath at 40^0 C for 16 hrs. Polymer was precipitated from the reaction mixture and filtered. Polymer was washed with diethyl ether and dried under vacuum.

Table 6.1: Synthesis of β-cyclodextrin or dimer β-cyclodextrin conjugated polymers

Polymer	**LCST ^{0}C**	**Mole % of β-CD**	**Mole % of NH_2**	**Below C* K_1 M^{-1}**	**Below C* K_2 M^{-2}**	**Above C* K_2 M^{-2}**	**C* g/dl**	**Type of binding**
β-CD	-	-	-	635		1.60×10^5	-	Stagewise
P_1 NIPA:2AEA 20:80	35	-	-	-	-	-	6.6	-
P_2	36	0.5	79.5	95.8	-	1.34×10^5	6.7	Stagewise Below C* 1:1 and above 2:1
P_3	35	2	78	59.38	-	1.91×10^5	6.9	
P_4	35	5	75	-	1.12×10^5	2.24×10^5	7.2	Chelate binding Below C* 2:1
P_5	36	7	73	-	1.23×10^7	2.60×10^7	7.4	
P_6	36	8	72	-	2.41×10^8	4.75×10^8	7.9	
P_7	37	22	58	-	3.17×10^8	5.85×10^8	11.9	
P_8	35	40.7	38.3	-	4.49×10^8	6.16×10^8	26.2	
P_9	38	51.2	27.8	-	6.75×10^8	7.21×10^8	29.1	
P_{10}	< 50	67.4	11.4	-	8.42×10^8	8.63×10^8	38.8	
Dimer conjugated polymers								
Dimer	-	-	-	-	1.84×10^8	-	-	Chelate
P_{11}	35	0.5	79.5	-	1.80×10^8	1.94×10^8	6.9	Chelate Below C* 2:1
P_{12}	36	3	77	-	1.91×10^8	1.96×10^8	7.9	
P_{13}	36	7	73	-	2.11×10^8	2.43×10^8	8.2	
P_{14}	37	12	68	-	2.37×10^8	2.51×10^8	8.5	
P_{15}	36	15	65	-	2.69×10^8	2.71×10^8	9.2	
P_{16}	34	34.0	45	-	2.72×10^8	2.79×10^8	10.2	
P_{17}	35	41.0	38	-	2.96×10^8	2.92×10^8	16.7	

^{1}H NMR [300 MHz D_20]: 5.48δ m [7H, C_1H], 2.27δ m [4H, CH_2CONH], 2.90δ m [1H, $CH-(CH_3)_2$], 2.01δ m [1H, CHCONH], 1.57δ m [1H, $CHCH_2$-CH], 1.14δ d s [6H, CH_3]

Yield: 65%

6.4.2 Synthesis of β-cyclodextrin or dimer β-cyclodextrin conjugated polymers

In a 250 ml conical flask 1 g NIPA:2-AEA copolymer was dissolved into methanol and depending upon the DS desired [Table 6.1] KOH equivalent to the hydrochloride was added. The solution was stirred for 1 hr at room temperature and then maintained at 5^0 C for 12 hours. Potassium chloride precipitated was filtered off and the filtrate evaporated to dryness. β-cyclodextrin and the dimer β-cyclodextrin were converted to tosylate derivative according to the procedures reported by Leckchiri et al., [1995] and Ueno et al., [1982] respectively and β-cyclodextrin tosylate was conjugated on to the polymer by the method reported by Seo et al., [1987]. Polymer was dissolved in a mixture of 30 ml of methanol and 15 ml of DMAc. Calculated amount of β-cyclodextrin tosylate was added with stirring at 50^0 C to the solution in small portions. After the reaction, 100 ml of distilled water was added and the water-soluble portion was evaporated off. After washing the residue with methanol and ether the final product was obtained as a white powder. The polymer was re-dissolved into water and precipitated by increasing temperature to ensure the complete removal of β-cyclodextrin tosylate. The polymer was filtered and repeatedly washed using ether and methanol. Incorporation of β-cyclodextrin in the copolymer was quantified colorimetrically using phenol sulphuric acid method [Dubois et al., 1956] and LCSTs of polymers were estimated by the turbidimetry method [Boutris et al., 1997].

^{1}H NMR [300 MHz D_20]: 5.48δ m [7H, C_1H], 2.27δ m [4H, CH_2COEDA-β-cyclodextrin], 2.90δ m [1H, $CH-(CH_3)_2$], 2.01δ m [1H, CHCONH], 1.57δ m [1H, $CHCH_2$-CH], 1.14δ d s [6H, CH_3]

Yield: 85%

6.5.0 Results and discussion

In chapter 5, we reported the association constants between β-cyclodextrin and polymer conjugates of β-cyclodextrin as well as dimer β-cyclodextrin with cholesterol. It was observed that in polymer β-cyclodextrin conjugates, the association constant increased

with DS of β-cyclodextrin, whereas the values were independent of DS in the case of polymer conjugates of dimer β-cyclodextrin. However this did not provide any insights into the mechanism as well as stoichiometry of binding. We therefore, investigated the mechanism of binding between β-cyclodextrin and pyrene using fluorescence spectroscopy.

Pyrene was selected as a model fluorescent probe and binding between pyrene and β-cyclodextrin conjugated polymers was investigated by fluorescence spectroscopy.

The intensity ratio of first [I_1 = 372 nm] and third [I_3 = 384 nm] vibronic transitions changes as a result of Ham effect. This allows estimation of association constants between pyrene and β-cyclodextrin even at low β-cyclodextrin concentrations [Karpovich et al., 1995, Munoz et al., 1991].

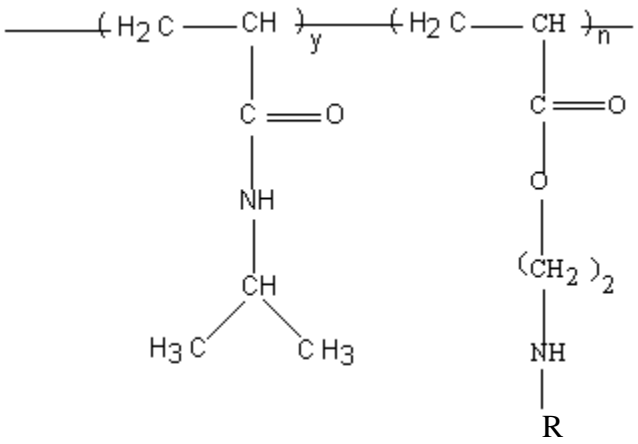
In subsequent sections we show that binding between polymer β-cyclodextrin conjugates and pyrene shifts from stagewise to chelate type as the DS of β-cyclodextrin increases. Polymers containing dimer β-cyclodextrin always show chelate type binding. In the case of β-cyclodextrin conjugated polymers, the association constant increases with DS. The contributions of intramolecular and intermolecular complexation to the binding constant were estimated and it was shown that the contribution of the latter decreases with increasing DS of β-cyclodextrin. In the case of dimer β-cyclodextrin conjugated polymers; the association constant is independent of DS.

6.5.1 Polymer synthesis and characterization

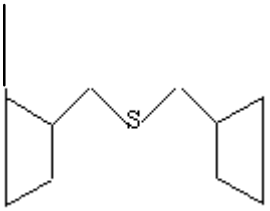
To study the binding between pyrene and β-cyclodextrin or dimer β-cyclodextrin conjugated polymers as a function of DS; copolymers of NIPA and 2-AEA were synthesized by free radical polymerization. NIPA was selected as a comonomer, as the copolymer exhibiting LCST behaviour could be used for recovery of cholesteol from aqueous medium in future. 2-AEA was synthesized as reported in experimental section and co-polymerized with NIPA. Protection of free amino group by the hydrochloride protects the O→N acyl migration [Notes 1970] and leads to the pendant amino end group. The hydrochloride group was deprotected using potassium hydroxide in methanol and resulting free amino group was conjugated with β-cyclodextrin or dimer β-cyclodextrin as reported in the experimental section [Figure 6.1]. Similar procedure was

followed with dimer β-cyclodextrin. Hollas et al., [1998] could synthesize β-cyclodextrin conjugated poly [allylamine] with a DS only upto 23%, due to the limited solubility of polymers. In contrast, we could synthesize a wide range of polymers in which DS could varied between 0.5 to 67% [Table 6.1].

Figure 6.1: Polymers conjugated by β-cyclodextrin and dimer β-cyclodextrin



R ═══ 1] Dimer



2] CD

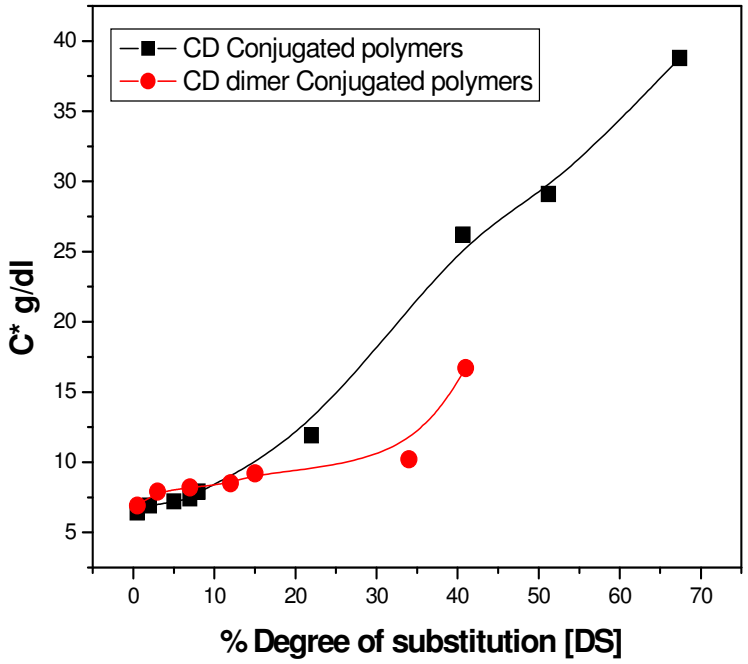
To avoid interference due to unreacted tosylate ion [Hollas et al., 1998], polymer was precipitated in water and repeatedly washed with ether and methanol. Complete removal of tosylate ion was confirmed by ^{1}H NMR analysis.

