Studies on Affinity Precipitation of Biomolecules Using Stimuli Sensitive Polymers

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Statement by the candidate

As required by the University ordinances 770 and 771.

I wish to state that the work embodied in this thesis titled **"Studies on Affinity Precipitation of Biomolecules Using Stimuli Sensitive Polymers"** forms my own contribution to the research work carried out under the guidance of **Dr. M. G. Kulkarni,** at National Chemical Laboratory, Pune. This work has not been submitted for any other degree of this or any other University. Whenever references have been made to previous works of other, it has been clearly indicated as such and included in the Bibliography.

Jayant Jagannath Khandare

Dr. Mohan Gopalkrishna Kulkarni
(Research Guide)
National Chemical Laboratory, Pune

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In the end I am glad to dedicate this thesis to my teachers, family and friends.

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Studies on Affinity Precipitation of Biomolecules Using Stimuli Sensitive Polymers

Introduction

Interactions between biomolecules and ligands play a critical role in medicine and biotechnology. Recovery of biomolecules in the pure form from a natural source and / or synthesized by a biotransformation route involves multiple steps and processes such as centrifugation, extraction, affinity chromatography and affinity ultrafiltration. Affinity based techniques are attractive as they provide higher selectivity and specificity and have emerged as an efficient bioseparation strategy for enzymes [Sii et al (1991)] and proteins [Senstad et al (1989)]. Affinity precipitation is a convenient bioseparation technique as it eliminates most of the drawbacks associated with affinity chromatography methods. Moreover, it offers ease of scale up, concentration and purification. The technique can be made into continuous operation process and the ligand used can be recycled. Affinity precipitation method usually consist of hetero bifunctional ligands coupled to the stimuli sensitive polymers e.g. poly *N*-isopropyl acrylamide (NIPA).

Polyvalent interactions involving proteins and carbohydrates play a critical role in biological systems. It has been realized that the polyvalent ligands bind more efficiently to the receptor than monovalent ligands [Sigal et al (1994)]. Ligand substrate interactions can thus be enhanced by designing polymeric affinity ligands which possess conformation for binding to the target biomolecule.

The present investigation was undertaken to design, synthesize and evaluate stimuli sensitive affinity polymers containing NAG for enhanced binding and recovery. Role of polymer composition, polyvalency and steric stabilization was also investigated. Molecularly imprinted polymers were also synthesized using lysozyme as a template. The molecularly imprinted polymers prepared in the presence of lysozyme exhibit enhanced binding than polymers prepared in the absence of lysozyme. The affinity precipitating polymers exhibiting optimum binding have been used for the recovery of lysozyme. The methodology can be replicated for other ligands e.g. inhibition of haemagglutination of host Red Blood Cells (RBC's) by influenza virus occurs through sialic acid ligands. The

polymeric NAG ligands investigated are stable, water soluble and are not hydrolyzed by lysozyme. The research work undertaken is presented in seven chapters and a brief outline of each is given below:

Chapter 1: Literature survey

This chapter provides an overview of various aspects of the stimuli sensitive affinity polymers especially, thermoprecipitating polymers. The effect of the monomer composition on phase separation is highlighted. The ligand-substrate binding is the key in biological processes. This has been illustrated with a discussion on protein carbohydrate interactions especially, the role of polyvalent ligands in enhanced interactions. Lastly, the advantages of designing tailored stimuli sensitive polymers containing target specific ligands is reviewed.

Chapter 2 : Objectives and scope of the work

The review of the literature reveals specific gaps. The objective of the present investigation is to bridge some of these gaps. While the objectives have been set in broader terms, the scope has been limited to illustrate the new methodologies proposed for enhanced binding with specific references to lysozyme as a biomolecule and NAG as the ligand.

Chapter 3: Experimental work : Designing of novel affinity precipitating polymers for biomolecules

This part deals with the design and synthesis of newer affinity thermoprecipitating polymers containing NAG. Spacer arm 6-Amino caproic acid (6-ACA) was incorporated into NAG monomer for enhanced access to the active site of lysozyme. Thermoprecipitating copolymers of Ac.NAG and NIPA; NIPA and methacryloyl NAG and NIPA and macromer were prepared. Monomer reactivity ratios were determined using Kelen-Tudos method to investigate their copolymerization behaviour and effect on copolymer structure. Block copolymers comprising varying NAG and NIPA sequences have been synthesized. Imprinted polymers are prepared in the presence of lysozyme as a template molecule and compared with the copolymers prepared in the absence of lysozyme. This chapter presents details of synthesis and characterization using IR, NMR, vapor pressure osmometry of various monomers, oligomers, macromer, random as well di and triblock polymers synthesized to evaluate the effect of polymer architecture on

substrate binding. It also describes various techniques such as enzyme inhibition, fluorescence spectroscopy used to evaluate the efficacy of the polymeric ligands.

Chapter 4: Results and discussion

The inhibition of lysozyme by NAG in the form of monomer, oligomer, macromer, copolymer and molecularly imprinted copolymer with *N*-isopropyl acrylamide was studied. Oligomers of NAG of varying molecular weights were evaluated for their efficacy. The oligomer of molecular weight 638 exhibited highest association constant (k_a = 5.3 x 10^5 M^{-1}) which was three orders of magnitude higher over NAG itself indicating the polyvalent effect. Binding between lysozyme and NAG is substantially enhanced in case of oligomers since the NAG units are in juxtaposition to one another. The copolymers of acryloyl NAG and NIPA prepared by the conventional free radical polymerization result in block copolymers as evidenced by the comonomer reactivity ratios. These copolymers exhibit substantially enhanced binding as compared to the copolymers of NIPA and methacryloyl NAG, in which methacryloyl NAG is randomly distributed along the polymer chain. Macromers comprising NAG form random copolymers with NIPA, yet exhibit enhanced binding with lysozyme. The competitive binding experiments in presence of Biebrich scarlet show that oligomers, macromers block copolymers and imprinted polymers containing NAG units in juxtaposition occupy the catalytically active site of lysozyme. The synthetic methodologies demonstrate alternative means of synthesizing polymers having tailored ligand architecture, which inhibit lysozyme far more efficiently than NAG. A detailed discussion of these findings is provided in this chapter.

Chapter 5: Recovery of lysozyme :

Since the objective of undertaking binding studies was to tailor polymer structures for enhanced binding to lysozyme for recovery, the recovery of lysozyme using tailored thermoprecipitating polymers was undertaken. The specific activity of lysozyme recovered using block copolymers was maximum (92 %) whereas for copolymers it was 73 %. These values are higher than any reported in the literature.

Chapter 6 : Summary and conclusions

This chapter summarizes the important conclusions of our studies. An attempt is made to enhance substrate-ligand interactions by designing polyvalent ligands. The role

of polymer architecture influences the binding and inhibition of lysozyme. Binding constants (K_b) was increased whereas the inhibition constants (I_{50}) decreased substantially for the oligomers, macromer, block copolymers and imprinted polymers containing polyvalent NAG. It also highlights the importance of tailoring ligand sequences along the polymer chain for enhancing the interactions. The investigations have resulted in the filing of six US patent applications as listed below and the publications based on this work are under preparation.

Chapter 7: Directions for further work

This investigation has helped to highlight the importance of tailoring polymer architecture for enhanced substrate binding and provided structural evidence in support of the results obtained and the explanations offered. It has also brought to attention new synthetic strategies and experimental techniques which can be exploited for further investigation into these and related areas. Polyvalent ligands appear promising for enhancing interactions and the bioseparation. Moreover the polyvalent ligands such as sialic acid, mannose, galactose can be used as alternative therapeutics to drugs and antibacterial as they are not expected to lead drug resistance. Possible applications of the materials so developed have been highlighted**.**

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1. Sii, D.; Sadana, A., J. Biotechnol., 19, 83, 1991
2. Senstad, C., Mattiasson, B., Biotech. Bioengg., 34,387,1989
3. Sigal, G. B.; Mammen, M.; Whitesides, G. M., J. Am. Chem. Soc., 118: 16, 3789-3800,1994

US Patents (Filed) :

1. Polymerizable monomers and preparation thereof (NF-336 / 2002)
2. Oligomers and preparation thereof (NF-363 /2002)
3. Polymerizable macromer and preparation thereof (NCL 34 / 2002)
4. Block co polymers and preparation thereof (NCL 35 / 2002)
5. Copolymers and preparation thereof (NCL 40 / 2002)
6. Triblock copolymers and preparation thereof (NCL 42 / 2002)
7. Polyvalent Imprinted polymers and preparation thereof (NCL / 2003)

1.0.0 Introduction:

Purification of enzymes and proteins is a major bottleneck in biotechnological research, especially in product development. Recovery of desired biomolecules in the pure form from a natural source and / or synthesized by a biotransformation route involves a series of purification and recovery steps, the rigor of which depends on intended applications whether in biotechnology, pharmaceuticals, or food industries. Although many techniques are available for the purification of the proteins and enzymes, the strategy for the recovery of the biomolecules needs to be selected judiciously since they are sensitive to temperature, pH and solvent. Moreover, the recovered protein / enzyme must retain its structure, specificity and activity. In general, the methods adapted for purification in the past are very specific to a particular protein and enzyme systems. Amongst a plethora of available techniques, affinity based separations are preferred in view of their selectivity in biomolecular separations [Sii et al (1991); Jane et al (1995); Senstad et al (1989)].

In particular affinity precipitation is a convenient bioseparation technique as it eliminates most of the drawbacks associated with affinity chromatography. Moreover, it offers ease of scale up, concentration and purification. The technique can be adapted to a continuous process and the ligand used can be recycled. It is now being looked upon as an alternative to expanded bed chromatography. Affinity precipitation involves binding between heterobifunctional ligands coupled to the stimuli sensitive polymers e.g. poly *N*-isopropyl acrylamide (NIPA) and the substrate, separation of the complex by altering the conditions such as temperature, pH etc. and decoupling the ligand and the substrate at a later stage. The binding between the ligand and the substrate plays a critical role and efforts are being made to enhance the binding [Pohl et al (1999)].

Amongst various systems of biological importance, the protein-carbohydrate interactions are of particular significance. However, such interactions must have a specific binding energy. Polyvalent interactions involving proteins and carbohydrates play a critical role in biological systems. It has been realized that the polyvalent ligands bind more efficiently to the receptor than monovalent ligands [Sigal et al (1994)].

Ligand-substrate interactions can thus be enhanced by designing polymeric affinity ligands, which possess the preferred conformation for binding to the target biomolecule.

The present investigation was undertaken to design, synthesize and evaluate stimuli sensitive affinity polymers containing *N*-acetyl glucosamine (NAG) for enhanced binding and recovery of lysozyme. Role of polymer composition, polyvalency and steric stabilization was investigated. Molecularly imprinted polymers were synthesized using lysozyme as a template. These polymers exhibited enhanced binding compared to polymers prepared in the absence of lysozyme. The affinity polymers exhibiting optimum binding have been used for the recovery of lysozyme. The methodology can be replicated for other ligands e.g. sialic acid since the inhibition of haemagglutination of host Red Blood Cells (RBC's) by influenza virus occurs through sialic acid ligands. Polymeric NAG ligands investigated are stable, water-soluble and are not hydrolyzed by lysozyme.

This chapter presents an overview of various aspects of the stimuli sensitive polymers, especially, thermoprecipitating polymers and their applications in affinity precipitation. The advantages of designing tailored stimuli sensitive polymers containing target specific ligands are reviewed. The ligand substrate binding especially, involving protein carbohydrate interactions plays a critical role in biological processes. We have therefore chosen to investigate means of enhancing binding between NAG and lysozyme as a model system representing these. Strategies for designing tailored stimuli sensitive polymers for the recovery of biomolecules are reviewed and their relative merits evaluated.

1.1.0 Stimuli sensitive polymers

Smart materials are considered as the most novel materials of the 21st century. The term 'stimuli sensitive polymers' refers to the polymeric systems, which demonstrate relatively large and sharp property changes in response to a small change in physical or chemical stimulus such as pH, temperature, ionic concentration, solvents, mechanical stress and electric or magnetic field. These microscopic changes are apparent at the macroscopic level as phase separation and are reversible [Galaev, Gupta and Mattiasson (1996)]. Stimuli sensitive polymers are either synthetic or natural polymers that exhibit

reversible conformational changes, which are reflected in their physico-chemical properties such as solubility in response to environment.

Stimuli sensitive polymers find extensive applications in diverse fields and significant progress has been made in designing such materials. They find applications in soft lenses, switches, membranes for bioseparation, transducers, matrices for drug delivery and implants in plastic surgery. The ability to undergo phase transition makes stimuli sensitive polymers unique.

Various molecular mechanisms that result in change in the conformation of stimuli sensitive polymers with change in environment are listed below:

Table 1.1 : Mechanisms of responses to various stimuli [Tanaka, 1978]

Stimulus	Response
1. Temperature	Phase separation
2. pH	Shape and Phase separation
3. Solvents	Mechanical, Phase separation
4. Electric field	Shape, Electrical
5. Magnetic field	Surface energy
6. Electromagnetic radiation	Reaction rates
7. Mechanical stress	Permeability

Fig.1.1 : Phase transition in theromosensitive polymer in solution

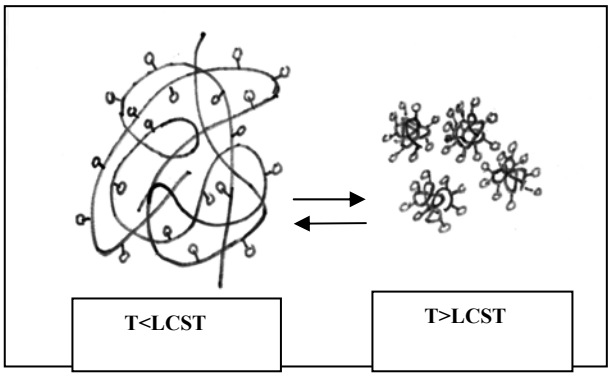


Table 1.2 : Stimuli Sensitive Polymers : Applications

[Galaev, Gupta and Mattiasson (1996)]

Area	Use
1. Biocatalysis	Immobilization of living cells
	Reversibly soluble biocatalyst
2. Medicine	Controlled drug release
	Targeted drug delivery
3. Selective separations	Controlled permeation
4. Downstream processing	Affinity precipitation
	Detachment of cells

Table 1.3: Types of Stimuli sensitive polymers and mechanism of phase transition

[Galaev, Gupta and Mattiasson (1996)]