Pyrene solubility in water is very low ~5 $\times 10^{-7}$ Mole/Lit [Kusumoto 1987]. For determination of stoichiometry and mode of binding, accurate estimation of pyrene concentration is important. Edwards and Thomas [1978] erroneously reported

dimerization of 1:1 complexes at pyrene concentrations higher than the solubility of pyrene in water [Nakajima et al., 1983, Schwarz et al., 1977]. In contrast, only 1:1 complexes were observed when the concentration ranges were selected appropriately. [Nakajima et al., 1983, Patonay et al., 1986]. Pyrene solutions in water were prepared according to the procedure reported by Munoz et al., [1991]. This avoided formation of pyrene micro crystals and yielded reproducible results. Pyrene concentration in the stock solution was 2 $\times 10^{-7}$ Mole/Lit. The absence of pyrene micro crystals in stock solution was confirmed by visual inspection as well as by low emission counts in the 500 nm region [Nakajima et al., 1977]. Sample solutions were purged with nitrogen to remove dissolved oxygen to avoid quenching due to the presence of oxygen [Patonay et al., 1986] and were protected from light to minimize photodecomposition.

The intrinsic viscosity [η] of polymers was estimated and critical overlap concentration of polymers [C*] calculated. The intrinsic viscosity for the β-cyclodextrin conjugated polymers decreased with increasing degree of substitution on NIPA-2-AEA polymer [Figure 6.2].

Figure 6.2: Critical concentration as a function of DS



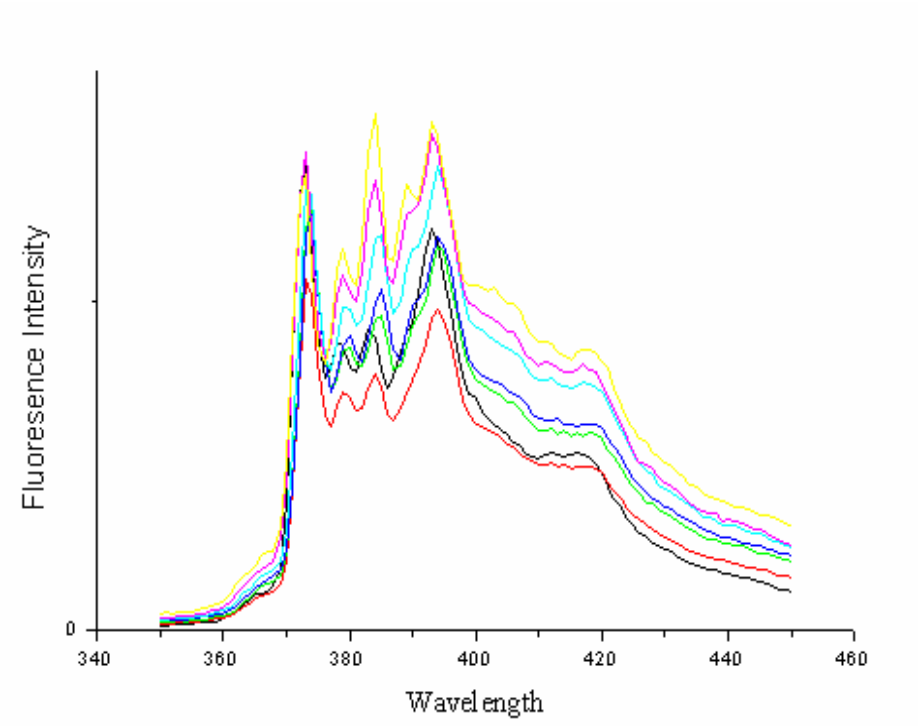
This decrease in viscosity indicates that β-cyclodextrin molecules do not contribute to the hydrodynamic radius of the polymer to the same extent as the polymer backbone [Hollas et al., 1998]. A second reason already discussed by Leckchiri et al., [1995] and Lewis et al., [1981] for poly [vinylamine] conjugated β-cyclodextrin, is the formation of numerous hydrogen bonds between β-cyclodextrin tosylate and amine groups of the polymer, which lead to a more compact polymer coil [Ruebner et al., 2000].

It was observed [Table 1] that LCST of β-cyclodextrin conjugated polymers increased with the DS of β-cyclodextrin. Above LCST, the approach to β-cyclodextrin cavities could be blocked by the aggregation of poly [NIPA] chains [Nozaki et al., 1997]. The fluorescence study was therefore carried out at 25^0 C, well below the LCST of any polymer.

6.5.2 Binding between β-cyclodextrin conjugated polymers and pyrene

Pyrene intensity changes on complexation with β-cyclodextrin play a key role in the evaluation of the fluorescence data. This requires a known pyrene concentration in all sample solutions. Pyrene spectra in aqueous solutions of varying β-cyclodextrin concentration are shown in Figure 6.3. The fluorescence intensity increased and the

Figure 6.3: Pyrene emission spectrum

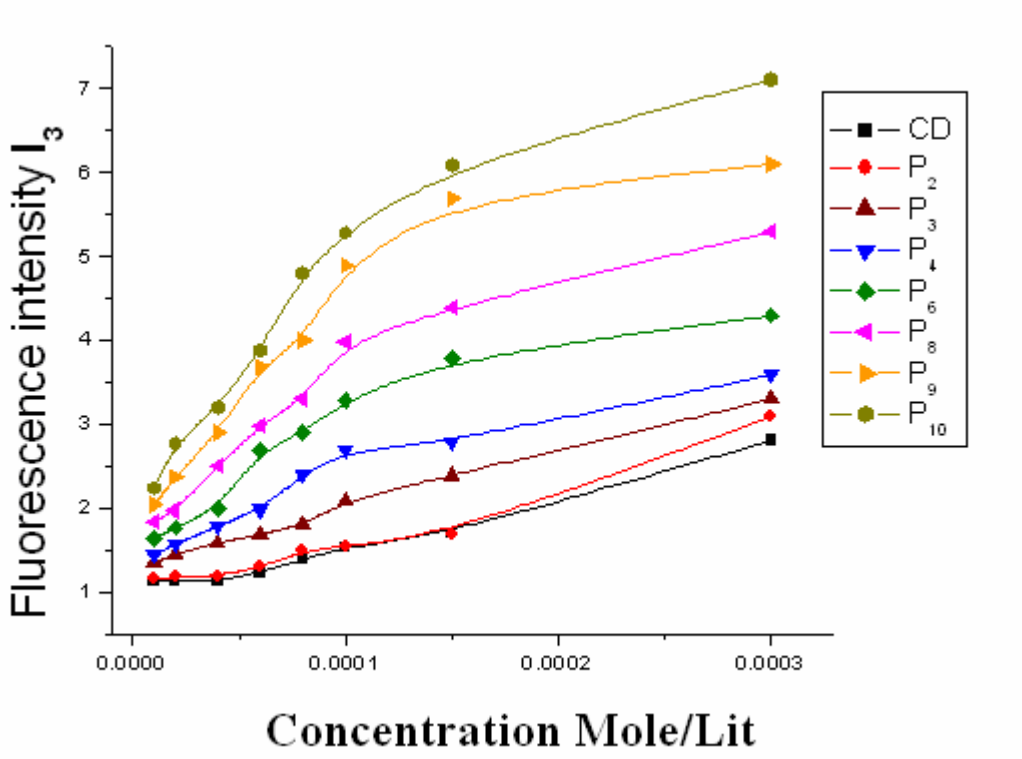


intensity ratio [R] I_1/I_3 decreased at higher β-cyclodextrin concentration. This indicates transition of pyrene molecule from polar solvent water into the hydrophobic β-cyclodextrin cavities [Hollas at al., 1998, Steven et al., 1996, Herkstroeter et al., 1986]. The initial decrease in intensity I_3 at lower β-cyclodextrin concentration indicates the formation of 1:1 complex. Subsequent rapid increase in intensity at higher β-cyclodextrin concentration is attributed to the formation of 2:1 complex. The spectrum does not show any excimer emission; which confirms that there are no pyrene micro crystals in the stock solution of pyrene [Steven et al., 1996]. Absence of excimer emission is also attributed to the formation of 2:1 complex at higher β-cyclodextrin concentration [Herkstroeter et al., 1986]. This confirmed stagewise formation of 2:1 complex between β-cyclodextrin and pyrene as a function of β-cyclodextrin concentration [Hollas et al., 1998, Xu et al., 1993, Kusumoto et al., 1986, 1987, Munoz et al., 1991, Adams et al., 1999, Patonay et al., 1986].

To study the stoichiometry and mode of binding between β-cyclodextrin conjugated polymers and pyrene; polymers P_2 to P_{10} were evaluated. Spectrum showed [Figure 6.4] initial decrease in fluorescence intensity of I_3 for polymer P_2 and P_3. Polymers P_2 and P_3 showed stagewise formation of 2:1 complex. As the concentration of polymer increases β-cyclodextrin from other polymer chains can form intermolecular 2:1 complex, therefore intensity I_3 increases rapidly [Xu et al., 1993, Hollas et al., 1998]. Polymer P_3 showed 1:1 binding below C* of polymer and 2:1 complex above C* indicating the formation of intermolecular 2:`1 complex. Clearly at low DS of β-cyclodextrin, the association constant for intermolecular 2:1 complex [1.3×10^5 M^{-2}] is much greater than that for intramolecular 1:1 complex [96 M^{-1}].