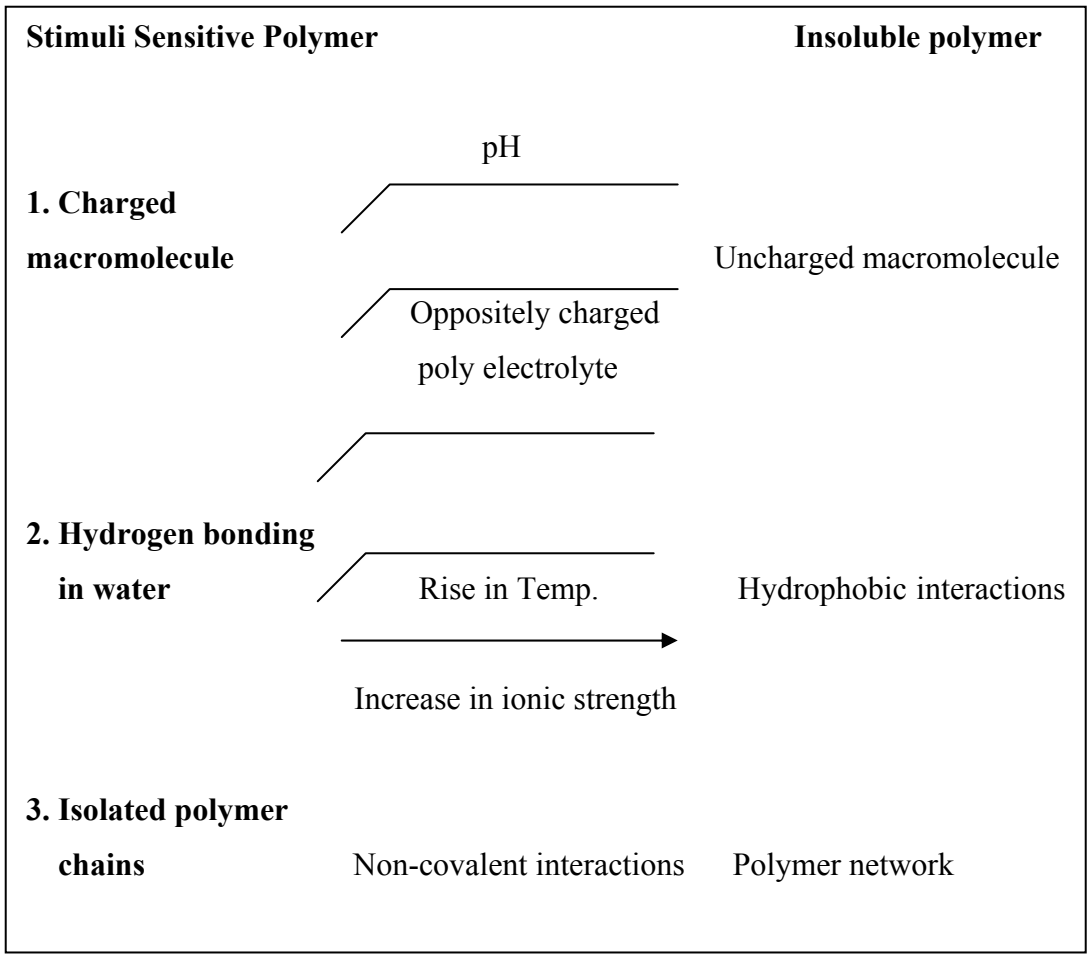


Table 1.4 : Phase transition temperature of aqueous poly (*N*-alkyl acrylamide)

	LCST ^{0} C
Poly(N-ethylacrylamide)	72.0
Poly(N-ethylmethacrylamide)	50.0
Poly(N-n-propylacrylamide)	21.5
Poly(N-n-propylmethacrylamide)	28.0
Poly(N-isopropylacrylamide)	32.0
Poly(N-isopropylmethacrylamide)	44.0
Poly(N-methyl-N-ethylacrylamide)	56.0
Poly(N-acryloylpiperidine)	5.50
Poly(N,N-diethylacrylamide)	50.0

1.1.1 Stimuli sensitive polymers: Overview

Extensive research has been reported on the stimuli sensitive polymers in last twenty years and the interest in such materials continues to grow. The unique aspect of the stimuli sensitive polymers is their unconventional solubility behaviour in aqueous medium, which makes them attractive for various biotechnological applications. Monomers or modified monomers are being used for the preparation of stimuli sensitive polymers to obtain desired properties. The changes in molecular structure of smart polymers either in solution or hydrogel form are reflected at a macroscopic level as reversible phase separation in solution or change in swelling equilibrium. These unique properties of stimuli sensitive polymers make them suitable for the purification and downstream processing of biological macromolecules.

The progress in stimuli-responsive polymers, with special emphasis on recent advances in understanding the structure–property relationship in poly (*N*-isopropylacrylamide) (PNIPA) based systems, reversible sol–gel systems, pH-sensitive polymers, hybrid hydrogels and electro-or light sensitive polymers has been extensively reviewed [Mattiasson et al (1996)] and [Byeongmoon et al (2002)].

In particular, Taipa et al (2000)] reported recovery of a monoclonal antibody from hybridoma culture by affinity precipitation with Eudragit S-100. An IgG1 monoclonal

antibody (MAB) was isolated from hybridoma culture by affinity precipitation with an Eudragit S-100-based heterobifunctional ligand. Affinity binding was performed in a homogeneous aqueous phase at pH 7.5 followed by precipitation of the bound affinity complex by lowering the pH to 4.8.

As mentioned in the foregoing, smart polymers, which respond to a wide range of stimuli, have been devised. A summary of stimuli sensitive polymers is presented below:

1.1.2 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 1.5: Monomers for pH responsive polymers

pH	Monomer	pH sensitive group
Acidic	Acrylic acid	-COOH
	Methacrylic acid	-COOH
	Sodium styrenesulphonate	-SO $_3$- Na $^+$
	Sulfoxymethacrylate	-SO_3 H
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^-

pH sensitive polymers find applications in oral drug delivery. Rohm and Haas commercialized a series of polymers under the trade name Eudragit. More recently another series of polymers for the same applications under the trade name Eastacryl (Eastman Kodak) and Kollokot (BASF) have been commercialized. Yet, the research for developing new polymers for the same applications continues. e.g. Torres and Peppas

(1999) reported synthesis and *in vitro* release studies from novel pH sensitive hydrogels for the oral delivery of calcitonin using graft PMAA–*g*–PEG. In this system, the release of calcitonin was relaxation-controlled.

pH-sensitive polymers have regained attention for applications in gene delivery and gene therapy research too. Transport of DNA into a cell is a difficult process because of negative charges and the large size of DNA molecules at physiological conditions. Therefore, positively charged polymers are used to balance out the charge and condense the DNA to nanoparticles of ~100 nm in size. Poly (L-lysine), poly (ethylene imine), amine containing dendrimers and amine-containing fractured dendrimers have been investigated for this purpose [Godbey et al (2001)].

Various other stimuli sensitive polymers have been reported for the isolation of biomolecules. Tyagi et al (1996) used chitosan (partially acetylated chitin) that bind to proteins such as lysozyme and lectin through *N*-acetyl glucosamine units. Chitosan is used as a affinity macroligand that forms a complex with lysozyme at pH 4.0. The protein is recovered by precipitation of polymer – protein complex by increasing pH to 8.0.

Taipa et al (2000) reported recovery of a monoclonal antibody from hybridoma culture by affinity precipitation with Eudragit S-100. An IgG1 monoclonal antibody (MAB) was isolated from hybridoma culture by affinity precipitation with an Eudragit S-100-based heterobifunctional ligand. An IgG1 monoclonal antibody (MAB) was isolated from hybridoma culture supernatant by affinity precipitation with a Eudragit S-100-based heterobifunctional ligand.

1.1.3 Temperature sensitive polymers:

The polymers, which undergo phase transitions as a function of temperature, are called "theromosensitive or thermoprecipitating polymers". A large number of theromosensitive polymers have been reported in the past for diverse applications. Temperature is one of the most significant and extensively studied stimulus that causes phase transition.

In general the solubility of the materials increases with increasing temperature. But the solubility of the polymers which exhibit lower critical solution temperature (LCST) behaviour decreases with increasing temperature. The best example is the aqueous solution of poly (*N*-isopropylacrylamide) (PNIPA) having LCST in the

temperature range of 31-33 ^{0} C. [Schild et al (1992)] and [Haeshin et al (1998)]. Below LCST the polymer is completely soluble whereas above LCST, the polymer phase separates.

1.1.4 LSCT and its origin: A brief overview:

The free energy of change ΔG_{mix} for mixing two pure substances at temperature T is given by the relationship:

$$\Delta G_{mix} = \Delta H_{mix} - T \Delta S_{mix} \qquad 1.1$$

The heat of mixing ΔH_{mix} is a positive quantity for most of the solutions, i.e. the formation of polymer solutions is usually an endothermic process. With rare exception the entropy of mixing ΔS_{mix} is positive, owing to the more random nature of solutions as compared to that of the unmixed components. Both ΔH_{mix}, and ΔS_{mix}, vary with temperature and concentration. A necessary, although not sufficient condition for the formation of a thermodynamically stable solution is

$$\Delta G_{mix} \leq 0$$

The Flory Hugins theory leads to the following equation for the energy of mixing for polymer solutions

$$\Delta G_{mix} = RT [n_1 \ln \Phi_1 + n_2 \ln \Phi_2 + \chi n_1 \Phi_2] \qquad 1.2$$

where,

Φ_1 and Φ_2 denote the volume fractions of the solvent and the polymer respectively, n1 and n2 denote number of moles of the solvent and the polymer and χ denotes the Flory- Huggins interaction parameter. The smaller the value of χ, the more stable is the solution relative to the pure components and the more likely is a system miscible over a wide range of concentration. The values of χ normally vary between 0.2 and 0.9. Furthermore, the temperature dependence of the parameter is given by

$$\chi = \alpha + \beta / T \qquad 1.3$$

where, α and β are system dependent. In most cases since the mixing is endothermic, β is positive. Above a certain temperature complete solution occurs. This temperature is referred as UCST. Conversely when mixing is exothermic, complete solution can occur only below a certain temperature referred to as the LCST.

Typical plot of $\Delta G_{mix} / m_{Tot}$ vs weight fraction of the polymer is shown in Fig. 1.1.1 .

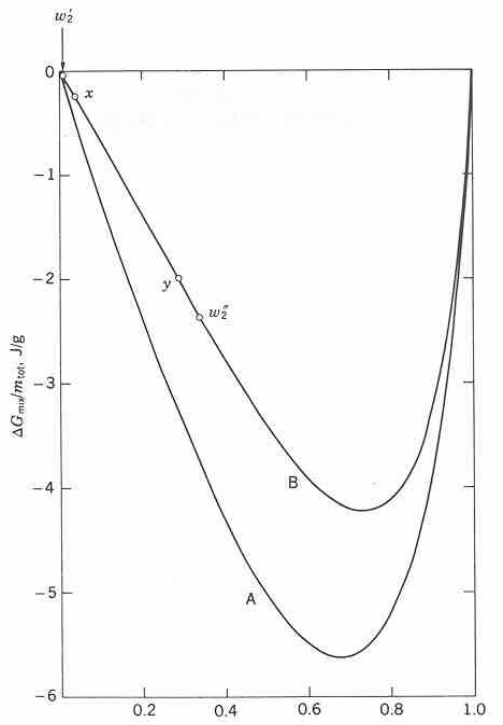


Fig. 1.1.1 Plot of $\Delta G_{mix} / m_{Tot}$ vs weight fraction of the polymer:

Curve A represents a system that is miscible in all proportions. The thermodynamic analysis necessitates that the curve is concave downwards. In the region x-y and phase systems is thermodynamically unstable as exemplified by curve B in fig. 1.1.2 which is a blown up portion figure 1.1.1 in the relevant composition range.

The plot of free energy per unit mass of g_{mix} vs. polymer fraction at critical temperature T_c is shown in fig 1.1.2 curve C.

At the critical point

$$\frac{[\delta^2 g_{mix}]}{[\delta \Phi_2{}^2]_{T,p}} = 0 \qquad 1.4$$

The temperature composition phase diagram for a system, which exhibits UCST, is shown in Fig. 1.1.3.

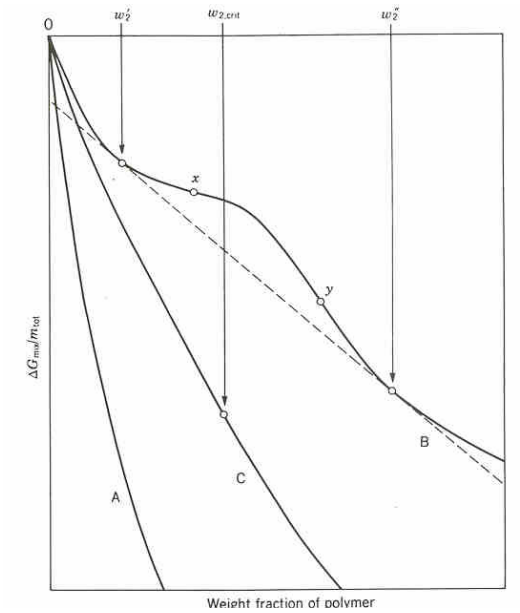


Fig. 1.1.2: Blown fig of 1.1.2 for $\Delta G_{mix} / m_{Tot}$ vs weight fraction of the polymer

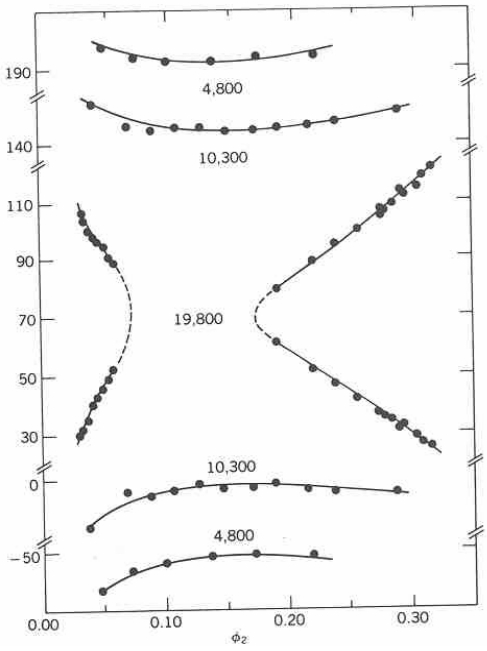


Fig .1.1.3: Temperature composition phase diagram

Here T_{crit} is the highest temperature at which the two phases can coexist. Polymer solvent systems which exhibit UCST have positive heats of mixing and intermediate to large entropies of mixing. In contrast, the polymer solvent system for which heat of mixing is negative i.e. mixing is exothermic and entropy of mixing is usually small, exhibit lower critical solution temperature. In case T_{crit} denotes the lowest temperature at

which two phases can coexists and below which a single phase exists. It must be noted that T_{LCST} does not necessarily have to be lower in magnitude than T_{UCST}. Further, critical miscibility results only when enthalpy and entropy values in eqn 1.1 are of magnitude such that following conditions are satisfied

$$\frac{[\delta^2 \Delta G_{mix}]}{[\delta \Phi_2{}^2]_{T,p}} = 0 \qquad 1.5$$

$$\frac{[\delta^3 g_{mix}]}{[\delta \Phi_2{}^3]_{T,p}} = 0 \qquad 1.6$$

These equations can be used to calculate T_{crit} and $\Phi_{2\,crit}$

The formation of polymer solution involves replacing solvent / solvent and polymer-polymer bonds by polymer–solvent bonds. If the solution formation is an endothermic process, an increase in temperature favors the formation of polymer solution. Such systems therefore exhibit UCST behaviour. When the solution process is exothermic, the solution formation is aided by lowering of the temperature. Consequently such a system exhibits LCST behaviour.

For this case

$$\frac{[\delta^2 \Delta H_{mix}]}{[\delta \Phi_2{}^2]} > 0 \qquad 1.7$$

whereas, a system exhibiting UCST behavior

$$\frac{[\delta^2 \Delta H_{mix}]}{[\delta \Phi_2{}^2]} < 0 \qquad 1.8$$

Lower critical solution temperatures are found in systems that have strong specific intermolecular interactions, especially, hydrogen bonding between polymer and solvent. When the components are mixed at low temperatures, heat is released ($\Delta H_{mix} < 0$) owing to the preferentially favorable attractions. However, heating increases molecular motion and cause a break down in the hydrogen bonding that makes mixing possible at lower temperatures. Thus ΔH_{mix} increases with increasing temperature, eventfully the phase separation occurs. Examples of phase separation polymers include: Poly (N-isopropyl acrylamide) and water, poly (methacrylic acid) and poly (ethylene oxide) and solutions of poly (methacrylic acid) in 1,4-dioxane.

The behaviour of a PNIPA in water reflects the balance of like and unlike interactions among its own segments and the surrounding molecules. In case of aqueous solutions, in water solvent – solvent interactions are particularly strong as indicated by partially ordered structure.

Ordering of solutes such as PNIPA in aqueous solution results from specific orientations required to hydrogen bond with the already arranged water molecules. This is important when water molecules must orient around nonpolar regions of solutes, being unable to hydrogen bond with them. This is also called as hydrophobic effect resulting in a decrease of entropy upon mixing (negative Δ S). At higher temperatures the entropy dominates the exothermic enthalpy of the hydrogen bonds formed between the polymer polar groups and water molecules that is the initial driving force for dissolution. Once the free energy change (ΔG) becomes positive upon mixing, the resulting consequence is phase separation is termed as lower critical solution temperature.

From the studies of PNIPA solutions discussed above it is clear that below the LCST, PNIPA exists as isolated, flexible but extended coils in dilute aqueous solution. At the LCST the individual polymer chains collapse prior to aggregation. This is true with more dilute solution. Thereafter scattering increases and a phase separation occurs. Finally, a second phase of lower polarity is formed that is suspended in the water rich phase. LCST of the polymer can be estimated by various methods e.g. Differential scanning colorimeter, Light scattering, Viscometry and Fluorescence.

Below the LCST, favorable interactions *via* hydrogen bonding between amide groups in polymer and water molecules lead to dissolution of polymer chains. Whereas above LCST, the hydrogen bonds are broken resulting water molecules to expel from the polymer [Kujawa et al (2001)]. Zareie et al (2000)] investigated stimuli responsive copolymer by atomic force microscopy. The coil-to-globule transition of poly *N*-isopropylacrylamide was demonstrated on a nanometer scale by using atomic force microscopy (AFM).

1.1.5 Applications of thermoprecipitating polymers:

Poly *N*-isopropylacrylamide has been extensively studied since it can form macroscopic gels, microgels, lattices, thin films, membranes, coatings, and fibers.

Moreover, poly *N*-isopropylacrylamide has been explored for applications in disciplines such as rheology, biology, photography and biotechnology.

Poly *N*-isopropylacrylamide has attracted applications in pharmaceutical and biomedical technology as its LCST is close to body temperature [Hoffman et al (1990)] and [Dong et al (1986)]. It can also be used for oil dewatering, food protein separations, metal removal, chromatography, transdermal drug delivery, flavor release and in robotics [Snowden et al (1996)]. Poly *N*-isopropylacrylamide is an attractive polymer for the chemical conjugation of affinity ligand, which can be used for the bioseparation. Various ligands have been conjugated covalently to poly *N*-isopropylacrylamide for the detection and purification of biomolecules such as double stranded DNA [Kadonga et al (1991)].

Water–soluble conjugates of double stranded DNA and poly (*N*-isopropylacrylamide) for single step affinity precipitation have been utilized. Umeno et al (1998) and Mori et al (2001) reported sequence-specific affinity precipitation of oligonucleotide using poly (*N*-isopropylacrylamide)-oligonucleotide conjugate. A sequence-specific precipitation separation system of oligonucleotide using a conjugate between poly (*N*-isopropylacrylamide) and oligonucleotide was developed.

Sharma and Gupta (2001) reported alginate as a macroaffinity ligand and used an additive for the enhanced activity and thermo stability of lipases. Alginate was used as a macroaffinity ligand to purify lipases from chromobacterium viscosum, porcine pancreas and Wheat Germ Agglutinin (WGA) by employing affinity precipitation technique.

Galaev et al (1999) reported copolymers of *N*-isopropylacrylamide with styrene derivatives of iminodiacetic acid (IDA) and 1-vinylimidazole (VI) by radical copolymerization and metal complexation. The thermoprecipitation property of the copolymers indicates the application of Cu (II) loaded copolymer of poly (VI/NIPAM) as a potential carrier for the metal chelate affinity precipitation of proteins. The studies carried out on the purification of protein inhibitors from different cereals, suggest the specific interaction of metal ions bound on the copolymer and the histidine residues on the surface of the targeted protein. The recovered copolymers could be reloaded with metal ions and can be reused number of times with high efficiency.

Ability to separate the conjugated poly (*N*-isopropylacrylamide) by thermal precipitation allows easy recovery of enzymes and recycling. Nguyen et al (1990)

reported use of poly (*N*-isopropylacrylamide) in standard two phase partitioning technique used in biotechnology to isolate enzymes.

Kondo et al (1994) developed thermo-sensitive immunomicrospheres for antibody purification. The latex particles composed of poly (styrene / *N*-isopropylacrylamide / glycidyl methacrylate) P (St / NlPAM / GMA) and poly (styrene / N-isopropylacrylamide / methacrylic acid) P (St / NlPAM / MAA) was prepared by emulsifier-free emulsion polymerization.

By controlling the polymer composition, the coil-to-globule transition can be thermodynamically controlled. Kunjawa et al (2001) copolymerized *N*-isopropylacrylamide with hydrophobic butylmethacrylate, which decreased the LCST of aqueous copolymer solution. Copolymerization with hydrophilic comonomers, such as acrylic acid and hydroxyl ethyl methacrylate, resulted in increase in LCST.

It is demonstrated in the past that addition of the hydrophilic nonionogenic monomers, for example acrylamide into polymers based on *N*-alkyl-substituted acrylamides (*N*-isopropylacrylamide and *N*-diethylacrylamide) increases LCST. On the contrary introduction of the hydrophobic units of *N*-n-octylacrylamide facilitates the occurrence of phase transitions due to enhancement of hydrophobic interactions [Popovich et al (1997)].

Bulmus et al (2000) studied site-specific polymer–streptavidin bioconjugates for pH controlled binding and triggered release of biotin. Related applications of poly *N*-isopropylacrylamide polymers were also investigated for on–off control of avidin–biotin binding. Below the transition temperature of 32 ° C, *N*-isopropylacrylamide copolymers are in a fully extended conformation in water because of favorable polymer–water interactions. The poly *N*-isopropylacrylamide with fully extended conformation interferes with the biotin-binding site on the avidin, whereas above the transition temperature, the polymers are collapsed and cannot interfere with the binding sites.

Garret et al (2000) used avidin (imino) biotin system as a general approach for affinity precipitation. They described a new type of thermo responsive affinity macroligand.

Tuncel et al (2002) reported a novel approach for albumin determination in aqueous media by using temperature and pH-sensitive N-isopropylacrylamide-co-N-[3-

(dimethylamino)-propyl] methacrylamide random copolymers. Albumin binds onto the copolymer chains by means of H-bond formation between the dimethylamino groups of the copolymer and the carboxyl groups of albumin. The phase-transition temperature exhibited a linear decrease with increasing albumin concentration. By utilizing this behavior albumin assay was developed. The results indicated that NIPA-co-DMAPM copolymer could be utilized as a new reagent for the determination of albumin concentration in the aqueous medium.

Hinrichs et al (1999) used theromosensitive copolymers as carriers for DNA delivery. The copolymers of 2-(dimethylamino) ethyl methacrylate (DMAEMA) and *N*-isopropylacrylamide (NIPA) of varying monomer ratios and molecular weights were synthesized and evaluated as carrier systems for DNA delivery.

1.2.0 Polymers: structural correlations

a) Homopolymer:

When a polymer is made by linking only one type of small molecule or monomer, the resulting polymer is called as a homopolymer.

------A----A----A------A-----A------A------A-----

Homopolymer

B) Copolymer :

When two different types of monomers are joined in the same polymer chain then such polymer is called as copolymer. Two monomers A and B can from a copolymer by arrangement as alternating, such polymer therefore is called as alternating copolymer:

—A—B—A—B—A—B—A—B—A—B—A—B—A—B—

Alternating copolymer

Whereas in random copolymer the two monomers may follow random order of arrangements

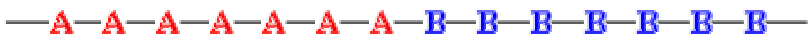
—A—A—B—A—B—B—A—B—A—A—B—B—B—A—

Random Copolymer

Copolymer can vary in their average composition and mean monomeric unit sequence length, as well as in the distributions in compositions and sequences. Since the arrangement of the ligand on the copolymer is very critical for interactions with biomolecules the determination of sequence is important and will be discussed later.

c) Block copolymer:

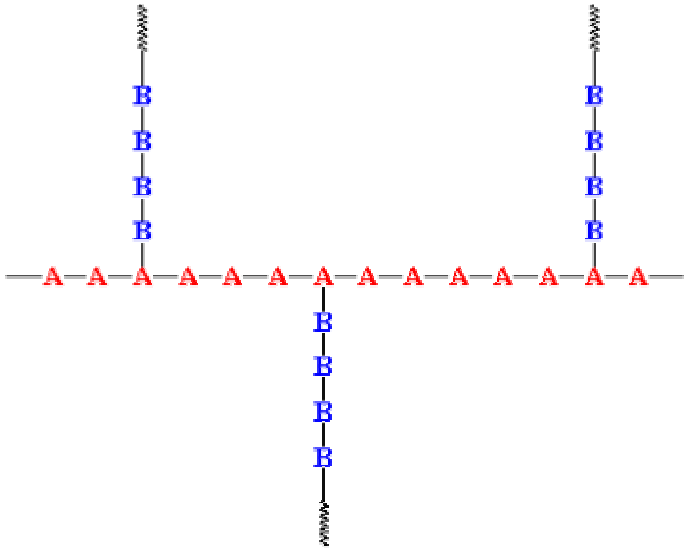
In block copolymer one type of monomers are grouped together with another type of monomers. Two reactive homopolymers can be condensed to form block copolymer.



Block copolymer

d) Graft copolymer:

When chains of a polymer made of monomer B are grafted onto a polymer chain of monomer A forms a graft copolymer:



Graft copolymer

1.2.1 LCST in polymers: structural correlations

Critical solution temperatures of a variety of homo polymers have been summarized in Table 1.3. Copolymerization offers a simple mean of tailoring LCST of polymers over a wide range.

Change in phase transition temperature in aqueous system is achieved either by the addition of hydrophobic or hydrophilic groups to a base polymer. Copolymerization is the most popular method to achieve the phase transition at desired temperature. However, the differences in reactivities of the monomers lead to formation of either alternative or block copolymers. Copolymerization leads to effective systematic changes in polymer properties [Zutty et al (1962)].

Klier et al (1990) reported graft copolymers of polyethylene glycol (PEG) on a poly methacrylic acid (PMAAC) backbone. The resulting polymer forms hydrogen bonded complexes between –O- and –COOH groups at lower pH 7.4. The gels gradually shrunk as the temperature was raised at pH 4, the temperature sensitivity does not occur at pH 7.4 where the gels were highly swollen owing to ionization of the carboxyl groups and the consequent disruption of the complex.

Yoshida et al (1995) prepared comb-type hydrogels with rapid de-swelling responses to temperature changes. A theromosensitive hydrogel was prepared with a comb structure in which poly *N*-isopropylacrylamide chains are grafted onto crosslinked networks. Within the gel, terminally grafted chains have freely mobile end of the poly *N*-isopropylacrylamide chains that are crosslinked and relatively immobilized. With increase in temperature grafted poly *N*-isopropylacrylamide chains begin to collapse from their expanded (hydrate) form to compact (dehydrated) forms. The collapse occurs before the poly *N*-isopropylacrylamide network begins to shrink because of the mobility of the graft chains.

Oya et al (1999) reported reversible molecular adsorption based on multipoint interaction by shrinkable gels. Polymer gels were prepared that recognize and bind a target molecule by multiple-point interaction and can reversibly change their affinity to the target by more than one order of magnitude. Adsorbing molecule was methacrylamidopropyltrimethylammonium chloride (MAPTAC), which carries one positive charge. It was envisioned that three or four MAPTAC groups would capture one pyranine molecule. A small amount of MAPTAC was embedded by copolymerization within a theromosensitive polymer network of *N*-isopropylacrylamide (NIPA) in the monomer ratio of less than 1/30.

1.2.2 Structural modifications: Copolymers

Attempts have been made to synthesize copolymers to change the LCST of the poly *N*-isopropylacrylamide by varying comonomer compositions and crosslinking. Various copolymers, graft copolymers and hydrogels have been reported in the past. Poly *N*-isopropylacrylamide polymers are useful as their LCST can be manipulated from 0 to 100 ^{0} C by tailoring structure for specific uses [Schild et al (1992)].

Taylor et al (1975) copolymerized *N*-isopropylacrylamide with other *N*-alkyl acrylamides over the entire composition range. Continuous changes in LCST were observed when the comonomer had a smaller or larger *N*-alkyl group than the *N*-isopropyl group (Table 6).

Table 1.6: Copolymers of varying compositions for hydroxypropylacrylate (HPA) / Acrylamide and NIPA / *N*-ethylacrylamide (NEA)

	Mole % composition	LCST ^{0} C
Hydroxypropylacrylate (HPA) / Acrylamide	100/0	16
	70/30	32
	60/40	51
	50/50	75
NIPA / *N*-ethylacrylamide (NEA)	100/0	32
	50/50	48
	30/70	56
	10/90	67
	0/100	74

Bulmua et al (2001) studied stimuli-responsive properties of conjugates of *N*-isopropylacrylamide-co-acrylic acid oligomers with alanine, glycine and serine mono-, di- and tri-peptides. At pH 7.4, LCSTs of the mono-and di-peptide conjugates were observed in the range of 41.6–43.9 ° C, and 46.2–60.2 ° C, respectively, while the co-oligomer at pH 7.4 did not show a LCST up to 60 ° C. Tri-peptide conjugates did not display LCST at pH 7.4, except the one with glycine–alanine–serine sequence.

Aoyagi et al (2000) reported molecular design of LCST polymers. The authors copolymerized 2-carboxyisopropylacrylamide (CIPAA) with *N*-isopropylacrylamide so as to contain 5 mole % of the former. The resulting copolymer exhibited little or no change in LCST, whereas 5 mole % of acrylic acid resulted in LCST increases of ~10°C, as measured in phosphate buffered saline (PBS) at pH 7.4. It was thought worthwhile to develop polymer systems with more than one transition. This can be obtained by controlling polymer architecture. For example, hydrogels containing different LCST oligomer grafts showed dual swelling transitions with increasing temperature. The first transition resulted from oligo *N*-isopropylacrylamide at 32 ^{O} C, and the second transition occurred at 36 ^{O} C owing to the presence of oligo (*N*-vinylcaprolactam) [Inoue et al (1997)].

1.2.3 Graft copolymers of PNIPA:

Graft and block copolymers are of interest as they retain the unique properties of individual components. Two different moieties in graft copolymers can provide a combination of desirable properties for a particular application. Graft copolymers contain one or more blocks of homopolymer, which are grafted as branches onto a main chain of homopolymer. The advantage of graft copolymer is that it contains monomer for further copolymerization and designed sequence of monomer.

Various modifications have been reported for synthesis of smart polymers. Block and graft copolymers having components with different phase transition capabilities are prepared. Applications of such copolymer systems include drug delivery, diagnostics, bioseparations, cell cultures and bioreactors. In addition to physical modifications, biomolecules are covalently conjugated onto the block or graft polymer chains with enhanced biological activity. [Chen and Hoffman (1995)].

Graft copolymers can be prepared by covalent coupling of the thermally–sensitive prepolymers containing functional group, onto the pendent functional group of the other monomer.

Okano et al (1993) designed novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly (*N*-isopropylacrylamide). Poly (*N*-isopropyl acrylamide) demonstrates a fully expanded chain conformation below 32 ^{O} C and collapses at high temperatures. Further, resulting temperature responsive polymer

was grafted onto surfaces of commercial polystyrene dishes and used as temperature switches for creating hydrophilic surfaces below 32 ^{O} C and hydrophobic surfaces above 32 ^{O} C.

1.2.4 Block copolymers of PNIPA:

Block copolymers can be prepared by coupling two or more chemically distinct homopolymer blocks, each having linear series of identical monomers. Multiple homopolymer blocks can produce a structured block of copolymers to produce tailored physical properties. Block copolymers are of various types such as a) diblock copolymers referred to as AB block copolymers, 2) triblock copolymers of the ABA type and 3) multiblock copolymers of the $(AB)_n$ type.

Among various copolymerization methods block copolymerization results in sequential placement of monomers in the resulting copolymer. The length of the blocks can be varied independently during synthesis. Thus polymerized blocks may have identical or different properties and are further coupled with other blocks.