Polymers P_4 to P_{10} containing 5 to 67% β-cyclodextrin substitution, showed continuous increase in intensity I_3. This could be attributed to the formation of 2:1 chelate complex. These polymers showed exclusively 2:1 stoichiometry even in the concentration range, where the β-cyclodextrin and polymers P_2 and P_3 showed 1:1 complexation. To evaluate the contributions of intramolecular and intermolecular complexation to the binding constant, the values of K_2 below as well as above C* were calculated. C* increased with the DS of β-cyclodextrin

Figure 6.4: Stagewise or chelate binding in β-cyclodextrin conjugated polymers



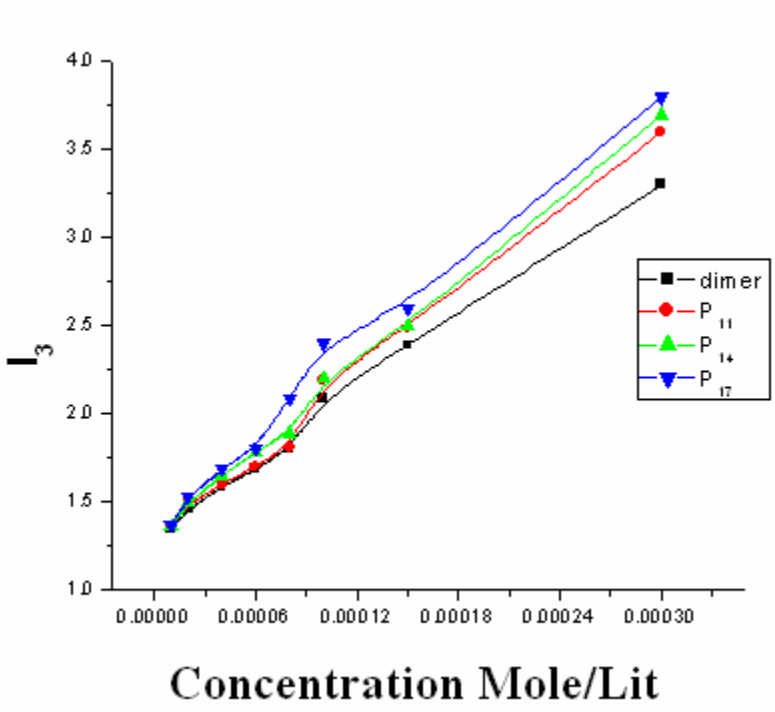
.The difference in values of K_2 below and above C*, decreased with increasing DS and is discussed later. This indicated that the contribution of the intermolecular complexation decreased with increasing DS.

Fluorescence intensity increased and the intensity ratio [$R = I_1/I_3$] decreased due to the transition of pyrene from polar medium to the non-polar β-cyclodextrin cavity [Figure 1]. Dong et al., [1984] studied intensity ratio of pyrene in aqueous solution and assigned empirical polarity scale 0.68 to a very nonpolar, 0.90 to a semipolar and 1.80 to a polar environment. Kusumoto et al., [1987] reported a value of 0.90 for 2:1 stoichiometry, whereas Hollas et al., [1998] and Munoz et al., [1991] reported values of 0.68 and 0.62 for 2:1 stoichiometry. In our case intensity ratio was found to be 0.65 for monomeric β-cyclodextrin, whereas for polymers the values were in the range 0.59 to 0.70. Thus our values for monomeric β-cyclodextrin as well for β-cyclodextrin conjugated polymers are typical of Pyrene in a non polar environment, resulting from complex formation and are in good agreement with those reported by Dong et al., [1984] and Kalyanasundaram et al., [1977]

6.5.3 Binding between dimer β-cyclodextrin conjugated polymers and pyrene

Earlier workers reported the binding between dimer β-cyclodextrin and potassium 2-p-toluidinylnapthalene-6-sulfonate [TNS] [Harada et al 1980, Haskard et al., 1996, Russell et al., 1991, Fokke et al., 1994, Fujita et al., 1984]. The dimer complexes showed exclusively 2:1 [β-cyclodextrin: TNS] stoichiometry; in the concentration range in which β-cyclodextrin showed 1:1 stoichiometry. In our earlier study [chapter 5] we have reported the complexes between dimer β-cyclodextrin and cholesterol using various mole ratios of dimer to cholesterol. These complexes were further characterized by ^{13}C NMR and DSC techniques and it was demonstrated that dimer β-cyclodextrin always formed 2:1 [β-cyclodextrin: cholesterol] complexes. Spectrum for dimer β-cyclodextrin showed enhanced fluorescence intensity as compared to β-cyclodextrin. A plot of intensity I_3 versus β-cyclodextrin concentration showed continuous increase in I_3 [Figure 6.5]. This indicated the formation of 2:1 complex.

Figure 6.5: Chelate binding in dimer β-cyclodextrin conjugated polymers



To study the effect of DS, the fluorescence spectra for polymers P_{11} to P_{17} containing dimer β-cyclodextrin conjugates were investigated. These polymers also showed

enhanced I_3 as observed in case of dimer β-cyclodextrin. This indicates the formation of 2:1 chelate binding [Figure 6.5], and is independent of the DS. For polymer P_{11} containing as low as 0.5% dimer β-cyclodextrin, the binding was chelate type and the value of K_2 was very high ~10^8 M^{-2}. The evaluation of values of K_2 showed that the values were practically the same both above and below C*. This indicates that the intermolecular complexation plays practically no role in binding at any DS. This is because pyrene forms a complex with the two β-cyclodextrin units, which constitute the dimer.

The intensity ratio [I_1/I_3] also confirmed the formation of inclusion complexes between dimer and pyrene. The intensity ratio for dimer β-cyclodextrin was 0.45, showing more hydrophobic environment as compared to β-cyclodextrin [0.80], whereas values for dimer β-cyclodextrin conjugated polymers ranged from 0.68 to 0.70. These values are in close agreement with those reported by Dong et al., [1984] and Kalyanasundaram et al., [1977] for β-cyclodextrin.

6.5.4 Study of association constants between β-cyclodextrin conjugated polymers and pyrene

The association constants indicate the stability of complexes and help to understand the factors affecting the complexation e.g. temperature, time, concentration, pH etc. Study of association constants by using thermodynamic stability of an inclusion complex [Hollas et al., 1998], calorimetric and solubility method [Breslow et al., 1996], Stern-Volmer method [Patonay et al., 1986], Intensity enhancement method [Kusumoto et al., 1987, Munoz et al., 1991] etc. have been reported earlier. After studying the stoichiometry of β-cyclodextrin conjugated polymers with pyrene, the strength of inclusion complexes was evaluated by investigating association constants. Intensity ratio [R] increases with increasing polarity of the medium [Kusumoto et al., 1987, Kalyanasundaram et al., 1977]. Evaluation of this ratio permits the estimation of association constants of the inclusion complexes. According to Munoz et al., [1991] and Kusumoto et al., [1987] the association constant [K_1] for 1:1 complex is determined by equation 1, whereas the association constant K_2 for 2:1 complex is given by equation 2.

$$\mathbf{1/(R_0-R) = 1/K_1\,[R_0-R_1]\,[\beta\text{-cyclodextrin}]_0 + 1/(R_0-R_1)} \quad \mathbf{[Equation\ 1]}$$

Where, R_0 and R_1 denote the ratios for pyrene in water and in the complex respectively. R is the ratio $[I_1/I_3]$ at given β-cyclodextrin concentration.

$$\mathbf{1/(R_0-R) = 1/K_2\,[R_0-R_2]\,[\beta\text{-cyclodextrin}]_0^2 + 1/(R_0-R_2)} \quad \mathbf{[Equation\ 2]}$$

After studying the stoichiometry of complexes, the strength of complexes was investigated by estimating association constant as a function of DS [Table 1].

In earlier report, association constant for β-cyclodextrin and pyrene system was found to be in the range 7.6 to 675 M^{-1} [Kusumoto et al., 1987, Nakajima et al., 1983, 1984, Patonay et al., 1986, Hashimoto et al., 1985, Hamai S 1989]. In our case K_1 was found to be 635 M^{-1} whereas K_2 at higher β-cyclodextrin concentration was found to be 1.60×10^5 M^{-2} [Table 1]. This value lies within the range 8.5×10^5 M^{-2} reported by Munoz et al., [1991], and 1.16×10^4 M^{-2} by Kusumoto et al., [1987]. The differences in association constant have been attributed to the difference in the I_1/I_3 ratio at different pyrene and β-cyclodextrin concentrations, pH, temperature etc. [Kusumoto et al., 1987, Munoz et al., 1991, Xu et al., 1993]. K_1 for polymers P_2 and P_3 was found to be lower. This could be due to the fact that at low DS, the two β-cyclodextrin moieties are far apart from each other and hence complete inclusion of pyrene was not possible. But as the polymer concentration increased, β-cyclodextrin from other polymer chains could form 2:1 intermolecular complexes.

Polymer P_4 to P_{10} [containing DS of β-cyclodextrin 5 to 67.4%] showed formation of 2:1 chelate complexes [Figure 4], therefore association constant K_2 was calculated according to equation 2. These polymers showed increase in association constant as the DS increased [Table 1]. This is because increasing DS and compact structure of polymer as indicated by lower [η] of polymers [Figure 2], reduces distance between β-cyclodextrin moieties and leads to an increase in local β-cyclodextrin concentration. Therefore two β-cyclodextrin rings from the same polymer chain come together and form 2:1 intramolecular chelate type complex. Due to this proximity of β-cyclodextrin to each other it could form hydrogen bond with hydroxyl group of another β-cyclodextrin and this extra stabilization could lead to enhanced K_2 as compared to β-cyclodextrin and polymers P_2 and P_3. Enhanced K_2 suggested that hydrogen bonding favors the second binding [Munoz et al., 1991, Xu et al., 1993]. In addition shorter distance between β-

cyclodextrin lowers the entropy loss due to a change in polymer chain conformation when encapsulating the pyrene by β-cyclodextrin molecule [Hollas et al., 1998].

6.5.5 Association constants between dimer β-cyclodextrin conjugated polymers and pyrene

We also studied the binding between dimer β-cyclodextrin conjugated polymers and pyrene as a function of DS. In the case of dimer two β-cyclodextrin units are linked to each other through covalent bonding, hence dimer always showed intramolecular 2:1 chelate type complexes [Figure 4]. Association constant K_2 was calculated according to equation 2 [Table 1] and compared with the value for dimer β-cyclodextrin [1.84×10^8 M^{-2}], which is thousand times higher as compared to β-cyclodextrin [1.60×10^5 M^{-2}]. This increase in association constant can be attributed to the presence of covalently linked neighboring β-cyclodextrin ring.

Polymer P_{11} to P_{17} , containing DS of dimer β-cyclodextrin 0.5 to 41%, showed marginal difference in K_2 ranging from 1.80×10^8 to 2.96×10^8 M^{-2} as compared to dimer β-cyclodextrin [1.84×10^8]. This indicates that association constants of dimer β-cyclodextrin conjugated polymers are independent of the DS.