Block copolymers of poly (*N*-isopropylacrylamide) (NIPAAm–co–acrylic acid) and poly (*N*-isopropylacrylamide) b–poly (ethylene glycol) (PNIPAAm–b–PEG) was reported by Topp at al (1997). The hydrophilic moieties in block copolymers led to sol-to-gel transition at 37 ^{O} C rather than precipitation. The LCST can be altered in smart polymers using various comonomers.

Block copolymers in which saccharides residues are incorporated as a pendent group are of interest because of their potential in advanced materials such as in cell specific cultures. Synthetic glycoconjugates are useful for studying carbohydrate–protein interactions analogous to those involved in the biological processes. [Kiessling et al (1996)]; Choi et al (1996)] and [Yamada et al (2001)].

In the area of polymer chemistry, amphiphilic block copolymers are known to exhibit various interesting properties such as surface activity and aggregate formation. Moreover, low molecular weight and macromolecular amphiphilies that exist in nature are of interest as most of them are hydrophilic. Therefore such components can be used to mimic naturally occurring amphiphiles for various biological processes in living systems [Yamada et al (1999)]. The homopolymers and block copolymers containing pendent NAG residues are reported to interact with WGA lectin. The activity in terms of

association constants (K_a) increased three orders of magnitude for block copolymers as compared to homopolymers containing NAG.

Chung et al (2000) synthesized AB block copolymers of poly *N*-isopropyl acrylamide with either poly (butyl methacrylate) (PBMA) or polystyrene (PS). The resulting copolymer forms a core-shell micellar structure. The hydrophobic drug adriamycin was loaded into the inner core of the polymeric micelles. The polymer exhibited reversible response to temperature cycles through an outer polymer shell LCST.

Zareie et al (2002)] investigated phase transition behavior of thermo-responsive poly (*N*-isopropylacrylamide)–polyethylenimine (PEI) block copolymers. Homopolymers of *N*-isopropylacrylamide (NIPA) having carboxylic acid-end groups using mercaptoacetic acid (MAA) as the chain transfer agent were prepared. Carboxylic acid-terminal poly (*N*-isopropylacrylamide was then condensed with polyethylenimine (PEI, MW-2000) using a water soluble carbodiimide (EDAC). In comparison with the carboxyl-end poly (*N*-isopropylacrylamide) polymer, block copolymers containing PEI exhibited a pH dependent-temperature sensitivity and higher LCST values in acidic pH.

1.2.5 Copolymer Synthesis: Reactivity ratios and copolymer structure:

The composition of a copolymer is usually different from the resulting composition of the comonomer feed from which it is prepared. This is because monomers have different tendencies to undergo copolymerization. Some monomers are more reactive in copolymerization than indicated by their rates of homopolymerization, whereas the other monomers are less reactive. Thus compositions of a copolymer cannot be determined from knowledge of the homopolymerization of the two monomers. The determinants of the chemical reactivity of the propagating chain in a copolymerization are dependent only on the identity of preceding the last monomer unit.

Copolymerization of the two monomers leads to two types of propagating species – Monomers M_1 and M_2 each can add either to a propagating chain ending in M_1 or to one ending in M_2. These can be represented by M_1* and M_2* where the asterisk represents a radical, a carbocation, or a carbanion as the propagating species depending on the particular case. Four propagation reactions are possible.

$$M_1^* + M_1 \xrightarrow{K_{11}} M_1^* \qquad 1.1$$

$$M_1^* + M_2 \xrightarrow{K_{12}} M_2^* \qquad 1.2$$

$$M_2^* + M_1 \xrightarrow{K_{21}} M_1^* \qquad 1.3$$

$$M_2^* + M_2 \xrightarrow{K_{22}} M_2^* \qquad 1.4$$

Where,

k_{11} is the rate constant for a propagating chain ending in M_1^* adding to monomer M_1,

k_{12} for a propagating chain ending in M_1 adding to monomer M_2, and so on. The propagation of a reactive centre by addition to the same monomer is termed as homo propagation or self propagation and propagation of a reactive centre by addition of the other monomer is referred to as cross propagation or a crossover reaction. All propagation reactions are assumed to be irreversible [Odian (1991)].

Methods for calculating reactivity ratios can be classified as linear least square (LLS) and non linear least square (NLLS) method. It is accepted that LLS method such as those proposed by Fineman and Ross can be applied to experimental data at sufficiently low conversions, because the calculation is based on the conventional copolymerization equation. Whereas the Fineman-Ross method for estimation of reactivity ratios extended by Kelen Tudos and can be applied to medium, high conversions experimental data without significant errors [Mao et al (1993)].

However, before describing the Kelen Todos method, we present a summery of copolymerization kinetics.

Monomers M_1 disappears by reactions 1.1 and 1.3, while monomer M_2 disappears by reactions 1.2 and 1.4. The rates of disappearance of the monomers, which are synonymous with their rates of entry into the copolymer are given by :

$$-\frac{d[M_1]}{dt} = k_{11}[M_1^*][M_2] + k_{21}[M_2^*][M_1] \qquad 1.5$$

$$-\frac{d[M_2]}{dt} = k_{12}[M_1^*][M_2] + k_{22}[M_2^*][M_2] \qquad 1.6$$

Dividing eq. 1.5 by eq. 1.6 yields the ratio of the rates at which the two monomers enter the copolymer, that is, the copolymer composition as

$$\frac{-d[M_1]}{-d[M_2]} = \frac{k_{11}\,[M_1^*][M_2] + k_{21}\,[M_2^*][M_1]}{k_{12}\,[M_1^*]\,[M_2] + k_{22}\,[M_2^*][M_2]} \qquad 1.7$$

In order to eliminate the concentration terms in M_1^* and M_2^* from Eq.1.7, a steady state concentration is assumed for each of the reactive species M_1^* and M_2^* separately. For the concentration of M_1^* and M_2^* to remain constant, their rates of interconversion must be equal. The rates of the reactions for 1.2 and 1.3 are equal,

$$k_{21}[M_2^*][M_1] = k_{12}[M_1^*][M_2] \qquad 1.8$$

Eq. 1.8 can be rearranged and combined with eq. 1.7 to form

$$\frac{d[M_1]}{d[M_2]} = \frac{\dfrac{k_{11}\,K_{21}\,[M_2^*][M_1]^2}{k_{12}\,[M_2]} + k21\,[M2^*][M1]}{k_{22}\,[M_2^*]\,[M_2] + k_{21}\,[M_2^*][M_1]} \qquad 1.9$$

Dividing the top and the bottom of the right side of eq. 1.9 by $k_{21}[M_2^*][M_1]$ and combining the results with the parameters r_1 and r_2, which are defined by

$$r_1 = \frac{k_{11}}{k_{12}} \quad \text{and} \quad r_2 = \frac{k_{22}}{k_{21}} \qquad 1.10$$

Finally, one can obtain

$$\frac{d[M_1]}{d[M_2]} = \frac{[M_1][r_1[M_1] + [M_2]}{[M_2][M_1] + r_2[M_2]} \qquad 1.11$$

Eq. 1.11 is called as the copolymerization equation or the copolymer composition equation.

M_1 and M_2 are molar ratio concentrations of the two monomer units in the copolymer.

r_1 and r_2 are defined as the reactivity ratios.

The tendency of two monomers to copolymerize is noted by ‘r’ values between zero and unity. r_1 value greater than unity implies that M_1 preferably adds to M_1^* instead

of M_2*. While an r_1 value less than unity means that M_1 preferentially adds M_2. An r_1 value of zero would mean that M_1 is incapable of undergoing homopolymerization.

The composition of a copolymer is usually different from the composition of the comonomer feed from which it is obtained. Some monomers may be more reactive in copolymerization. Different types of behaviour are observed for the monomers in the formed copolymers depending on the values of the reactivity ratios. The feed composition may change in final comonomer composition if one of the monomer is greater in reactivity than to another. Thus, the variation may occur in the composition on copolymerization.

A) Ideal copolymerization: $r_1 r_2=1$

A copolymerization is called ideal when the product is of monomer reactivity ratios is unity ($r_1r_2 =1$). This type of copolymerization occurs when both the monomers exhibit equal preference for adding one or other of the two monomers.

B) Alternating copolymerization: $r1 < 1$ and $r_2 < 1$ and $r_1 r_2 < < 1$

When $r_1 < < 1$ and $r_2 < < 1$ the two monomers form copolymers in equimolar amounts in a nonrandom, alternating arrangement along the copolymer chain. This type of copolymerization is known as alternating copolymerization. Each of monomers preferably adds to the other radical.

C) Block copolymerization: $r_1 >1$ and $r_2>1$

When both r_1 and r_2 are greater than unity i.e. r_1 and $r_2 >1$, there is a tendency to form block copolymers in which there will be blocks of both monomers in the chain. In such copolymerization the monomer has a tendency on the part of the radical to add to the same monomer thereby forming the blocks.

Various methods have been devised to quantify the propensity of the monomer to form the copolymer with a given monomer. This is represented by the monomer reactivity ratios such as non linear least squares and linear methods [Powers et al 1950) and Tidwell et al (1965)].

1.2.6 Reactivity estimation by Kelen-Tudos method

Kelen-Tudos (1975) presented a simple graphical linear equation for estimating reactivity ratios.

$M_1 / M_2 = x$ and

$d\ M_1 / d\ M_2 = y$

The composition equation may be written as :

$Y = x\ [1 + r_1 x] / r_2 + x$

The transformed variables of Finnman and Ross as

$G = r_1\ (F - r_2)$ 1.12

And / or

$G / F = -r_2\ (1/F) + r_1$ 1.13

Where the transformed variables are:

$G = x\ (y-1)\ /\ y$ and 1.14

$F = x^2 / y$

Graphical plotting of eq. 1.12 r_1 as the slope and r_2 as the intercept, while the plot of eq. 1.13 presents r_2 as the slope and r_1 as the intercept.

The data obtained under extreme conditions at low M_2 and very low M_1 comonomer concentrations have the greatest influence on the slope of a line calculated by the usual linear least – squares procedure. The calculated r_1 and r_2 values depend on arbitrary factors, such as which monomer is selected as M_1.

The method is more precise as the values of the comonomers composition were extended by calculating mean of the comonomers in the copolymer. Xue et al (2000) obtained reactivity ratios for *N*-isopropylacrylamide with Acrylic acid, Methacrylic acid, 2-methyl-2-acrylamidopropane sulphonic acid. The reactivity values are given below:

Table 1.7 Reactivity ratios for *N*-isopropylacrylamide (NIPA), Acrylic acid (AA), Methacrylic acid (MAA) and 2-methyl-2-acrylamidopropoane sulphonic acid (AMPS)

	r_1	r_2
NIPA-AA	14.0	0.07
NIPA-MAA	10.2	0.01
NIPA-AMPS	2.40	0.03

1.3.0 Affinity based separations using polymers:

Affinity chromatography:

Affinity chromatography technique was developed by Cuatrecasas and co-workers in late 1960s and was specific for the particular proteins. [Cuatrecasas et al (1968)]. The technique has advantage, as the interactions are specific between the biological ligand and its complementary binding site such as coenzyme, hormone, antibody or nucleic acid. [Irwin et al (1995)]. In subsequent years many other affinity based separation techniques [Luong et al (1992)] such as affinity precipitation, affinity partitioning, and affinity ultrafiltration were introduced.

Affinity chromatography, also known as bioselective adsorption, is a protein purification technique developed forty years ago. The fundamental principal comprises the method of isolation of a particular protein by using immobilized inert matrix. The purification by affinity chromatography is achieved in single step and the concentration factor is of the order of several thousand folds. Affinity chromatography is a powerful technique, which allows protein purification in a relatively short amount of time with a higher yield. It relies on the specificity of a protein-binding site for a particular ligand.

Affinity chromatography can be used for purifying wide range of proteins. For example, using a specific DNA sequence as a ligand, the proteins can be isolated. In affinity chromatography enzymes can be used as ligands for isolating biomolecules. For example, adenosine monophosphate (AMP) can be immobilized and used to bind those proteins exhibiting an affinity for AMP, ADP, or ATP. Extra cellular and other receptor proteins can also be purified by affinity chromatography.

1.3.1 Affinity precipitation:

Affinity precipitation has a greater potential as an alternative and complement to affinity chromatography. Affinity precipitation involves utilization of ligands, which have high affinity for the biomolecule. In affinity precipitation an affinity ligand is coupled to a water-soluble smart polymer if necessary through a spacer thereby forming a macroligand. These polymers have a number of advantages as they exhibit reversible solubility in water (Fig. 1.2).

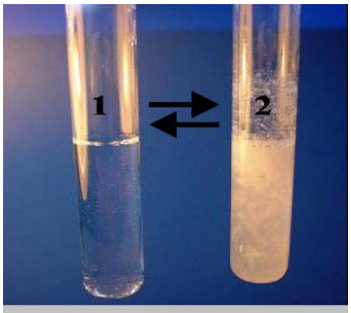


Fig. 1:
Reversible thermo-precipitation (**2**) of an aqueous solution of poly-N-isopropylacrylamide (**1**).

Fig. 1.2 Phase separation

In order to exploit reverse solubility effect such polymers can be chemically conjugated to a small affinity ligand, which is capable of recognizing the target molecule. The conjugate is then called as affinity macroligand (AML).

Stimuli sensitive affinity ligands (i) are homogeneous in nature and size, (ii) are uniform in affinity environment, (iii) offer optimum ligand accessibility, and (iv) offer minimal non-specific interaction. These features of affinity ligands ensure the specificity and efficiency of the target biomolecules.

Affinity precipitation technique involves specific affinity interactions with a receptor biomolecule and separation is achieved by the conventional precipitation method. The affinity interactions in affinity precipitation can involve ionic, van der Waals, and / or hydrophobic interactions. The soluble ligand binds to the target molecule in a homogeneous solution to form a complex. The complex formed is precipitated by increasing the temperature. The complex of ligand and biomolecule is redissolved in a medium, preferably water. Further, the complex is decoupled by decreasing pH. Polymer is further precipitated by increasing temperature and separated from the solution. The solution containing target biomolecule is separated either by dialysis or freeze drying method. The desired target biomolecule is subsequently eluted for higher purity.

Affinity precipitation is superior to conventional precipitation method and affinity chromatography due to the greater affinity interactions. Moreover, the binding between the complementary ligand to the target molecule is performed in a solution. Therefore, it is free from the mass transfer limitations and offers better accessibility to substrates *vis a vis* affinity chromatography, and is easy to scale up.

1.3.2 Advantages of affinity precipitation technique:

Affinity precipitation has an edge over the other bioseparation techniques as it offers following advantages:

1. High productivity due to reduced number of process steps, reduced process volume and consistently high yields.
2. Reproducible process.
3. Ease of handling on large scale.
4. Low capital investment.

1.3.3 Affinity ligands for precipitation:

In order to isolate a product, small affinity ligand, which is capable of specifically recognizing the target molecule, is chemically conjugated to the polymer. The resulting affinity macroligand (AML) can be used to first bind and subsequently co-precipitate the target molecule. Thermo-responsive AMLs are water-soluble, have well defined chain length, transition temperature and affinity group, which offer optimal binding to the target biomolecule.

Affinity precipitation is superior to conventional precipitation as well as affinity chromatography as it includes the features of high selectivity, which results due to affinity interactions. Moreover, it is easy to scale up, devoid of mass transfer limitations associated with solid adsorbents since interaction of the target molecule with its complementary ligand is performed in solution.

Affinity precipitation is performed in two modes according the technique of the ligand:

1) Homo-bifunctional or
2) B) Hetero bifunctional affinity precipitation.

1.3.4 Homo-bifunctional precipitation:

It contains twin ligands, which are linked by a spacer arm (Fig.1.3). The bis ligand can bind to two biomolecules simultaneously at different sites if the spacer linked is sufficiently flexible. Precipitation by homobifunctional ligand results when the concentration of both the macroligand and target molecule is optimal, and the macroligand-target protein complex aggregates which eventually precipitates. The ratio of ligand has to be optimum for the formation of the lattice structure with the biomolecule sites for the precipitation. If the ratio of the bis ligand is high, binding sites

may be occupied by one end of a bis ligand molecule with insufficient intermolecular crosslinking which is necessary for the precipitation.