β-cyclodextrin conjugated polymer P_6 to P_{10} showed higher association constant as compared to dimer β-cyclodextrin and polymer P_{11} to P_{17}. This is because of formation of intramolecular complexes between pyrene and β-cyclodextrin units from the same chain but also the contribution from poly N-isopropylacrylamide P [NIPA] segments attached to the β-cyclodextrin units [Nozaki et al., 1995, 1997]. Nozaki et al., [1995] conjugated β-cyclodextrin with [P [NIPA] and estimated the association constants for 8-anilino-1-napthalenesulfonic acid ammonium salt [ANS]. In this case more than one P [NIPA] chain was conjugated with β-cyclodextrin unit but there were no β-cyclodextrin repeat units along the chain. Below the LCST of the polymer, the association constant for the system ANS-β-cyclodextrin-EDA-P [NIPA] was two orders of magnitude higher than that for ANS-β-cyclodextrin. P [NIPA] attached to β-cyclodextrin had more hydrophobic microenvironment than free P [NIPA] chains because of the enhanced concentration of P [NIPA] chains in the vicinity of β-cyclodextrin. The enhanced association constants resulted from the cooperativity between β-cyclodextrin and the P [NIPA] chains to bind

ANS. As a result the association constant for polymer P_6 to P_{10} exceeds that for the dimer and dimer conjugated polymers.

6.6.0 Concluding remarks

The mode of binding and stoichiometry between β-cyclodextrin conjugated polymers and pyrene was investigated as a function of DS by fluorescence spectroscopy. β-cyclodextrin conjugated polymers showed intermolecular 2:1 binding at low [below 5%] DS, whereas intramolecular chelate type binding at high [above 5%] DS. Dimer β-cyclodextrin conjugated polymers showed intramolecular 2:1 chelate type binding, which is independent of DS. β-Cyclodextrin conjugated polymers showed increase in binding constant [from 1.34×10^5 to 8.42×10^8 M^{-1}] as the DS of β-cyclodextrin increased [from 0.5 to 67%]; whereas dimer β-cyclodextrin conjugated polymers the binding constant is relatively independent of DS [ranging from 1.8×10^8 to 2.9×10^8 M^{-1}].

Chapter 7

Conclusions and Suggestions for Future Work

The key conclusions arrived at from present investigations are as follows.

Chapter 3

- Cholesterol bearing monomers i.e. monocholesteryl itaconate and cholesteryl methacrylate and crosslinkers glyceryldicholesteryl itaconate and monocholesteryl itaconate glycerol methacrylate enhanced the capacity for cholesterol [43.7 mg/g] as well as selectivity [α = 3.7] as compared to the non imprinted polymer. [Page no. 68]
- The non specific binding was suppressed when crosslinkers containing hydrophilic groups i.e glycerol dimethacrylate [α = 1.3 to 1.7] and monocholesteryl itaconate glycerol methacrylate [α = 1.4 to 3.7] were used. [Page no. 68]
- The imprinting effect is reflected in higher capacity [38.5 mg/g] and selectivity [α = 2.75] values for cholesterol as compared to stigmasterol [17 mg/g, α = 1.1] and testosterone [8.1 mg/g, α = 1.2]. [Page no. 73]

Chapter 4

- The two stage polymerization / crosslinking approach was developed for MIPs using MBAM as a crosslinker and allylamine hydrochloride as a functional monomer.
- The two stage approach resulted in enhanced capacity [587 mg/g] and selectivity [α = 1.72] for NaC as compared to the simultaneous polymerization / crosslinking approach [475 mg/g, α = 1.35]. [Page no. 88]
- The two stage approach also resulted in enhanced active site utilization [84%] as compared to the simultaneous polymerization / crosslinking approach [33%]. [Page no. 88]
- Selectivity study reveals that with increasing mole ratio of template to functional monomer [from 0.1 to 2], selectivity for NaC over NaT increased from 1.64 to 3.55. [Page no. 88]

Chapter 5

- Various β-cyclodextrin containing monomers and β-cyclodextrin dimers were synthesized. β-cyclodextrin dimers showed higher binding constant [2.4×10^6 to 3.3×10^6 M^{-1}] as compared to monomeric β-cyclodextrin [0.63×10^4 to 0.87×10^4 M^{-1}]. [Page no. 107]
- New affinity thermoprecipitating polymers were synthesized by conjugating β-cyclodextrin and dimer β-cyclodextrin as affinity ligands for cholesterol.
- In β-cyclodextrin conjugated polymer, binding constant increases [1.4×10^4 to 2.2×10^7 M^{-1}] as the DS increases [2 to 67.4%], whereas in dimer β-cyclodextrin conjugated polymers it is relatively independent [1.4×10^6 to 4.0×10^6 M^{-1}] of DS. [Page no. 112]
- Binding between β-cyclodextrin conjugated polymers and cholesterol could be stage wise at low DS [below 7.4%], while chelate type at higher DS [above 7.4%].
- Dimer β-cyclodextrin conjugated polymers always showed chelate type binding, independent of DS. [Page no. 113]

Chapter 6

- Mode of binding and stoichiometry between β-cyclodextrin conjugated polymers and pyrene was investigated by fluorescence spectroscopy.
- β-Cyclodextrin conjugated polymers showed intermolecular 2: 1 binding below 5% DS, and intramolecular chelate type binding above at 5% DS.
- Dimer β-cyclodextrin conjugated polymers showed intramolecular 2: 1 chelate type binding, which is independent of DS.
- β-Cyclodextrin conjugated polymers showed increase in binding constant [1.34×10^5 to 8.42×10^8 M^{-1}] as the DS of β-cyclodextrin increased [0.5 to 67%]; whereas in dimer β-cyclodextrin conjugated polymers it was relatively independent of DS, ranging from 1.8×10^8 to 2.9×10^8 M^{-1}.

This work has led to methodologies, which enhance capacity and selectivity for cholesterol or bile salts, based on MIPs and affinity based separation. It has also opened up further possibilities, which could be exploited to enhance the capacity and selectivity

of cholesterol or bile salts during recovery from aqueous media. These are summarized below.

1. The investigation carried out in chapter 3 demonstrates that cholesterol bearing monomers and crosslinkers enhance the capacity and selectivity for cholesterol. Further hydrophilic crosslinker minimized the non specific binding of cholesterol. Thus, it would be logical to investigate bile salt bearing monomers and crosslinkers for the MIPs to sequester bile salts.
2. The two stage approach used for sequestering bile salts [chapter 4] showed enhanced active site utilization for MIPs. The use of cholesterol bearing monomers for the sequential polymerization and crosslinking will lead to enhanced cholesterol binding capacities. Experiments could be undertaken to quantify the extent of enhancement.
3. The investigation carried out in chapter 4 demonstrated the use of allylamine hydrochloride as a functional monomer and leads to high percentage utilization of active sites in the binding of bile salts. Thus partial alkylation and quaterization of allylamine hydrochloride followed by imprinting will lead to further enhanced capacity for bile salts.
4. Allylamine hydrochloride was used as a functional monomer for bile salts [chapter 4]. Incorporation of allylamine hydrochloride into the polymer was very low due to its low reactivity. If the incorporation of functional monomer increased, binding with guest molecule will increase. 2-aminoethyl methacrylate hydrochloride can be used to further enhance the capacity for bile salts.
5. In chapter 5, affinity thermoprecipitating polymer was synthesized for the separation of cholesterol. Similarly polymerizations which undergo precipitation as a result of changes in pH, ionic strength; use of organic solvent etc. for the precipitation of poly [NIPA] could be investigated.

References

Chapter 1:

Asano T, Pollard M, Madsen D. C, Proc. Soc. Exp. Biol Med., **150**, 780, 1975.

Asanuma H, Kakaza M, Shibata M, Hishiya T, Komiyama M, Chem. Comm., 1971, 1997.

Asanuma H, Hishiya T, Komiyama M, Adv. Mat., **14**, 1019, 2000.

Asanuma H, Kakaza M, Shibata M, Hishiya T, Komiyama M, Supra. Sci., **5**, 417, 1998.

Baille W. E, Huang W. Q, Nichifor M, Zhu X. X, J. M. S. Pure Appl. Chem., **A37**, 677, 2000.

Belokon Y, Taranov V. I, Savel'eva T. F, Belikov V. M, Macromol. Chem., **181**, 2183, 1980.

Belokon Y, Taranov V. I, Savel'eva T. F, Lependina O. L, Timofeyeva G. I, Belikov V. M, Macromol. Chem., **183**, 1921, 1982.

Benrebouh A, Avoce D, Zhu X. X, Polymer, **42**, 4031, 2001.

Benson G. M, Alston D. R, Hickey D. M. B, Jaxa-Chamiec A. A, Whittaker C. M, Haynes C, Glen A, Blanchard S, Cresswell S. R, J. Pharm. Sci., **86**, 76, 1997.

Bergen S. S, Itallie T. B, Tennent D. M, Schell W. H, Proc. Soc. Exp. Biol. Med, **102**, 676, 1959.

Bergeron R. J, Cycloamylase-Substrate Binding, In J. L Wood, J. E. D. Davies and D. D. MacNicol [eds.], Inclusion Compounds, Academic Press, London, Vol. 3, PP. 443, 1984

Bilheimer D. W, Grundy S. M, Brown M. S, Goldstein J. L, Proc. Natl. Acad. Sci., USA, **80**, 4124, 1983.

Bonnet P, Jaime C, Morin-Allory L, J. Org. Chem., **66**, 689, 2001.

Bonnet P, Jaime C, Morin-Allory L, J. Org. Chem., **67**, 8602, 2002.

Boonpangrak S, Prachayasittikul V, Bulow L, Ye L, J. Appl. Poly. Sci., **99**, 1390, 2006.

Borzatta V, Cristofori M, Brazzi A, Eur. Pat. Appl., 12804, 1980.

Breslow R, Chung S, J. Am. Chem. Soc., **112**, 9659, 1990.

Breslow R, Greenspoon N, Guo T, Zarzyeki R, J. Am. Chem. Soc., **111**, 8296, 1989.

Breslow R, Halfon S, Zhanh B, Tetrahedron, **51**, 377, 1995.