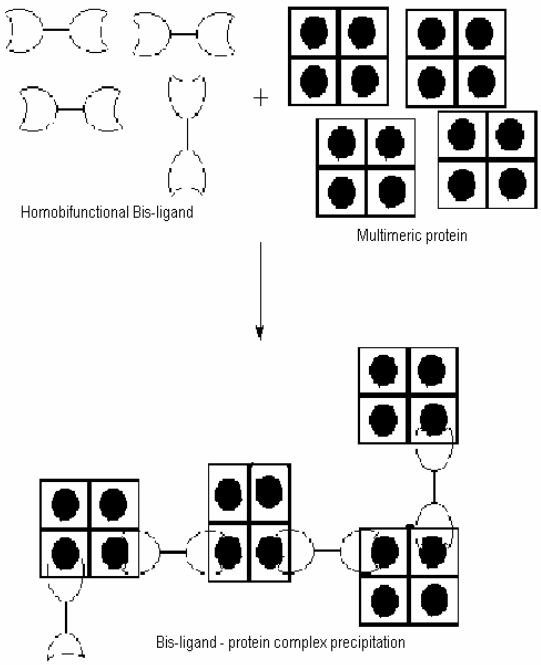
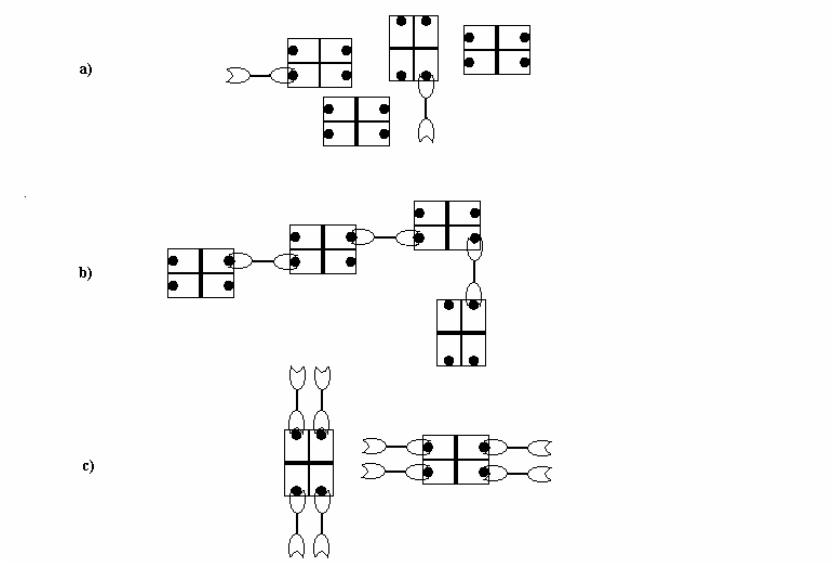


Fig. 1.3: Homobifunctinal ligands

Examples of various ligands:

1. NAD^{+},
2. Procin red HE – 3 B, Triazine dye,
3. Cibacron blue,
4. Metal ions like Cu^{+2}, Zn^{+2},
5. *N*-acetyl glucosamine (NAG), sialic acid, galactose, mannose, etc,
6. P-amino benzamidine(PABA),
7. DNA and RNA,
8. Protein A and,
9. IgG.



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 than tetrameric protein, a decrease in precipitation will occur because each binding site on protein is occupied by different bis-ligand molecule.

Fig. 1.4 : Effect of bis–ligand on affinity precipitation on multimeric proteins

1.3.5 Heterobifunctional ligands

Heterobifunctional ligands are chemically linked to the smart polymers. Precipitation of the polymer-ligand-target protein complex is effected by the change in environmental conditions such as temperature, pH, ionic strength, electric field etc. Hence one part of affinity macroligand binds to the targeted biomolecule while the other plays role in solubility and insolubility.

Heterobifunctional ligands offer following advantages over homobifunctinal ligands:

1. They can be used for the purification of monomeric as well as multimeric proteins.
2. The system is more convenient to scale up for the bioseparations.
3. The polymers can be designed to respond to specific stimulus.
4. The operation is carried out in homogenous environment, thus eliminates the problems associated in affinity chromatography and affinity ultrafiltration.
5. The ligands can be recycled for the recovery of biomolecule.

The precipitation can be achieved at low pH or temperature that will retain the structure and the specificity of the recovered biomolecule.

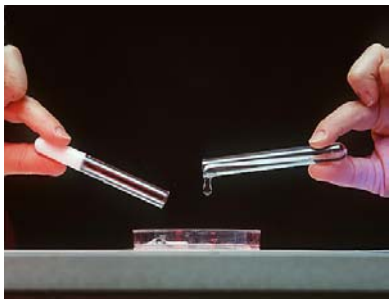


Fig. 1.5 Precipitation of smart polymer

1.4.0 Monovalent and polyvalent interactions

Nature of ligand substrate binding

Adhesion of the ligands present on the surface of the pathogen to the surface of the receptive host is the first step involved in infection. Receptor recognition and binding of ligands are events through which the numerous interactions are controlled in the body. Especially, carbohydrates play a central role in biological phenomena such as protein carbohydrate interactions. Despite known role of carbohydrates in biology, relatively few investigations are reported on methods of enhancing these interactions. New targets for carbohydrates such as enzymes, proteins and viruses are being identified which can have numerous applications in therapeutics. Carbohydrates play critical role in various biological processes such as cell recognition, cell adhesion, cell differentiation, inflammation, viral and bacterial infection, tumerigenesis, and metastasis [Rouhi et al (1996)].

Sharon et al (1989) reported that the carbohydrate portions of glyco-conjugate molecules are important entity in carbohydrate biology. Advantage of carbohydrate modification lies in that it may impart change in physical characteristics such as solubility, stability, activity, antibody recognition and susceptibility to enzyme.

Site-specific ligand conjugates are interactive molecules, and are useful in immunoassays and biomolecule separations. The interacting molecules can be proteins or peptides, antibodies, enzymes, polysaccharides or glycoproteins that specifically bind to

other substrate receptors in the suitable environment. A ligand so bound can be displaced from the binding site by altering environmental conditions.

Recent literature highlights the advantages of polyvalent interactions and their application in medicine and biotechnology. The fucoside sialic acid moities can be linked to polymer for the treatment of rotavirus [(Mandeville et al (2001)]. These moieties can inhibit or prevent rotavirus infection in mammals and humans.

The interactions in biology are either monovalent or polyvalent in character. Monovalent ligands display weak affinities and poor specificity towards the receptor binding sites. In contrast, a saccharide in a multivalent / polyvalent form can bind to the same substrate with greater affinity and specificity. The binding of cell surface receptors to multivalent carbohydrate molecules exhibits wide variety of biological responses and has unique edge over monovalent interactions Mammen et al (1995 and (1998) reported polyacrylamides bearing pendent alpha sialoside groups as efficient inhibitors in agglutination of erythrocytes by influenza virus, demonstrating the role of polyvalency.

The affinity of the polyvalent inhibitor towards a surface of the virus is greatly enhanced compared to a monovalent sialic acid inhibitor. In addition high molecular weight polymers containing ligands inhibit binding between the virus and its receptor through steric exclusion. Polyvalent binding is characterized by the simultaneous interactions between multiple ligands on one entity and multiple receptors on the target biomolecule .

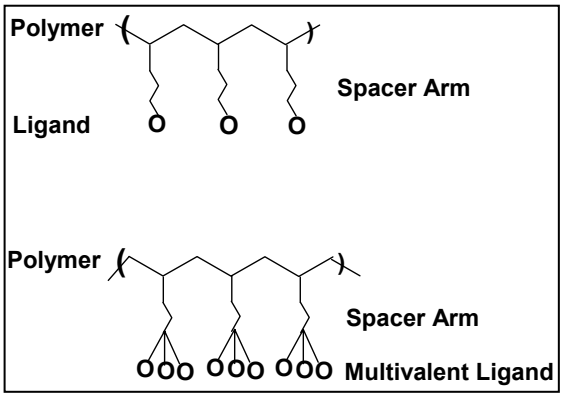


Fig 1.6 Monovalent and Multivalent ligands with spacer arm for orientation around the receptor of the biomolecule

Sigal et al (1996) reported the efficacy of polymers containing sialoside groups in inhibiting the adhesion of influenza virus to erythrocytes. The authors delineated the contributions of enhanced substrate ligand binding and steric considerations to efficiency of inhibition. These investigators reported sialic acid ligands, which can be exploited for the inhibition of the influenza virus. Monomeric inhibitor requires a higher concentration for inhibition since it is required to occupy at least half of the sialic acid binding sites on the virus, whereas the high molecular weight inhibitors need only a few attachments to achieve the same.

Carbohydrates can be utilized as binding entity to the receptors by conjugating with the monomer and polymerized to form a multivalent conjugate. Multivalent ligand may include shorter oligomers having pendant functional groups that may impart specific properties such as water solubility, hydrolytic stability etc. to the polymer.

1.4.1 Role of Steric stabilization:

In addition to the enhanced binding, steric exclusion resulting from the higher molecular weight of the polymeric ligands plays an important role in enhancing inhibition [Spaltenstein et al (1991)]. The novel approach is a model for pathogen-host interactions and was reported by Mammen et al (1995). It was demonstrated that agglutination of erythrocytes caused by influenza virus can be prevented by use of polyvalent sialic acid inhibitors and the contribution of the two factors mentioned above namely 1) High-affinity binding through polyvalency and 2) Steric stabilization as mechanisms for inhibition between the surfaces of virus and erythrocytes were quantified.

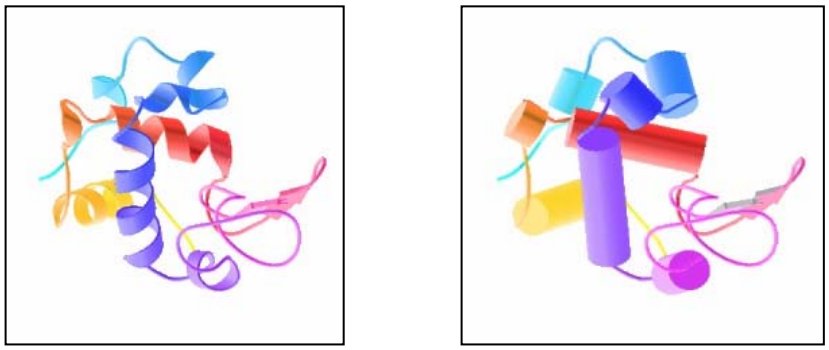
Sialic acid containing polyacrylamide exhibited significant increase in prevention of the agglutination of red blood cells by influenza virus. The differences in inhibition constants between monomeric and polymeric inhibitors were demonstrated on the basis of the total sialic acid groups in solution. It is expected that the synthesis of polymers containing ligands will provide a general route for higher affinity polyvalent ligands for a variety of receptors on the surfaces of viruses or cells [Sigal et al (1996)].

1.4.2 Lysozyme

Lysozyme is one of the few clearly understood enzymes. In 1922, Alexander Fleming discovered a substance in his own nasal mucus that had a capacity to lyse certain bacteria. The substance was later proved to be an enzyme and was named as “lysozyme.”

Moreover it was also found that the lysozyme existed widely in nature. Initially, Fleming found Lysozyme to possess antibacterial activity. Later he discovered the penicillin, the first true antibiotic. Subsequently lysozyme was studied thoroughly and many researchers investigated various aspects of the structure of lysozyme.

Lysozyme is widespread in animals and plants. Lysozyme is also found in mammalian secretions and tissues, saliva, tears, milk, cervical mucus, leucocytes, kidneys, etc. The most extensively studied lysozyme is from hen egg white (lysozyme "c").



Ribbons **Cylinder**

Fig. 1.7: Lysozyme structures

David Philips elucidated X ray structure of HEW in 1965. The protein molecule is roughly ellipsoidal in shape with dimensions of 30 x 30 x 45 Angstrom.

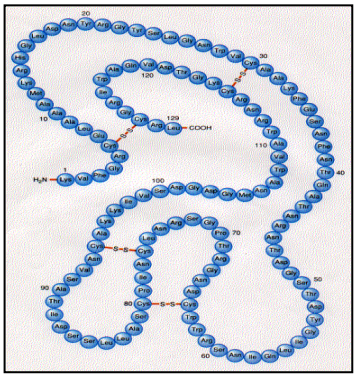


Fig. 1.8. Lysozyme structure

Lysozyme is a globular protein and contains a deep cleft on its surface. Chitohexose derived from substrate e.g. chitosan can fit into six sites of lysozyme. X-ray crystallography has shown that as lysozyme and its substrate unite, each is slightly

deformed. The fourth hexose in the chain becomes twisted out of its normal position. This imposes a strain on the C-O bond on the ring-4 side of the oxygen bridge between rings 4 and 5. A molecule of water is inserted between two hexoses, which breaks the chain. The energy needed to break this covalent bond is lower since the atoms connected by the bond have been distorted from their normal position [Voet and Voet (1994)].

The amino acid residues in the vicinity of rings 4 and 5 provide a probable mechanism for completing the catalytic action. Residue 35, glutamic acid (Glu-35), is about 3Å from the-O- bridge that is to be broken. The free carboxyl group of glutamic acid is a hydrogen ion donor and available to transfer H^+ to the oxygen atom. This would break the already-strained bond between the oxygen atom and the carbon atom of ring 4.

Lysozyme has the ability to catalyze the hydrolysis of complex polysaccharides that form bacterial cell walls. The protein has 129 amino acid residues, a MW of 14,600 D, and four -S-S- bonds. The natural substrate is a linear polymer of alternate residues of *N*-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM). The links that are hydrolyzed by the enzyme are the NAM-NAG bonds and not NAG-NAM.

Lysozyme (muramidase) hydrolyzes preferentially the [beta]-1,4 glucosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine which occur in the mucopeptide cell wall structure of certain microorganisms, such as *Micrococcus lysodeikticus.* A somewhat more limited activity is exhibited towards chitin oligomers [Holler et al. (1975)].

Characteristics of Lysozyme from Chicken Egg White:

Molecular weight: 14,388

Optimum pH: 9.2

Isoelectric point: pH 11.0

Stability: Lysozyme stored as a dry lyophilized or crystalline powder at 2–8 ^{0}C is stable for years. Solutions at pH 4-5 are stable for several weeks refrigerated and for days at ambient temperatures.

1.4.3 Nature of the binding site:

NAG oligosaccharides of less then five residues are very slowly hydrolyzed by HEW lysozyme although these substrate analogs bind to the enzyme's active site and are thus its competitive inhibitors. The X ray structure of the $(NAG)_3$–lysozyme complex

reveals that $(NAG)_3$ binds on the right side of the enzymatic binding cleft for sites A, B, and C. NAG associates with the enzyme through strong hydrogen bonding interactions, some of which involve the acetamido groups of residues A and C, as well through close – fitting hydrophobic contacts.

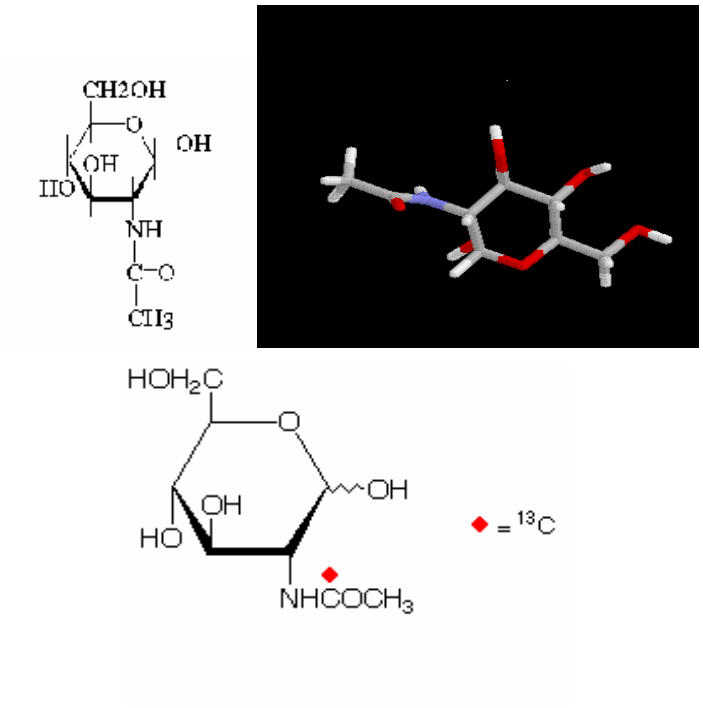


Fig. 1.9: *N*-acetyl glucosamine (NAG)

1.4.4 Lysozyme Catalyzed Hydrolysis:

$(NAG)_3$ takes several weeks to hydrolyze under the influence of lysozyme. It was thus presumed that the complex revealed by X ray analysis is unproductive. Therefore, the catalytic site of the enzyme is located at neither the A-B nor the B-C bonds. In order to locate catalytic site of Lysozyme, model was built to investigate how a larger substrate could bind to the enzyme.

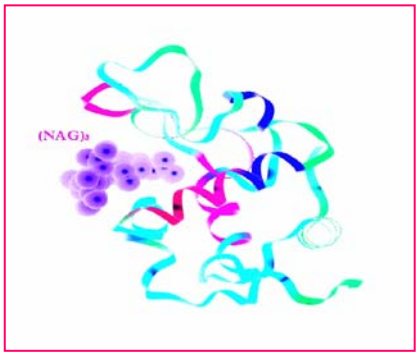


Fig. 1.10: Lysozyme-$(NAG)_3$ complex

Active site cleft of lysozyme is long enough to accommodate $(NAG)_6$, which the enzyme can rapidly hydrolyze.

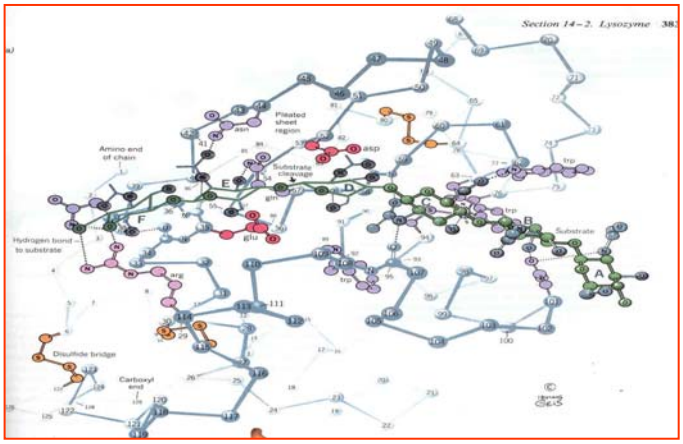


Fig. 1.11.- X ray structure of lysozyme bound to $(NAG)_6$ substrate (with green)

1.5.0 Catalytic mechanism of lysozyme:

(Residue numbers are for human lysozyme):

The mechanism of catalytic activity of lysozyme can be summarized as follow: [Voet and Voet (1994)].

1. A total of six NAG units fit in the active site cleft (subsites are designated "A-F").
2. A large number of interactions in the 'specificity pocket' define specificity for NAG.
3. Binding for NAG_3 = -24 kJ/mol
4. Binding for NAG 4 = +12.1 kJ/mol.
5. Therefore NAG bound in the active site is in a strained conformation – transition state stabilization.
6. This distortion is due to a hydrogen bond between the main chain of Val 110 and the O6 atom of NAG.
7. Glu 35 (pKa = 6.3) protonates the oxygen in the glycosidic bond (between NAG D and NAG E (acid catalysis)
8. The glycosidic bond is broken, resulting in a carbocation on the C1 of NAGD.
9. The carbocation is stabilized by formation of an oxonium ion. This requires the formation of a planar, half-chair conformation of NAGD.

10. The carbocation is stabilized by the negative charge on Asp 53 (pKa=3.5, quite normal), but a covalent intermediate is not formed. This is an example of transition state stabilization in enzyme catalysis.
11. The reaction is completed by attack of a water molecule (hydroxide) on the oxonium ion and the reprotonation of Glu 35.

1.5.1 Chitosan: Natural ligand for lysozyme binding

NAG trimer $(NAG)_3$ is known to be a powerful competitive inhibitor of lysozyme that binds to the same site on the enzyme as the substrate. In order to find where on the enzyme it binds, trimer was added to enzyme crystals in their mother liquor and allowed to diffuse into the crystals. These crystals were studied by X-ray diffraction. The comparison between the enzyme models with and without $(NAG)_3$ showed that the inhibitor bound to the cleft, but occupied only half of its length. This showed that the cleft was at least part of the substrate's binding site. Physical models of the molecules of enzyme and of NAG-NAM polymers resulted in following findings:

1. The cleft is completely filled by six sugar residues.
2. The weak interactions (hydrogen bonds, etc,) that probably determine the substrate binding and the amino acid residues could be identified.
3. Starting from one end of the cleft chosen arbitrarily, the sites where six consecutive sugar residues bind were numbered. It was found that binding of NAM to the third site was not possible because the bulkiness of the lactyl group could not be made to fit in it. NAG, on the other hand, has no difficulty fitting in it.

This leads to conclusion that site 3 must be filled with NAG and the only way in which the natural substrate can bind is as below:

1 2 3 4 5 6

.... NAG - NAM - NAG - NAM - NAG - NAM - NAG - NAM - NAG - NAM...

Since the glycosidic bond that gets hydrolyzed is the NAM-NAG bond, this hydrolysis take place between sites 2-3 or 4-5. In addition, most abundant products of the hydrolysis of $(NAG)_6$ are $(NAG)_4$ and $(NAG)_2$.

The catalytic site is not located in 2 -3 is because $(NAG)_3$ binds to 1 - 2 - 3 thereby preventing the binding of longer polymers, but the rate at which it is hydrolyzed

is negligible. Consequently, 4 - 5 emerges as the only reasonable binding. In addition, the standard free energy of binding of the sugar residues to the six sites and the rates of hydrolysis obtained with polymers of different lengths provide supportive information:

Table 1.8: Binding free energies for sugar residues

Site	1	2	3	4	5	6
ΔG_0 of binding (KJ/mole)	-8	-13	-20	+16	-10	-12
Number of NAG Residues in polymer	1	2	3	4	5	6

1. The +16 KJ/mole of free energy of binding to site 4 indicates a possible distortion of the substrate to force it to fit the site. In other words, the negative free energy of binding to the other sites is partially used to force a distortion of the fourth residue so that it fits. Observations of physical models of the molecules show that distortion consists of a flattening of one end of the chair configuration of the sugar ring. Furthermore, a comparison of the binding constants of $(NAG)_4$ and of its lactone (whose molecule has a flat shape) shows that the binding constant for the latter is 3600 times higher.
2. The tetramer hydrolyzes very slow, because of the greater probability due to its binding to the active (catalytic) site of the enzyme resulting in 500-fold increase in binding to that of pentamer. The hexamer has one more residue to contribute to binding strength, while longer polymers do not change this situation because the extra residues do not fit in the binding site.

1.5.2 Productive and non-productive binding:

Polyvalent ligands exhibit greater binding with the host receptor, however the interactions are random and unaccountable for their efficacy in terms of productive complex. Ligand-substrate interactions can be either productive or non productive, involving the occupancy of the active sites for the ligand. Productive binding is studied using competitive binding agent such as Biebrich Scarlet. Holler, et al (1975) described the method of estimating productive and non-productive lysozyme–chitosaccharide complexes. The addition of lysozyme and Biebrich Scarlet produces a number of changes

in the difference spectra. Biebrich scarlet–lysozyme complex against Biebrich Scarlet alone exhibits a different spectrum due to a red shift of the absorbance maximum from 505 nm for the free dye to 510 nm when bound to the enzyme. The difference spectrum has a small maximum at 459 nm and an isobestic point at 550 nm. The difference coefficient for the minimum at 495 nm was obtained from the intercept of a Hildebrand–Benesi plot.

Biebrich scarlet is a competitive inhibitor of lysozyme. Binding does not affect the dissociation constant of the lysozyme-Biebrich Scarlet complex, but perturbs the absorption spectrum of Biebrich scarlet. The experiments show that Biebrich scarlet is a competitive inhibitor for cell wall hydrolysis and that it binds to catalytic part of the active site from which it can be displaced by chitosaccharides that interact with the site.

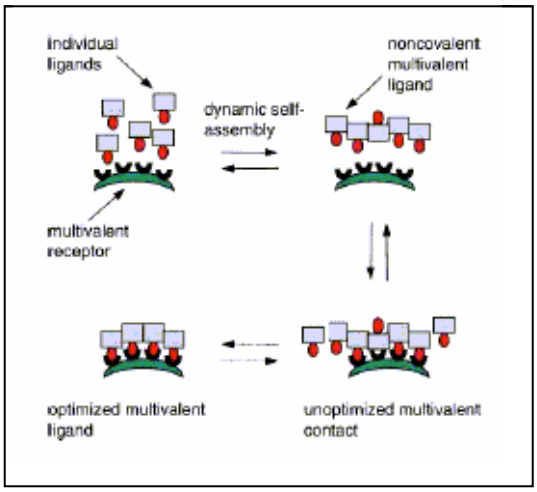


Fig. 1.12 : Optimized multivalent ligands for productive binding

1.6.0 Design of polymers for affinity precipitation of biomolecules

1. The ligands must have specific affinity for the interacting molecule.
2. They must have functional group for conjugation with stimuli sensitive polymers.
3. Have greater binding constant.
4. The binding efficiency of the ligand should be retained when incorporated in the polymer.
5. The polymer should phase separate at a temperature well below the thermolabile temperature of the biomolecules.
6. The ligand should not affect the nature and structure of the biomolecules.

7. The polymer should not be too hydrophobic as it can affect the activity of the recovered protein / enzyme [Galaev and Mattiasson (1993 b)].
8. Polymer should redissolve after the complex is formed between the ligand and the biomolecules.
9. The precipitation cycles must retain the efficiency of interactions.

Thermoprecipitating polymers containing NAG ligands must contain appropriate amount of ligands for the effective interactions and binding. The polymer must phase separate at lower temperature without affecting the nature of the recovered biomolecule.

Literature review provides an overview of the present status of the affinity precipitation and the critical components required for the effective separation. The review highlights certain gaps. The objective of the present investigation is to bridge these gaps and to enhance our understanding of the factors which influence ligand-substrate interactions so that the choice of ligands to be used in affinity based separations can be made on the basis of this improved understanding.

The binding of lysozyme to chitosan is always initiated with the binding between the NAG unit in chitosen with site A in lysozyme. The dissociation constants are nearly the same for all possible bound complexes. The ligand selected in the present study is NAG that can bind to the site C of lysozyme. Raftery and Dahlquist (1968) studied the association equilibria of NAG anomers and lysozyme by N.M.R. spectroscopy. They demonstrated the associations by estimating binding constants by proton N.M.R. spectroscopy.

Natural ligands containing carbohydrates are susceptible to microbial growth and hydrolysis by the enzymes. Hence, there is a need to synthesize ligands similar to repeat units of chitosan, which will not be hydrolyzed by lysozyme and stable. Nishimora et al (1994) reported the newer glycosides containing glycopolymers especially homopolymers for interactions with WGA and erythrocytes. Further insights into the design of ligands can be obtained from Kristiansen et al (1999) who reported the binding of the *N*-acetylated sugar residues in chitosan primarily to the sites of WGA rather than to chitin–oligosaccharides and Kobayashi et al (1997) who reported the newer artificial glycoconjugates polymers for enhanced interactions with lectins.

1.6.1 Monomer and oligomers of *N*-Acetyl glucosamine (NAG) for enhanced binding:

The first comprehensive analysis of binding problems applied to biomolecules was presented by Scatchard (1949). A quantitative methodology involved estimating dissociation constants, the number of binding sites for different classes of biomolecules and ligand-ligand interactions were studied. Experimental results of the concentration of bound complexes as a function of the free ligand concentrations was reported.

Chitosan is most powerful ligand, which binds to lysozyme through NAG residues and is obtained by deacetylation of chitin. On industrial-scale, chitin is produced by treating seafood waste, especially shells from crustaceans (shrimps, crabs, lobsters, krills, etc). Chitosan are acid soluble polysaccharide [Kristiansen et al (1999)] and consist 2-acetamido-2-deoxy-β-D-glucose (GlcNAc;A-unit) and 2–amino-2-deoxy-β-D-glucose (GlcNAc, D-unit). NAG can bind to the active sites of the lysozyme which comprises subsites designated A-F. Specific binding of chitosan sequences to lysozyme begins with binding of the NAG units in the site C.

Binding between lysozyme and purified oligosaccharides as well as binding with highly *N*-acetylated chitosan where hexamer sequences containing A units interact with lysozyme has been extensively studied [Lumb et al (1994)]. Lysozyme can bind to six consecutive sugar units, and therefore our objective is to prepare ligands identical to NAG units similar to that of natural chitin and chitosan.

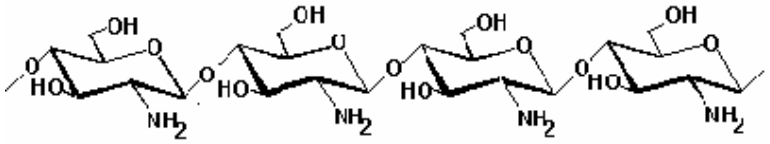


Fig. 1.13 Chitosan

1.6.2 Spacer effect:

The interacting ligand can bind more efficiently to biomolecule by incorporation of the spacer. The advantage of incorporating spacer arms is the enhanced accessibility of the ligand to the active sites of the host receptor. Vaidya et al (1999) reported series of copolymers containing spacers; the value of K_i decreased from 275 x 10^{-6} M (*N*-isopropyl acrylamide co p-aminobenzamidine (PABA) to 25 x 10^{-6} M (*N*-isopropyl acrylamide –

co- 6-Amino Caproic acid. PABA) as the spacer chain length increased from 0 to 5 carbon atoms.

The spacers of suitable carbon atoms will be incorporated to the monomer containing NAG for enhanced flexibility and accessibility to the active sites of the lysozyme.

1.6.3 Designing copolymers for lysozyme recovery:

The present work was undertaken after a critical literature survey to design affinity precipitating polymers containing NAG for enhanced interactions. The effects of various architecture on LCST will be studied as to design polymers for lysozyme recovery.

1. They can be used for the purification of monomeric as well as multimeric proteins.
2. The method is more convenient to scale up the bioseparation.
3. Polymers can be designed for the specific stimuli such as pH or temperature.
4. The process is carried out in homogenous environment, thus eliminates the problems associated in affinity chromatography and affinity ultrafiltration.
5. The process requires lesser equipments.
6. The ligands can be recycled after the recovery of biomolecule [Vaidya et al (1999)].

Although there is vast literature available on affinity precipitating polymers there is hardly any report on designing polyvalent ligands for enhanced interactions. The thermoprecipitating copolymers containing increasing concentration of NAG will therefore be synthesized. The random, block and graft copolymers containing varying amount of the NAG ligands will also be synthesized.

1.6.4 Block copolymers :

In the field of polymers new synthetic strategies and potential technologies are being applied to obtain polymers of the desired characteristics. Much attention is being given to the synthesis of block copolymers, especially for the monomers having dramatically different reactivity ratios. Benoit et al (2002) reported one step formation of functionalized block copolymers using free radical polymerization method. Block

copolymers reported were of maleic acid / styrene obtained by a single step, whose molecular weights are controlled up to 10000 while retaining low polydispersities.