Breslow R, Zhang B, J. Am. Chem. Soc., **118**, 8495, 1996.

Chad C. H, Mathew J. B, William H. B, Randall S. H, Mandeville W. H, Petersen J. S, Polomoscanik S. C, Sacchiro R. J, Chen X, Dhal P. K, Macromolecules, **34**, 1548, 2001.

Chen J. P, Ferment J, Bioengg., **70**, 199, 1990a.

Chen J. P, Hoffman A. S, Biomaterials, **11**, 631, 1990b.

Choi Y. H, Yang C. H, Kim H. W, Jung S, J. Incl. Pheno. Macro. Chem., **39**, 71, 2001.

Ciardelli G, Borrelli C, Silvestri D, Cristallini C, Barbani N, Giusti P, Biosensors and Bioelectronics, **21**, 2329, 2006.

Class S. D, J. Pharm. Sci., **80**, 128, 1991.

Claudy P, Germain P, Letoffe J M, Bayol A, Bosserdet P, Gonzalez B, Colloque Bilan Aliment 2000, Paris 29-30 Janvier 1990.

Claudy P, Letoffe J. M, Germain P, Bastide J. P, Bayol A, Blasquez S, Rao R. C, Gonzalez B, J. Therm. Anal., **37**, 2497,1991.

Cook D. A, Godfrey J. C, Schneider D. L, Rubinfeld J, Ger Offen, 2822546, 1978.

Davidson L, Blencowe A, Drew M G B, Freebairn K. W, Hayes W, J. Mater. Chem., **13**, 758, 2003.

Davidson L, Hayes W, Curr. Org. Chem., **6**, 265, 2002.

DePalma R. G, Bellon E. M, Kotetsky S, Schnider D. L, Exp. Mol. Pathol., **31**, 423, 1979.

Dhal P. K, Arnold F. H, J. Am. Chem. Soc., **113**, 7417, 1991.

Dhal P. K, Arnold F. H, Macromolecules, **25**, 7051, 1992.

Dhal P. K, Vidyasankar S, Arnold F. H, Chem. Mater., **7**, 154, 1995

Donnelley R and Sons Co, The Dow Chemical Company, Dowex: Ion-Exchange, Chapter 1, Principles of Ion Exchange, Lakeside Press, Chicago, 1958.

Drugs of the Future, **18**, 15, 1993.

Dublin P. L, Bock J, Davies R. M, Schulz D. N, Thies C, In Macromolecular Complexes in Chemistry and Biology, Springer-Verlag, Berlin, Germany, 1994.

Edwards H, Thomas J. K, Carbo. Res., **65**, 173, 1978.

Egawa Y, Shimura Y, Nowatari Y, Aiba D, Juni K, Inter. J. Pharm., **293**, 165, 2005.

Eggert, M, Baltes, T, Garret-Flaudy F, Freitag, R, J. Chro., **827**, 269, 1998.

Ekberg B, Mosbach K, Trends. Biotech., **7**, 92, 1989,

Fokke V, Chantal M. B, Erik D, Bianca H. M. R, Martinus C, Feiters J, Engbersen F. J, David N. R, Roeland J, Nolte M, Tet. Lett., **35**, 1773, 1994.

Frijlink H. W, Eissens A. C, Hefting N. R, Poelstra K, Lerk C. F, Meijer D. K, Pharm. Res., **8**, 9, 1991.

Fromming K, Fridrich R, Mehnert W, Eur. J. Pharm. Biopharm., **39**,148, 1993.

Fujii Y, Matsutani K, Kikuchi K, J. Chem. Soc. Chem. Comm., 415, 1985.

Fujita K, Ejima S, Imoto T, J. Am. Chem. Soc., 1277, 1984.

Galaev I. Y, Gupta M. N, Mattiassson B, Chemtech., December 19, 1996.

Galaev I. Y, Mattiasson B, Enzyme Microb. Technol., **15**, 354, 1993.

Galaev I. Y, Mattiasson B, Biotechnol. Bioengg., **41**, 1101, 1993a.

Galaev I. Y, Mattiasson B, Biotech. Tech., **6**, 353, 1992.

Gaoming W, Brown G. R, St-Pierre L. E, Langmuir, **12**, 466, 1996.

Ghosh S, Mattiasson B, Isolation and Purification of Proteins in Thermostability of Enzymes, Gupta M. N. Ed., Springer-Verlag, Germany and Narosa Publishers, India, PP 44-73, 1993.

Gisser K. R. C, Geselbracht M. J, Capellari A, Hunsberger L, Ellis, A. B, Perepezko J, Lisensky G. C, J. Chern. Edu., **71**, 334, 1994.

Grier N, Shen T. Y, Wagner A. F, US 4205064, 1980.

Guoqiang D, Lali A, Kaul R, Mattiasson B, J. Biotechnol., **37**, 23, 1994.

Gupta M. N, Mattiasson, B. Affinity Precipitation in Highly Selective Separations in Biotechnology G. Street ed., Blackie Academic and Professional, Glasgow, UK, PP. 7-33, 1994.

Harada A, Furue M, Nozakura S, Macromolecules, **10**, 676, 1977.

Harada A, Masaoki F, Shun-ichi N, Poly. J., **12**, 29, 1980.

Hashim S. A, Itallie T. B, J. Am. Med. Assoc., **192**, 289, 1965.

Haskard C. A, Easton C. J, May B. L, Lincoln S. F, J. Phys. Chem., **100**, 1457, 1996.

Havel R. J, Kane J. P, Ann. Rev. Pharm., **13**, 287, 1973.

Hishiya T, Shibata M, Kakazu M, Asanuma H, Komiyama M, Macromolecules, **32**, 2265, 1999.

Hishiya T, Asanuma H, Komiyama M, Poly. J., **35**, 440, 2003.

Hishiya T, Asanuma H, Koiyama, J. Am. Chem. Soc., **124**, 570, 2002.

Hollas M, Chung M, Adams J, J. Phy. Chem. B, **102**, 2947, 1998.

Hollas M, Chung M, Jorg A, J. Phy. Chem. B, **102**, 2947, 1998a.

Hollas M, Chung M, Jorg A, Poly. Prep., **39**, 780, 1998b.

Holtmeier H. J, Cholesterin-Zur physiologie, Pathophysiologie and KliniL, Springer-Verlag, Berlin, 1996.

Homma Y, Goto Y, Okajima S, Handa S, Nakaya N, Takeuchi I, Koizumi K, Kawamura Y, Sekita T, Hottori S, Tamachi H, Watanabe T, Miyamoto K, Namiki M, Shima Y, Ozawa H, Nutr. Metab. Cardio. Dis., **6**, 211, 1996.

Hoshino K, Taniguchi M, Kitao T, Morohashi S, Sasakura T, Biotechnol. Bioeng., **60**, 568, 1998.

Hwang C, W. Lee, J. Chro. A, **962**, 69, 2002.

Idziak I, Gravel D, Zhu X. X, Tet. Let., **40**, 9167, 1999.

Ilingsworth D. R, Bacon S, Am. J. Cardiol., **60**, 336, 1987.

Irwin J. A, Tipton K. F, Essays in Biochem., **29**, 137, 1995.

Irwin J. A, Tipton K. F, Meth. Mole. Bio., **59**, 217, 1996.

Jansen G. R, Zanetti M. E, J. Pharm. Sci., **54**, 863, 1965.

Jaxa-Chamiec A. A, Hickey D. M. B, Shah P. V, EP402062, 1990.

Jorg A, Hollas M, Chung M, Macromol. Symp., **142**, 159, 1999.

Kane J. P, Malloy M, J. Med. Clin. North Am., **66**, 537, 1982.

Kane J. P, Malloy M. J, Tun P, Phillips N. R, Freedman D. D, Williams M. L, Rowe J. S, Havel R. J. N, Engl. J. Med., **304**, 251, 1981.

Kempi V, Linden W, Int. Z Klin Pharmakol. Ther. Toxikol, **4**, 424, 1971.

Kobayashi K, Hirata O, JP07126175, 1995a.

Kobayashi K, Hirata O, CA12374918K, 1995b.

Kondo H, Nakatani H, Hiromi K, J. Biochem., **79**, 393, 1996.

Kugimiya A, Kuwada Y, Takeuchi T, J. Chro. A, **938**, 131, 2001.

Kumar A, Galaev I. Y, Mattiasson B, Bioseparation, **7**, 129, 1998.

Kusumoto Y, Chem. Phys. Let., **136**, 535, 1987.

Kusumoto Y, Shizuka M, Chem. Phys. Let., **125**, 64, 1986.

Lauterburg B. H, Dickson E. R, Pineda A. A, Carlson G. L, Taswell H. F, J. Lab. Clin. Med., **94**, 585, 1979.

Manes J. D, Schnieder D. L, J. Lipid Res., **12**, 376, 1971.

Martel B, Leckchiri Y, Pollet A, Morcellet M, Eur. Poly. J., **31**, 1083, 1995.

Mattiasson B, Dainyak M. B, Galaev I. Y, Poly. Plast. Tech. Engg., **37**, 303, 1998.

Mattiasson B, Olsson U, Senstad C, Kaul R, Ling T. G. I, Proc. 4th European Congress on Biotechnology, Vol.4, Edited by O. M. NeijsseI, RR Van der Meer and KC. A. M. Luyben, Elsevier Science Publishers, B.V, Amersterdam, PP. 667-676, 1987.

Mattiasson B, Ramstorp M, J. Chro., **263**, 323, 1984.

Monji N, Hoffman A. S, Appl. Biochem. Biotech., **14**, 107, 1987.

Mosbach K, Ramstrom O, Technology, **14**,163,1996.

Mosbach K, Trends. Biochem. Sci., **19**, 9, 1994.

Natalia P, Whitcombe M. J, Vulfson E. N, J. Appl. Poly. Sci., **77**, 1851, 2000.

Nesanas N, Lou J, Breslow R, Bio.Medi. Let., **10**, 1931, 2000.

Nguyen A. L, Luong J. H. T, Biotech. Bioengg., **34**, 1186, 1989.

Nichifor M, Zhu X. X, Baille W, Cristea C, J. Pharm. Sci., **90**, 681, 2001a.