Block copolymers can be prepared by attaching homopolymers to another block of the homopolymer or the statistical copolymer structure in which there is a regular distribution of groups along the copolymer chains. Block copolymers thus prepared will have well defined structure compared to the random copolymers. Therefore, in the present investigation it was thought worthwhile to synthesize block copolymers containing NAG ligand in juxtaposition for enhanced interactions. Moreover, the resulting block copolymers will be temperature sensitive and can be used for the recovery of lysozyme.

Similarly, the triblock copolymers can be designed by synthesizing difunctional homopolymers of the poly *N*-isopropylacrylamide using 4-azobis cyanovaleric acid and condensing with another polymer synthesized in the presence of hydroxyl or amine chain transfer agents [Takai et al, (1993)] such as mercapto propionic acid. The monofunctional reactive NAG oligomer prepared using mercapto ethanol can be coupled to reactive poly *N*-isopropylacrylamide using coupling agents. The resulting copolymer will be BAB block copolymers. It is expected that the polyvalent NAG units will bind effectively to the active sites of the lysozyme, where the central segment will provide steric exclusion.

1.7.0 Molecular imprinting (MIP): Selective recognition method for biomolecules

Introduction:

Controlling molecular order up to the meso and macroscopic scale constitutes one of the significant advancements in materials research [Dhal et al (2001)]. Molecular imprinting is a technique whereby functional monomers are allowed to self-assemble around a template molecule and subsequently crosslinked. The template molecule is removed, leaving behind a cavity complementary in shape, size and functionality, which will bind another molecules identical to the template.

Molecular imprinting finds applications in diagnostics, drug discovery processes, biotechnology, chemo-enantio and bio-selective separations and site-specific drug delivery etc [Hoss et al (1994)]. Design of host specific molecules using molecular imprinting technique has been investigated since long time as it finds applications in

bioseparation, enzyme, mimics, chiral separations and antibody mimics. The technique creates selective binding sites in synthetic polymers [Mosbach, et al (1994)].

1.7.1 General principle of molecular imprinting:

Two basic approaches for molecular imprinting are followed (1) the pre-organization approach, developed by Wulff and coworkers, where the aggregates in solution prior to polymerisation are maintained by (reversible) covalent bonds, and (2) the self-assembly approach, developed by Mosbach and coworkers, where the pre-arrangement between the print molecule and the functional monomers is formed by non-covalent or metal coordination interactions. Both imprinting approaches make use of a high percentage of crosslinker. The resulting polymers are of substantial rigidity and completely insoluble. The template-assisted assembly, leads to an artificial recognition matrix.

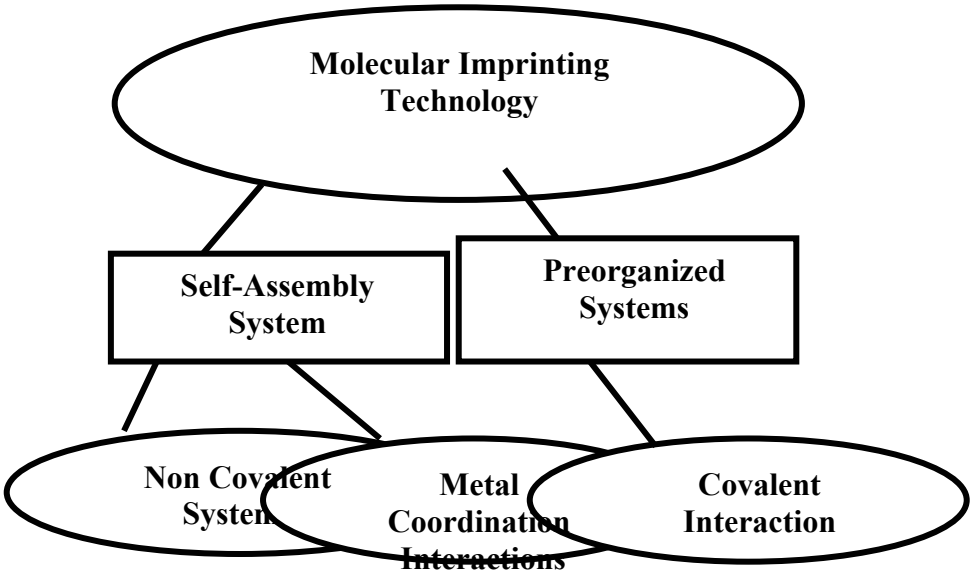


Fig. 1.14 Approaches to molecular imprinting technology

Variety of molecules such as drugs, amino acids, carbohydrates, proteins, nucleotide bases, hormones, pesticides and co-enzymes have been used for imprinting. These compounds have been successfully used for the preparation of selective recognition matrices. However, imprinting strategies involving, non-covalent interactions between the print molecule and the functional monomers are more versatile. The apparent weakness of these interactions may be overcome by allowing a multitude of interaction

points simultaneously. Furthermore, the use of non-covalent interactions in the imprinting step closely resembles the recognition pattern observed in nature.

Monomers, which can be involved in noncovalent interactions, are brought together using template molecules and the crosslinking agent to form well defined aggregates of template and monomer. Wulff (1995) reported molecular imprinting in cross linked materials using molecular templates to form artificial antibodies. Highly specific binding sites were obtained. A definite shape containing functional groups in a predetermined orientation in a polymerizable form of a phenyl-α-D–mannopyranoside as a template was obtained, to which two molecules of 4 vinyl phenylboronic acid were bound by esterification with two hydroxyl of the sugar. Boronic acid was chosen as the binding group as it undergoes a rapid and reversible reaction with diols. The monomer was subjected radical copolymerization with large amounts of a cross linking agent such as ethylene dimethacrylate in the presence of an inert solvent.

In biological systems, molecular complexes are formed by a plethora of non-covalent interactions such as hydrogen bonds and ion pairing. These interactions are weak in nature compared to covalent bonds, however the simultaneous action of several of these weaker bonds often leads to complexes with greater stability.

Another method of bio imprinting was investigated by Stahl and Mosbach (1991). α- chymotrypsin was precipitated in the presence of *N*-acetyl-D-phenylanine with 1-propanol and was further dried. After removal of templates, modified α-chymotrypsin exhibited good selectivity for the formation of D-phenylalanine ethyl ester in non aqueous solution, whereas an enzyme analogously treated in the absence of a template showed virtually no selectivity for the reaction.

Recently Yu et al (2002) reported preparation of a class of enzyme inhibitors including a chiral compound, by using imprinted polymers or biomolecules as molecular scale reaction vessels. In the first approach kallikrein inhibitor was imprinted. After extraction of the template inhibitor, the condensation reaction was carried out within the cavities of the MIP. The products of condensation reactions were evaluated to inhibit kallikrein. The products thus formed in the MIP cavity are ‘double imprints’. The process is also called anti–idiotype approach as it relates to the known biological phenomenon of the formation of anti antibodies to existing immunoglobulins.

In the second approach, chiral template L-phenylalanine methyl ester derivative form enantio specific synthesis inside the MIP binding cavities and used as a template. (2-Trifluromethyl) acrylic acid and divinyl benzene were used as the functional monomer and crosslinker, respectively. After preparation of MIP the template was removed. The nucleophilic reaction was allowed to take place between the positively charged triazine derivatives and various hydrophobic amines inside the cavities to give either the same or related inhibitors, depending on the added amine. Condensation in the polymer cavities form larger products which was not possible with an imprinted polymer prepared for smaller template in a previous study – presumably because of the steric hindrance.

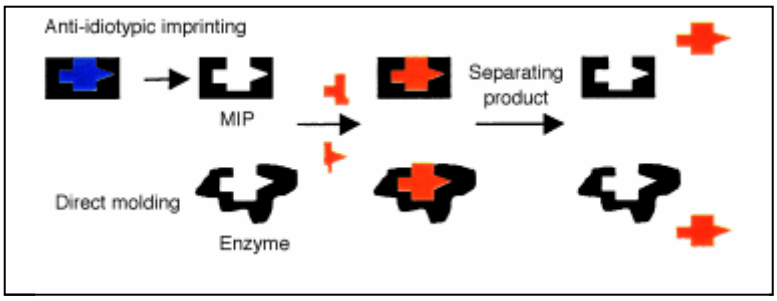


Fig. 1.15 Schematic representation of the anti-idiotypic and direct molding approaches [Yu et al (2002)]

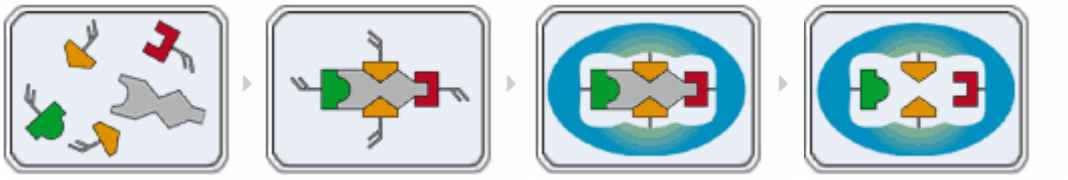


Fig. 1.16. Schematic representation of molecularly imprinted polymers

Hokfelt et al (1980)] reported peptide-macromolecule interactions, which are ubiquitous in nature such as enkephalins, assisted in signaling, peptide hormones (e.g. corticotropin, vasopressin), and peptide antibiotics (e.g., gramicidins). A critical element of the above processes is recognition of a specific peptide by a macromolecular receptor. It was therefore thought worthwhile to prepare artificial binding sites for such peptides that may provide insight into recognition processes. In addition these artificial receptors

may facilitate the screening of peptide mixtures or assist in the evaluation of peptidomimetics that can be used to either enhance or inhibit receptor responses.

Bradley et al (2001) reported protocols for molecular imprinting that create macromolecular receptors for small peptides. Both polymerization and recognition steps are carried out in an aqueous environment. The peptide recognition sites have been achieved by incorporating two types of interactions. One consists of strong, specific binding between an *N*-terminal His residue and Ni (II) bound to the polymer. The second bonding comprises multiple weaker interactions between the network polymer chains and the imprinting peptide molecule that are established during the polymerization. The polymerizable methacrylamide-NTA-Ni^{2+} mixed complex was prepared by combining aqueous solutions of nitrilotriacetic acid complex, monomer with $NiSO_4$. The pre-polymerization complex was then formed by addition of the *N*-terminal histidine peptide His-Ala. Copolymerization of this complex (5 mol %) with *N,N*-ethylenebisacrylamide cross-linking monomer (82 mol and acrylamide (13 mol %) provided a pale blue monolith. The polymer, which was formed in quantitative yield, was triturated and washed with water to remove the template. The binding, which relies on a metal-ligand interaction, is not compromised by water or other solvents.

Shi et al (1999) reported template-imprinted nano structured surfaces for protein recognition. The investigators used radio-frequency glow-discharge plasma deposition to form polymeric thin films around proteins coated with disaccharide molecules. The disaccharides are covalently attached to the polymer film, creating polysaccharide-like cavities that exhibit highly selective recognition for a variety of template proteins, including albumin, immunoglobulin G, lysozyme, ribonuclease and streptavidin. Direct imaging of template recognition is achieved by patterning a surface at the micrometer scale with imprinted regions.

Molecularly imprinted polymers (MIPs) are reported by our group for a specialized separation technique using various biomolecules. The US patent granted to our group [Vaidya et al 2002)] describes the process for preparation of trypsin and chymotrypsin imprinted polymers useful for separation of enzymes. The invention describes polymerization of complex comprising enzyme and affinity monomer, a comonomer and a crosslinker. Molecularly imprinted polymers synthesized in the

presence of biomolecules as templates impart the advantages of higher affinity and selectivity. Monomer *N*-acryloyl 4-phenyl butylamine and chymotrypsin form chymotrypsin-*N*-acryloyl 4-phenyl butyl amine complex. The comonomer used was acrylamide along with methylenebis acrylamide as a crosslinker. Template chymotrypsin was extracted from the particles by 3-4 alternative treatments of acetone and chloroform. The resulting chymotrypsin imprinted polymers were further used for enzyme separation.

Imprinted polymers in general display good recognition properties and are usually prepared in non-polar organic solvents such as chloroform or toluene. Biological recognition mainly occurs in hydrophilic environment and therefore it is important to synthesize MIPs containing ligands capable of interactions with a receptor molecule in the aqueous medium. Preparation of imprinted polymers in aqueous system has proven to be a difficult task, since the water molecule destroys the hydrogen-bonding interactions between functional monomer and the template molecule. Moreover, commonly used cross-linkers do not dissolve in water.

Biomolecules such as enzymes and proteins are thermolabile and may undergo structural changes under the experimental conditions used for polymerization in the presence of these templates and lose their biological activity. The choice of such biomolecules as templates in organic solvents may alter their conformation and cause loss of specificity. Thus there is a need to synthesize imprinted polymers in aqueous media so that the biomolecules used are compatible and stable. Such systems can find wide range of applications in biomolecular recoveries and medicine.

Using controlled chemical synthesis methods such as molecular imprinting, it would be possible to control the spacing, steric accessibility, number of ligand molecules in the polymer. Moreover, molecular weight, density, solubility and structure of the imprinted polymeric conjugates can be manipulated as desired.

With a view to enhance ligand substrate interactions it was thought worthwhile to synthesize molecularly imprinted polymers containing polyvalent ligands. Molecularly imprinted ligands for enhanced interactions have not been reported in the past to our knowledge. The approach reported here could be a model to develop molecular imprinted polymers for other ligands such as; sialic acid used for the inhibition of influenza and rotavirus infections.

1.7.2 Concluding remarks:

Literature survey highlights the importance of affinity-based separations in biotechnology. However, there are certain shortfalls and gaps in the technology. The interactions of the ligand conjugated in polymers are weak and there is tremendous scope to enhance host receptor interactions using polyvalent ligands. Design of polyvalent ligands of various architectures is undertaken in the present study. These polymerizable monomers containing NAG can be homopolymerized or copolymerized with NIPA to form stimuli sensitive polymers. An ideal polymer would be one that does not show non-specific adsorption, apart from being readily available and being non-toxic.

Therefore, the objective of the present study was to develop affinity-precipitating polymers containing NAG, which is a part of natural polymers such as chitin and chitosan for enhanced lysozyme recovery. The resulting polymers will be more stable and aqueous soluble than NAG containing natural chitosan and hopefully will exhibit stronger binding to lysozyme.

This investigation emphasizes the role of polyvalent binding of the NAG to lysozyme and merits *vis a vis* the monovalent counterparts. The scope and the objectives of the present investigation are discussed in the next chapter.

2.0 Objectives and scope of work

The literature review presented in the first chapter has highlighted the necessity of designing affinity ligands for enhanced binding with the biomolecules and the progress made so far. The enhanced ligand-protein binding in biology has implications in cell adhesion, cell recognition, fertilization and biorecognition. The present investigation has been undertaken with a view to tailor polyvalent ligands comprising stimuli sensitive polymers, which will exhibit enhanced binding towards biomolecules and demonstrate their utility in the recovery of biomolecules.

The objectives of the present investigation are summarized below:

1. To design and synthesize stimuli sensitive polymers, especially thermoprecipitating polymers that will exhibit enhanced binding with the biomolecules.
2. To exploit knowledge of active sites present on the enzymes for tailoring polyvalent ligands.
3. To design monomers and oligomers containing carbohydrates that will be more stable than natural ligands.
4. To synthesize and evaluate the efficacy by incorporating spacer in the ligands.
5. To synthesize thermoprecipitating copolymers containing polyvalent ligands for bioseparations.
6. To design and synthesize oligomers, graft and block copolymers containing polyvalent ligands for enhanced binding.
7. To establish the role of polyvalency and the steric stabilization effect in inhibition.
8. To evaluate the efficacy of synthesized ligands in terms of binding constant (K_b) and inhibition concentration (I_{50}) for enzymes.
9. To study the interactions of polyvalent ligands at the molecular level by productive and non-productive binding studies.
10. To synthesize molecularly imprinted polymers using biomolecules as a template for enhanced affinity. To establish specificity of imprinted copolymers prepared

in the presence of biomolecule with the polymers prepared in the absence of biomolecule.