Nozaki T, Maeda Y, Ito K, Kitano H, Macromolecules, **28**, 522, 1995.

Nozaki T, Maeda Y, Kitano H, J. Poly. Sci. A, **35**, 1535, 1997.

Patonay G, Mae E R, Warner I M., Anal. Chem., **57**, 569, 1985.

Patonay G, Shapira A, Diamond P, Warner I. M, J. Phys. Chem., **90**, 1963, 1986.

Pena A, M, Ndou T. T, Anigbogu V. C, Warner I. M, Anal. Chem., **63**, 1018, 1991a.

Pena A. M, Ndou T. T, Zung J. B, Warner I. M, J. Phys. Chem., **95**, 3330, 1991b.

Perez N, Whitcombe M. J, Vulfson E. N, Macromolecules, **34**, 830, 2001.

Pierre L. E, Brown G. R, Wu G, CA2038852, 1992.

Pierre L. E, Brown G. R, Henning D. S, Bouvier M, US4593073, 1986.

Pierre L. E, Brown G. R, Tan Z, Class S. D, CA2063499, 1993.

Raghavan K. S, Chang R. K, Pang J, Figuly G. D, Hussain M. A, Pharm. Dev. Tech., **2**, 233, 1997.

Ramaswamy R, Divakar S. J. of Incl. Pheno.Mole. Reco. in Chem., **30**, 253, 1998.

Ramirez-Vick J. E, Garcia A. A, Separation and Purification Methods, **25**, 85, 1997.

Ramstrom O, Andersson L. I, Mosbach K, J. Org. Chem., **58**, 7562, 1993.

Russell C. P, Christopher T. David S, Waldeck H, J. Am. Chem. Soc., **113**, 2325, 1991.

Schlenk H, Sand D, Tillotson J. A, US2827452, 1958.

Schneider D. L, Gallo D. G, Sarrett H. P, Proc. Soc. Exp. Biol. Med., **121**, 1244, 1966.

Schreiber M. A., Moyer K. L, Mueller B. J, Ramos M. A, Green J. S, White L, Hedgepeth W, Juliano K, Scull J. R, Hovsepian P. K, J of Pharm. Biomed. Anal., **25**, 343, 2001.

Sellergren B, Wieschemeyer J, Boos K. S, D. Seidel, Chem. Mater., **10**, 4037, 1998.

Sreenivasan K, J. Appl. Poly. Sci., **80**, 2795, 2001a.

Sreenivasan K, Poly. Int., **42**, 169, 1997.

Sreenivasan K, Analy. Let., **34**, 307, 2001c.

Sreenivasan K, J. Appl. Poly. Sci., **70**, 15, 1998a.

Sreenivasan K, J. Appl. Poly. Sci., **68**, 1863, 1998.

Sreenivasan K, J. Appl. Poly. Sci., **82**, 889, 2001b.

Sreenivasan K, J. Therm. Anal., **45**, 573, 1995.

Steven De F, Jan V S, Noel B, Frans C. Schryver D, Chem. Phys. Let., **249**, 46, 1996.

Szejli J, Osa T, "Comprehensive Supramolecular Chemistry", Pergamon, Oxford, Vol. 3, 1996.

Szejtli J, "Cyclodextrin Technology", Kluwer Academic Publisher, Dordrecht 1998.

Terinent D. M, Siegel H, Zaneth M. E, Kuron G. W, Ott W. H, Wolf F. J, J. Lipid Res., **1**, 469, 1960.

Thale M, Faergeman O. P, Scand J, Gastroenterol., **13**, 353, 1978.

Tkahashi M, Sarwal A. N, Raicht R. F, Cohen B. I, Lipids, **15**, 434, 1980.

Umeno D, Mori T, Maeda M, Chem. Comm., 1433, 1998.

Vaidya A. A, Lele B. S, Kulkarni M. G, Mashelkar R. A, Biotech. Bioengg., **64**, 418, 1999.

Wagner A. F, Ger Offen, 2806707, 1978.

Wagner A. F, Grier N, Shen T. Y, US4161581, 1979.

Wang S, Xu J, Tong Y, Wang L, Chaobin He, Poly. Int., **54,** 1268, 2005.

Wen J. S, Hedges A. R, J. Mol. Sci. Pure Appl. Chem., A, **33**, 673, 1996.

Whitcombe M. J, Rodriguez E, Villar P, Vulfson E. N, J. Am. Chem. Soc., **117**, 7105, 1995.

Williams O. R, Mahaguna V, Sriwongjanya, Eur. J. Pharm. Biopharm., **46**, 355, 1998.

Wulff G, Angew Chem., Int. Ed. Engl., **34**, 1812, 1995.

Wulff G, In Polymeric Reagents and Catalysts [Ford, W.T. ed], ACS Symposium Series, American Chemical Society, Washington D. C, **308**, 186, 1985.

Wulff G, Sahan A, Angew. Chem. Int. Ed. Engl., **11**, 341, 1972.

Wulff G, Akelah A, Macromol. Chem., **179**, 2647, 1978.

Xu W, Demas J. N, DeGraff B. A, Whaley M, J. Phys. Chem., **97**, 6546, 1993.

Yang Z, Breslow R. Tet. Let., **38**, 6171, 1997.

Zhang B, Breslow R, J. Am. Chem. Soc., **115**, 9353, 1993.

Zhong N, Byun H. S, Bittman R, Tet. Let., **42**, 1839, 2001.

Zhu X. X, Brown G. R, St-Pierre L. E, J Pharm Sci., **81**, 65, 1992.

Zhu X. X, Brown G. R, St-Pierre L. E, J. Macromol. Pure Appl. Chem., A, **29**, 711, 1992.

Zhu X. X, Brizard F, Piche J, Yim C. T, Brown G. R, J. Colloid Interface Sci., **232**, 282, 2000.

Zhu X. X, Moskova M, Denike J. K, Polymer, **37**, 493, 1996.

Chapter 3:

Akimitsu K, Yasuyuki K, Toshifumi T, J. Chro. A, **938**, 131, 2001.

Asanuma H, Hishiya T, Komiyama M, Adv. Mater., **14**, 1019, 2000.

Asanuma H, Kakaza M, Shibata M, Hishiya T, Komiyama M, Chem. Comm., 1971, 1997.

Davidson L, Blencowe A, Drew M. G. B, Freebairn K. W, Hayes W, J. Mater. Chem., **13,** 758, 2003.

Davidson L, Hayes W, Curr. Org. Chem., **6**, 265, 2002.

Hishiya T, Shibata M, Kakazu M, Asanuma H, Komiyama M, Macromolecules, **32**, 2265, 1999.

Holtmeier H., Cholesterin-Zur Physiologie, Pathophysiologie and KliniL: Springer-Verlag, Berlin, 1996.

Huval C. C, Mathew J. B, William H. B, Randall S. H, Mandeville W, Petersen J. S, Polomoscanik S. C, Sacchiro R. J, Chen X, Dhal P. K, Macromolecules, **34**, 1548, 2001.

Hwang C, Lee W, J. Chro. A, **962**, 69, 2002.

Mayes A. G, Mosbach K, Trends Anal. Chem., **16**, 321, 1997.

Natalia P, Whitcombe M. J, Vulfson E. N, J. Appl. Poly. Sci., **77**, 1851, 2000.

Perez N, Whitcombe M. J, Vulfson E. N, Macromolecules, **34**, 830, 2001.

Perrin W. L, Armarego D. D. Purification of Laboratory Chemicals Perrin, D. R. Oxford Publication 1996.

Sellergren B, Trends Anal. Chem., **16**, 310, 1997.

Sellergren B, Wieschemeyer J, Boos K. S, Seidel D, Chem. Mater., **10**, 4037, 1998.

Shea K. J, Trends Poly. Sci., **2**, 166, 1994.

Sreenivasan K, J. Appl. Poly. Sci., **68**, 1863, 1998.

Sreenivasan K, J. Appl. Poly. Sci., **82**, 889, 2001.

Sreenivasan K, J. Appl. Poly. Sci., **70**, 15, 1998.

Sreenivasan K, Poly. Int., **42**, 169, 1997.

Sreenivasan K, J. Appl. Poly. Sci., **80**, 2795, 2001.

Whitcombe M. J, Rodriguez E, Villar P, Vulfson E. N, J. Am. Chem. Soc., **117**, 7105, 1995.

Wulff G, Angew. Chem. Int. Ed. Engl., **34**, 1812, 1995.

Zhong N, Byun H. S, Bittman R, Tet. Let., **42**, 1839, 2001.

Chapter 4:

Baille W. E, Huang W. O, Nichifor M, Zhu X. X, J. Macro. Sci. A, **37**, 677, 2000.

Benson G. M, Alston D. R, Hickey D. M. B, Jaxa-Chamiec A. A, Whittaker C. M, Haynes C, Glen A, Blanchard S, Cresswell S. R, J. Pharm. Sci., **86**, 76, 1997.

Bergthaller P, US432944, 1982.

Born M, Koch T, Ritter H, Acta Poly., **45**, 68, 1994.

Born M, Ritter H, Angew. Chem., **107**, 342, 1995.

Born M, Ritter H, Macromol. Rapid Comm., **12**, 471, 1991.

Borzatta V, Cristofori M, Brazzi A, Eur. Pat. Appl., 12804, 1980.

Christensen F. N, Davis S. S, Hardy J. G, Taylor M. J, Whalley D. R, Wilson C. G, J. Pharm. Pharmacol., **37**, 91, 1985.

Class S. D, J. Pharm. Sci., **80**, 28, 1991.

Cook D. A, Godfrey J. C, Schneider D. L, Rubinfeld J, Ger Offen, 2822546, 1978.

Fears R, Brown R, Ferres H, Grenier F, Tyrrell A. W. R, Biochem. Pharm., **40**, 2029, 1990.

Fordtran J. S, Locklear T. W, Am. J. Dig. Dis., **11**, 503, 1966.

Gore M. A, Karmalkar R. N, Kulkarni M. G, J. Chro. B, **804**, 211, 2004.

Grier N, Shen T. Y, Wagner A. F, US4205064, 1980.

Harada A, Acta Poly., **49**, 2216, 1998.

Harada S, Kiyoshi S, US4528347, 1985.

Homma Y, Goto Y, Okajima S, Handa S, Nakaya N, Takeuchi I, Koizumi K, Kawamura Y, Sekita T, Hottori S, Tamachi H, Watanabe T, Miyamoto K, Namiki M, Shima Y, Ozawa H, Nutr. Metab. Cardio Dis., **6**, 211, 1996.

Huval C. C, Holmes-Farley S. R, Mandeville W. H, Sacchiero R, Dhal P. K, Eur. Poly. J., **40**, 701, 2004.

Huval C. C, Mathew J. B, William H. B, Randall S. H, Mandeville W. H, Petersen J. S, Polomoscanik S. C, Sacchiro R. J, Chen X, Dhal P. K, Macromolecules, **34**, 1548, 2001.

Jaxa-Chamiec A. A, Hickey D. M. B, Shah P. V, EP402062, 1990.

Jeromin J, Ritter H, Macromol. Rapid Comm., **19**, 377, 1998.

Johns W. H, Bates T. R, J. Pharm. Sci., **59**, 329, 1970.

Kobayashi K, Hirata O, CA12374918K, 1995b.

Kobayashi K, Hirata O, JP0712675, 1995a.

Kulkarni M. G, Karmalkar R. N, Satav S. S, PCT/IB03/03593, 2004a.

Kulkarni M. G, Karmalkar R. N, Satav S. S, PCT/IB03/05070, 2004b.

LaRosa J, Cardiology, **76**, 55, 1989.

Lele B. S, Gore M. A. Kulkarni M. G, Synth. Comm., **29**, 1727, 1999.

Li Z, Day M, Ding J, Faid K, Macromolecules, **38**, 2620, 2005.

Mandeville W. H., Goldberg D. I., Curr. Pharm. Res., **3**, 15, 1997.

Matsui J, Minamimura N, Nishimoto K, Tamaki K, Sugimoto N, J. Chro. B, **804**, 223, 2004.

Matsui J, Tamaki K, Sugimoto N, Analy. Chim. Acta, **466**, 11, 2002.

Nau P. P, Kritchevsky D, The Bile Acids, Vol. 2, Physiology and Metabolism. New York: Plenum Press, 1973.

Nichifor M, Zhu X. X, Baille W, Cristea D, Carpov A, J. Pharm. Sci., **90**, 681, 2001.

Parkinson T. M, Gundersen K, Nelson N. A, Atherosclerosis, **11**, 531, 1970.

Perrin D. D, Armarego W. L. F, Purification of Laboratory Chemicals, Oxford Publication, 1996.

Pierre L. E, Brown G. R, Wu G, CA2038852, 1992.

Pierre L. E, Brown G. R, Henning D. S, Bouvier M, US4593073, 1986.

Polli J. E, Amidon G. L, J. Pharm. Sci., **84**, 55, 1995.

Raghavan K. S, Chang R. K, Pang J, Figuly G. D, Hussain M. A, Pharm. Dev. Tech., **2**, 233, 1997.

Regen S. L, Stedronsky E. R, Zhang L, Janout V, Macromolecules, **31**, 5542, 1998.

Schneider H. J, Hacket F, Rudiger V, Ikeda H, Chem. Rev., **98**, 1755, 1998.

Satav S. S, Karmalkar R. N, Kulkarni M. G, Mulpuri N, Sastry G. N, J. Am. Chem. Soc., **128**, 7752, 2006.

Schreiber M. A, Moyer K. L, Mueller B. J, Ramos M. A, Green J. S, White L, Hedgepeth W, Juliano K, Scull J.R, Hovsepian P. K, J. Pharm. Biomedi. Anal., **25**, 343, 2001.

Shepherd J, Cardiology, **76**, 65, 1989.

Stedronsky E. R, Biochim. Biophys. Acta., **210**, 255, 1994.

Szejli J, Osa T, "Comprehensive Supramolecular Chemistry", Pergamon, Oxford Vol. 3, 1996.

Szejtli J, "Cyclodextrin Technology", Kluwer Academic Publisher, Dordrecht 1998.

Terinent D. M, Siegel H, Zaneth M. E, Kuron G. W, Ott W. H, Wolf F. J, J. Lipid Res., **1**, 469, 1960.

Thale M, Faergeman O, Scand J, Gastroentreol., **13**, 353, 1978.

The Dow Chemical Company, Dowex: Ion-Exchange, Chapter 1, Principles of Ion Exchange, Lakeside Press, R. Donnelley and Sons Co., Chicago, 1958.

Tkahashi M, Sarwal A. N, Raicht R. F, Cohen B. I, Lipids, **15**, 434, 1980.

Wagner A.F, Ger Offen, 2806707, 1978.

Wenz G, Angew. Chem., **106**, 851, 1994.

Wu G, Brown G. R, St-Pierre L. E, Langmuir, **12**, 466, 1996.

Zhu X. X, Brown G. R, Pierre L. E, J. Macromol. Pure Appl. Chem. A, **29**, 711, 1992.

Zhang L, Janout V, Renner J. L, Uragami M, Regen S. L, Bioconjugate Chem., **11**, 397, 2000.

Chapter 5:

Asanuma H, Kakazu M, Shibata M, Hishiya T, Komiyama M, Supramol. Sci., **5**, 417, 1998.

Asanuma H, Kakazu M, Shibata M, Hishiya T, Komiyama M, Chem. Comm., 1971, 1997.

Asanuma H, Kakazu M, Shibata M, Hishiya T, Komiyama M, Macromolecules, **32,** 2265, 1999.

Bonnet P, Jaime C, Morin-Allory L, J. Org. Chem., **67**, 8602, 2002.

Boutris C, Chatzi E, Kiparissides C, Polymer, **38**, 2567, 1997.

Breslow R, Greenspoon N, Guo T, Zarzyeki R, J. Am. Chem. Soc., **111**, 8296, 1989.

Breslow R, Halfon S, Zhanh B, Tetrahedron, **51**, 377, 1995.

Breslow R, Zhang B, J. Am. Chem. Soc., **118**, 8495, 1996.

Cao J, Zhiying P, Kexue S [Beijing], **31**, 1794, [Chinese], 1996.

Choi Y. H, Yang C. H, Kim H. W, Jung S. J, Incl. Pheno. Macro. Chem., **39**, 71, 2001.

Claudy P, Letoffe J. M, Germain P, Bastide J. P, Bayol A, Blasquez S, Rao R. C, Gonzalez B, J.Therm. Analy., **37**, 2497, 1991.

Dubois M, Gilles K. A, Hamitton J. K, Rebers P. A, Smith F, Anal. Chem., **28**, 350, 1956.

Feil H, Bae Y. H, Feijen J, Kim S. M, Macromolecules, **26**, 2496, 1993.

Fersht A, Enzyme Structure and Mechanism; 2ed; W. H Freeman and Company; New York, 1985.

Frenkel M, Marsh M. N, TRC Data Series Spectral Data for Steroids, College Station. Thermodynamics Research Center, Texas TX, PP 386, 1994.

Frijlink H. W, Eissens A. C, Hefting N. R, Poelstra K, Lerk C. F, Meijer D. K, Pharm. Res., **8**, 9, 1991.

Fromming K, Fridrich R, Mehnert W, Eur. J. Pharm. Biopharm., **39**, 148, 1993.

Fujita K, Ejima S, Imoto T, J. Am. Chem. Soc., 1277, 1984.

Fujita K, Tahara T, Imoto T, Koga T, J. Am. Chem. Soc., **108**, 2030, 1986.

Galaev I. Y. U, Mattiasson B, Biotechnol Bioengg., **41**, 1101, 1993.

Harada A, Furue M, Nozakura S, Macromolecules, **9,** 701, 1976.

Harada A, Furue M, Nozakura S, Macromolecules, **10**, 676, 1977.

Hollas M, Chung M, Adams J, J. Phy. Chem. B., **102**, 2947, 1998.

Jayaraman, J, In Laboratory Manual in Biochemistry, Wiley Eastern Ltd, New Delhi. 1985.

Leckchiri Y, Martel B, Morcellet M, Pollet A, Eur. Polym. J., **131**, 1083, 1995

Lele B. S, Gore M. A, Kulkarni M. G, Synth. Comm., **29**, 1727, 1999.

Nesanas N, Lou J, Breslow R, Bio.Med. Let., **10** 1931, 2000.

Notes, J. Poly. Sci. A-1, **8**, 783, 1970.

Nozaki T, Maeda Y, Ito K, Kitano H, Macromolecules, **28**, 522, 1995.

Oakenfull D, George S, Singh G, Laurence M, PCT Int. Appl. W09111114 A1, 1991.

Ogura N, JP07155140 A2, 1995.

Perrin W. L, Armarego D. D. Purification of Laboratory Chemicals Perrin, D.R. Oxford Publication 1996.

Rajendraprabhu R, Muthuraman S, 'Notes on Common Clinical Biochemical Techniques' 1980.

Ramaswamy R, Divakar S, J. of Incl. Pheno.Mole. Reco. Chem., **30**, 253, 1998.

Risch S. J, Reineccius G. A, Encapsulation and Controlled Release of Food, [Chapter 6], 1995.

Seo T, Taketoshi K, Iijima T, Macro. Chem., **188**, 2071, 1987.

Siegel B, Breslow R, J. Am. Chem. Soc, **97**, 6869, 1975.

Sigma Library of FT-IR Spectrum, 1st edn., Sigma, St. Louis, MO, pp 1207, 1986.

Sreenivasan K, Analy. Let., **34**, 307, 2001.

Ueno A, Breslow R, Tet. Let., **23**, 3451, 1982.

Wallimann P, Marti T, Fuhrer A, Diederich F, Chem. Rev., **97**, 1567, 1997

Yang Z, Breslow R, Tet. Let., **38**, 6171, 1997

Chapter 6:

Bonnet P, Jaime C, Morin-Allory L, J. Org. Chem., **66**, 689, 2001.

Bonnet P, Jaime C, Morin-Allory L, J. Org. Chem., **67**, 8602, 2002.

Boutris C, Chatzi E, Kiparissides C, Polymer, **38**, 2567, 1997.

Breslow R, Zhang B, J. Am. Chem. Soc.,**118**, 8495, 1996.

Choi Y. H, Yang C. H, Kim H. W, Jung S, J. Incl. Pheno. Macro. Chem., **39**, 71, 2001

Dong, D. C, Winnik, M. A, Can. J. Chem., **62**, 2560, 1984.

Dubois M, Gilles K. A, Hamitton J. K, Rebers P. A, Smith F., Anal. Chem., **28**, 350, 1956.

Edwards H, Thomas J. K, Carbohydrate Research, **65**, 173, 1978.

Fokke V, Chantal M. B, Erik D, Bianca H. M. R, Martinus C. F, Johan F. J. E, David N. Roeland R, J. M. N, Tete. Lett., **35**, 1773, 1994.

Fromming K. H, Szejtli J, Cyclodextrins in pharmacy, 1st ed.; Kluwer Academic Publishers: Dordrecht, Chapter 1, 1994.

Fujita K, Ejima S, Imoto T, J. Am. Chem. Soc., 1277, 1984

Hamai, S, J. Phys. Chem., **93**, 2074, 1989.

Harada A, Masaoki F, Shun-ichi N, Polymer J., **12**, 29, 1980.

Hashimoto S, Thomas J. K, J. Am. Chem. Soc., **107**, 4655, 1985.

Haskard C, A, Easton C. J, May B. L, Lincoln S. F, J. Phys. Chem., **100**, 14457, 1996.

Herkstroeter W. G, Martic P. A, Evans T. R, Farid S, J. Am. Chem. Soc., **108**, 3275, 1986

Hollas M, Chung M, Jorg A, J. Phy. Chem. B, **102**, 2947, 1998a.

Hollas M, Chung M. A, Adams J, Polymer Preparations, **39**, 780, 1998.

Jorg A, Hollas M, Chung M, Macromol. Symp., **142**, 159, 1999

Kalyanasundaram K, Thomas J. K, J. Am. Chem. Soc., **99**, 2039, 1977.

Karpovich, D. S, Blanchard G. J, J. Phys Chem., **99**, 3951, 1995.

Kusumoto Y, Chemical Physics Letters, **136**, 535, 1987.

Kusumoto Y, Shizuka M, Satake I, Chemical Physics Letters, **125**, 64, 1986.

Leckchiri Y, Martel B, Morcellet M, Pollet A, Eur. Polym. J., **131**, 1083, 1995.

Lele B. S, Gore M. A, Kulkarni M. G, Synthetic Communication, **29**, 1727, 1999.

Lewis E. A, Barkley J, St. Pierre T, Macromolecules, **14**, 546, 1981.

Li S, Purdy W. C, Chem Rev., **92** 1457, 1992.

Munoz. A de la, Ndou T., Zung J.B, and Warner I. M., J. Phys. Chem., **95**, 3330, 1991.

Nakajima A, J. Lumin., **15**, 277, 1977.

Nakajima, A. Spectrochim. Acta, **39A**, 913, 1983.

Notes, J. Poly. Sci. A-1, **8,** 783, 1970.

Nozaki T, Maeda Y, Ito K, Kitano H, J of Polymer Sci. Part A, **35**, 1535, 1997.

Nozaki T, Maeda Y, Ito K, Kitano H, Macromolecules, **28**, 522, 1995.

Patonay G, Shapira A, Diamond P, Warner. I. M, J. Phys. Chem., **90**, 1963, 1986.

Perrin W. L, Armarego D. D. Purification of laboratory chemicals Perrin: D.R. Oxford publication, 1996.

Ruebner A, Stattom G. L, James M. R, Macromol. Chem. Phys., **201**, 1185, 2000.

Russell C. P, Christoper T. S, David H. W, J. Am. Chem. Soc, **113**, 2325, 1991.

Schwarz F. P, J. Chem. Eng. Data, **22**, 273, 1977.

Seo T, Taketoshi K, Iijima T, Macromolecule Chem., **188**, 2071, 1987.

Steven De F, Jan V S, Noel B, Frans C, Schryver D, Chem. Phys. Lett., **249**, 46, 1996.

Szejli J, Osa T, "Comprehensive Supramolecular Chemistry", Pergamon, Oxford, Vol. 3, 1996.

Szjtli J, Cyclodextrins and their inclusion complexes, Academiai Kiado, Budapest, 1982.

Udachin K. A, Ripmeester J. A, J. Am. Chem. Soc., **120**, 1080, 1998.

Ueno A, Breslow R, Tetrahedron Letters, **23**, 3451, 1982.

Xu Wenying, Demas J. N, DeGraff B. A, Whaley M, J. Phys. Chem., **97**, 6546, 1993.

Zung J. B, Nelson G, Warner I. M, J. Inclusion Phenom., **6**, 277, 1988.

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- Senior Research Fellowship by Council of Scientific and Industrial Research (CSIR), Government of India, India. 2001 to 2004.
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Research Experience

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Preamble of Thesis

1. Enhanced Capacities and Selectivities for Cholesterol in Aqueous Media by Molecular Imprinting: Role of Novel Cross linkers

Molecularly imprinted polymers (MIPs) are being increasingly investigated as selective sorbents and catalyst. Selective sorption of cholesterol from aqueous medium using MIPs has been plagued by low capacity as well as low selectivity due to non specific hydrophobic binding. Here we report synthesis of monomers and cross linkers bearing cholesterol for the synthesis of MIPs. Binding sites were created by hydrophobic interactions between cholesterol bearing monomers and template added during the polymerization. Imprinted polymers prepared using equivalent amounts of functional monomer and template exhibited enhanced capacity in aqueous medium.

The non specific binding was suppressed when cross linkers containing hydrophilic groups were used. The templating effect of cholesterol is clearly seen in the strong and selective retention of cholesterol as compared to stigmasterol and testosterone in case of imprinted polymers.

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2. Enhancing Active Site Utilization in Molecularly Imprinted Polymers :

A New Synthetic Approach

This paper presents a two stage approach for the synthesis of molecularly imprinted polymers [MIPs]. It involves selective polymerization of a divinyl monomer, methylene bis acrylamide with allylamine hydrochloride to yield a water soluble polymer which is subsequently crosslinked in the presence of the template sodium cholate. This leads to enhanced utilization of active sites from 33 to 84%, enhanced binding capacity as well as selectivity towards NaC as compared to sodium taurocholate during rebinding as compared to imprinted polymers prepared by conventional simultaneous polymerization, imprinting and crosslinking technique. The approach is generic one and can be exploited for the synthesis of molecularly imprinted polymers, which would offer, enhanced binding capacity and selectivity.

Communicating shortly.

US patent filled: NCL/NF-218/2005.

3. Enhancing Cholesterol-β-Cyclodextrin Binding: Role of Polymer Structures and Implications

Binding between cholesterol and β-cyclodextrin, polymer containing β-cyclodextrin, β-cyclodextrin dimers and polymer conjugates comprising β-cyclodextrin dimers were investigated. Four cyclodextrin containing monomer by varying spacer length and binding site were synthesized and characterized. Additionally three cyclodextrin dimers were synthesized varying spacer length between two cyclodextrin through different binding site. The binding constant between cholesterol and polymers containing β-cyclodextrin as a function of cyclodextrin content was studied. The results showed binding constant was increased with increasing β-cyclodextrin content. This indicates intermolecular stage wise binding between cyclodextrin and cholesterol at lower cyclodextrin content, whereas chelate type binding at higher content of cyclodextrin. Binding constant of dimers and dimer containing polymers was independent of the content of cyclodextrin dimer, which indicates that in case of dimers only

intramolecular chelate complexes formed hence binding constant remains constant.

Communicating shortly.

4. Complexation Between Pyrene and Cyclodextrin Polymers: Fluorescence Study

Cyclodextrins are increasingly being used for the encapsulation of drugs, synthesis of supramolecular complexes and recovery of biomolecules. The binding between cyclodextrin and the guest molecule plays a critical role in these processes and can be enhanced by conjugating cyclodextrin with the polymers. In order to investigate the role of degree of substitution [DS] on binding with the guest molecule, complexation between pyrene and β-cyclodextrin [CD] in aqueous solution has been investigated using fluorescence spectroscopy over a wide range of polymer concentrations. A stagewise formation of 1:1 [CD: pyrene] and intermolecular 2:1 complexes at low DS [below 5%] and intramolecular 2:1 chelate type complexes at higher DS [above 5%] was confirmed, the association constant increases with DS. On the other hand dimer CD and dimer CD conjugated polymers always formed 2:1 intramolecular chelate type complexes, irrespective of the DS and it is independent of DS.

Communicating shortly.

List of publications

1. Lele, B. S, Gore, M.A, Kulkarni, M.G.
Direct esterification of poly (ethylene glycol) with amino acid hydrochlorides.
Synthetic Communications, 29(10), 1727-1739 (1999).

2. B. S. Lele, M. A. Gore, M. G. Kulkarni
Friedel-Craft N-alkylation and N-acylation of acrylamide: A novel approach for synthesis of alkylacrylamides.
Journal of Applied Polymer Science, 73, 1845-1850 (1999).

3. M. A. Gore, R.N. Karmalkar, M.G.Kulkarni.
Enhanced Capacities And Selectivity for Cholesterol in Aqueous Media by
Molecular Imprinting: Role of Novel Cross linkers
J. of Chromatography B**,** 804 (1)**,** 211-221 (2004).

4. M. A. Gore and M. G. Kulkarni
Enhancing Active Site Utilization in Molecularly Imprinted Polymers: A New Synthetic Approach
Communicating shortly.

5. M. A. Gore and M. G. Kulkarni

Enhancing Binding of Cholesterol and β-Cyclodextrin Inclusion Complex: Role of Polymer Architecture

Macro 2002, Seventh National Conference of the Society for Polymer Science, India. Held on Dec. 9-11, 2002.

Communicating shortly.

6. M. A. Gore and M.G. Kulkarni

Complexation Between Pyrene and Cyclodextrin Polymer: Fluorescence Study International Seminar on Advances in Polymer Technology, India. Held on Jan 16-17, 2004

Communicating shortly.

Patent:

1. M. A. Gore and M. G. Kulkarni

Novel bile acid sequestrants and a process for the preparation thereof.

US Patent filled No.: NCL/NF 218/ 2005