11. To establish the effect of the copolymer composition and architecture in affinity precipitation.
12. To asses the overall efficacy of the affinity thermoprecipitating polymers and recover biomolecules.

Although, the objectives of the present invention are set in broader terms, the scope has been limited to illustrate the new methodologies for enhanced binding with reference to lysozyme as a biomolecule and NAG as the ligand.

The investigation has helped to highlight the importance of tailoring polymer architecture for enhanced substrate binding and provided structural evidence in support of the results obtained and explanations offered. It has also brought to attention new synthetic strategies and experimental techniques, which can be exploited for, further investigation into these and related areas. Since polyvalent interactions involving proteins and carbohydrates have wide implications in biology, the synthetic methods reported in the present investigation can be adapted for other affinity ligands to enhance interactions e.g. polymeric sialic acid ligands that inhibit haemagglutination of host red blood cells caused by influenza virus. Polyvalent ligands seem to be promising for enhanced interactions and bioseparation. Moreover, the ligands such as sialic acid, mannose and galactose can be used as alternative therapeutics to drugs and antibacterial as they are not expected to lead to drug resistance.

The scope of the present investigation is defined more precisely as follows:

1) To design and synthesize stimuli sensitive polymers, especially thermoprecipitating polymers containing NAG for enhanced interactions with lysozyme.
2) To exploit the knowledge of active sites present on lysozyme for designing polyvalent NAG ligands.
3) To design monomers and oligomers containing NAG that will be more stable than natural ligands containing NAG e.g. chitin and chitosan.
4) To synthesize and evaluate the effect on binding by incorporating spacer in NAG monomer.

5) To synthesize thermoprecipitating copolymers of NIPA and Ac.NAG in varying feed ratio for the recovery of lysozyme.
6) To incorporate spacer 6-amino caproic acid and establish the spacer effect in enhanced interactions.
7) To design and synthesize graft and block copolymers containing polyvalent NAG for enhanced binding.
8) To establish the role of polymer in steric stabilization.
9) To evaluate the efficacy of synthesized ligands in terms of binding constant (K_b) and inhibition concentration (I_{50}) for lysozyme.
10) To study the interactions of polyvalent NAG ligands at the molecular level by competitive binding studies.
11) To compare competitive lysozyme-NAG complex at catalytic and non catalytic binding sites of lysozyme using Biebrich Scarlet dye.
12) To synthesize molecularly imprinted polymers containing NAG using lysozyme as a template and compare the polymers so prepared *vis a vis* the polymers prepared in the absence of lysozyme.
13) To asses the overall efficacy of the affinity thermoprecipitating polymers containing NAG and recover lysozyme.

3.0.0 Experimental section:

Materials:

Acrylic acid, *N*-isopropylacrylamide (NIPA), methacrylic acid, 6-amino caproic acid, mercaptoethanol (ME), mercapto propionic acid (MPA), dicyclohexyl carbodiimide (DCC), *N, N, N', N''*-Tertramethylethylenediamine (TEMED), Biebrich scarlet were purchased from Aldrich Chemicals Inc. and used without further purification. *N*-acetylglucosamine (NAG), Lysozyme (3x crystallized, dialyzed and lyophilized, activity 47000 units per mg solid), one unit activity of lysozyme corresponds to a change in absorbance (ΔA_{450}) of 0.001 per minute at pH 6.2 at 25 ^{0} C using suspension of *Micrococcus lysodeikticos* (*Micrococcus luteus* ATCC No. 4698) were obtained from Sigma and used as supplied, 1,4-dioxan, acetone, Sodium hydroxide, Sodium bi carbonate, ethyl acetate, methanol, dimethylformamide (DMF), Ammonium per sulphate (APS), Azobisisobutronitrile (AIBN), Diethyl ether, Thionyl chloride ($SOCl_2$) etc. were supplied by local suppliers. All the solvents were distilled and then used for the reactions.

3.1.0 Instrumentation

Molecular weights were determined by using Knauer Vapor Pressure Osmometer (VPO) K-7000.

Fluorescence measurements were carried out on Perkin-Elmer LS-50B luminescence spectrophotometer with excitation at 290 nm. The solutions were placed in 10 mm quartz cells maintained at 18 ^{o} C.

Lysozyme inhibition and productive binding studies using Biebrich Scarlet dye were carried out using Shimadzu UV spectrophotometer.

NMR:

High resolution ^{1}H NMR spectra were measured with a Bruker–DRX 500 spectrometer.

^{1} H spectra were obtained on Bruker DRX-500 super conducting Fourier Transform NMR spectrometer operating at 500-13 MHz for proton. For ^{1} H spectrum a 45 ^{O} pulse width of 4.8 μs and a repetition time of 1 S were used to accumulate 64 transits. The ^{1} H chemical shifts were scaled (calibrated) by the proton signals left at 4.80 ppm in D_2O (taken as reference).

Synthesis of ligands:

3.1.1 Synthesis of Acryloyl *N*-Acetyl Glucosamine (Ac. NAG)

NAG (11.1 gm) and sodium bicarbonate (4.2 gm.) were dissolved in distilled water (80 ml). The resulting clear solution of pH 8-9 was stirred continuously on a magnetic stirrer at 5-10 ^{0} C. Acryloyl chloride (5 ml) in dichloromethane (5 ml) was taken in addition funnel and added dropwise to the solution. The reaction mixture was maintained in the pH range of 7.4–7.8 with addition of saturated solution of sodium bicarbonate. Unreacted acryloyl chloride was extracted in ethyl acetate (100 ml.) and the clear aqueous layer was separated and acidified to pH 5.0 by concentrated HCl. Finally Ac. NAG was precipitated in distilled acetone and dried under vacuum. ^{1} H NMR spectra were characterized using Bruker 500 MHz.

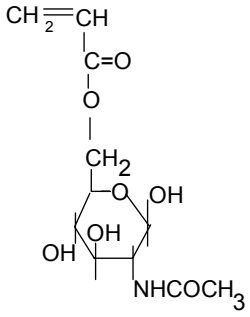


Fig. 3.1 Schematic representation of Acryloyl NAG

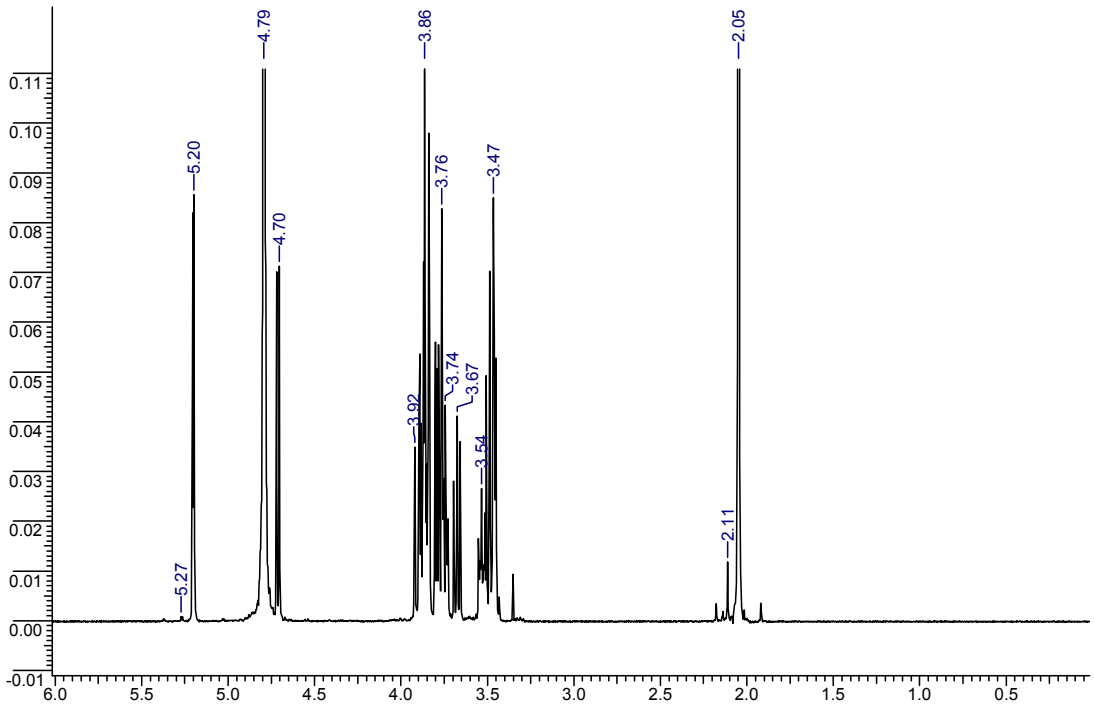


Fig. 3.2: ^{1} H NMR spectra of NAG

IR (nujol)

1560 cm^{-1} (acrylic double bond), 1635 cm^{-1} (amide carbonyl), 3323 cm^{-1} (-OH,-NH stretching)

^{1} H NMR (D_2O)

2.0 δ 3 H (-CO-CH_3), 2.6 δ 2H (-O-C$\underline{H}_2$-), 3.4 δ 2H ($\underline{H}$ at C_3 and C_5 of glucose ring), 3.8 δ 3 H ($\underline{H}$ at C_1,C_2 and C_4 of glucose ring), 5.2 δ 1H (-$\underline{H}_2$C=C$\underline{H}$-).

A generalized scheme for the synthesis of various ligands containing NAG is shown in fig.3.3

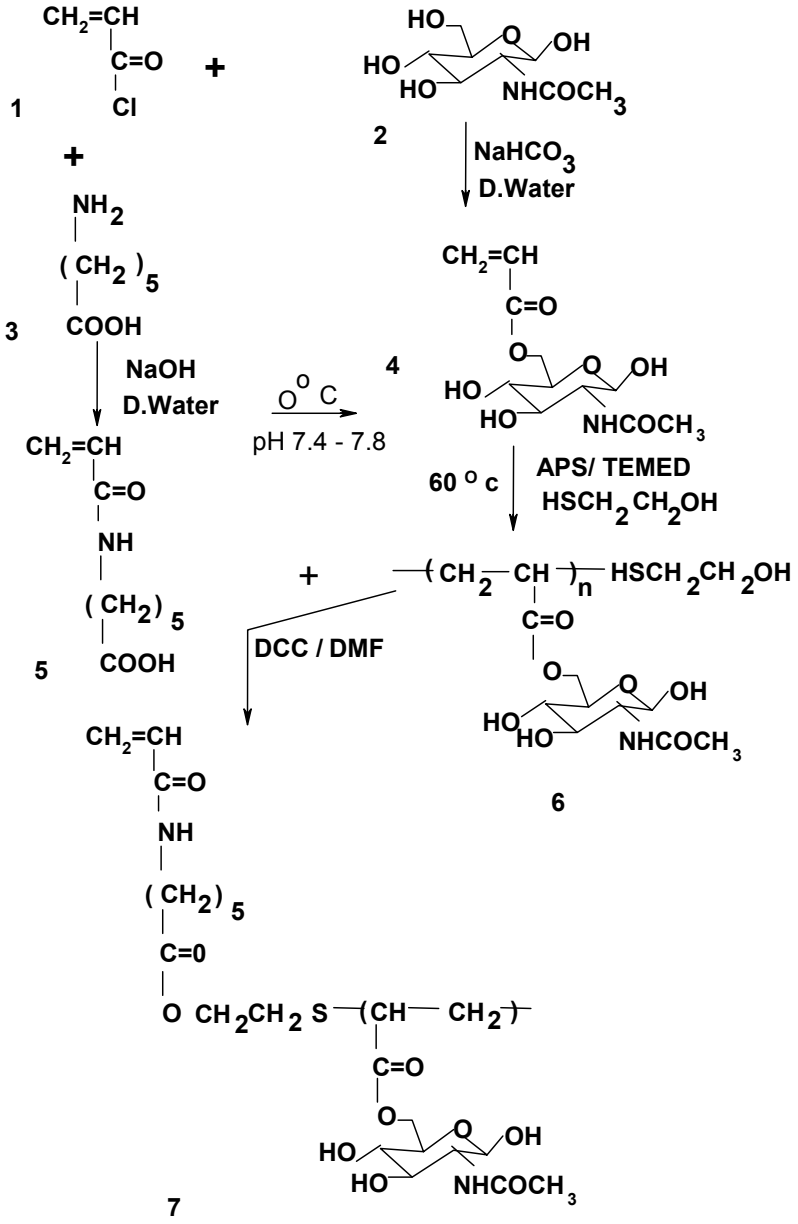


Fig. 3.3: Scheme for synthesis of NAG ligands

Where,

1. = Acryloyl chloride,
2. = *N*-acetyl glucosamine (NAG)
3. = 6- Aminocaproic acid (6-ACA)
4. = Acryloyl NAG (Ac.NAG)

5. = Acryloyl 6-ACA (Ac.6-ACA)
6. = PAc.NAG Mercaptoethanol
7. = [Acryloyl NAG]-6-(-*N* acryloyl-amino) caproate (Macromer)

3.1.2 Synthesis of *N*- Acryloyl- 6-amino caproic acid (Ac.6-ACA)-(spacer)

250 ml capacity beaker was equipped with a dropping funnel and pH meter. 6-amino caproic acid (ACA) (4 gm.) and sodium hydroxide (6 gm) were dissolved in 80 ml distilled water. The resulting solution was stirred continuously at 5-10 ^{o} C on a magnetic stirrer. Acryloyl chloride (3 ml) was mixed in dichloromethane (3 ml) and added dropwise to the solution. The pH of reaction mixture was maintained in the range 7.4 – 7.8 by the addition of 10 M NaOH solution. Unreacted acid chloride was extracted in ethyl acetate (100 ml). The clear aqueous solution was acidified to pH 5.0 by concentrated HCl and the product was extracted in ethyl acetate (3 x 100 ml). The organic layer was dried on anhydrous sodium sulfate and concentrated under vacuum. The viscous liquid was added to 500 ml petroleum ether. The solid Acryloyl 6-amino caproic acid was obtained and dried under vacuum for 48 hrs.

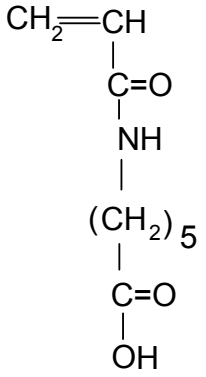


Fig. 3.4: Schematic representation for Acryloyl 6-ACA

IR (Nujol)

3284 cm $^{-1}$ (-NH and –OH stretching) , 2978 cm $^{-1}$ and 2852 cm $^{-1}$ (-CH stretching) , 1697 cm $^{-1}$, (-CO stretching of COOH), 1650 cm $^{-1}$ (-CO stretching of –$CONH_2$), 1622 cm $^{-1}$ (-C=C stretching), 1546 cm $^{-1}$ (-NH bending)

^{1} H NMR (D_2O)

2.3 δ 2 H (-C$\underline{H}_2$-COO), δ1.7 (-C$\underline{H}_2$-)$_2$, 1.4 δ (-C$\underline{H}_2$)$_2$-C$\underline{H}_2$), 3.3 δ 2 H (-N_2-C$\underline{H}_2$), 5.6 δ 1H (-C$\underline{H}_2$=C$\underline{H}$), 6.2 δ 2H (-C$\underline{H}_2$=CH-).

3.1.3 Synthesis of *N*-Acryloyl- 6-amino caproyl *N*- acetyl glucosamine (Ac.6-ACA.NAG)

Acryloyl 6-amino caproic acid (Ac. 6-ACA) (5 gm) and. *N*- acetyl glucosamine (NAG)(6.4 gm) were dissolved in 20 ml dimethylformamide (DMF). The clear solution was obtained by continuous stirring and added dicyclohexyl carbodiimide (DCC) (5.50 gm) was added as a coupling reagent. The reaction mixture was stirred continuously for 24 hrs. at room temperature. Dicyclohexyl urea (DCU) was filtered off and *N*- Acryloyl-6-amino caproyl *N*- acetyl glucosamine was precipitated in distilled acetone and was vacuum dried for 48 hrs.

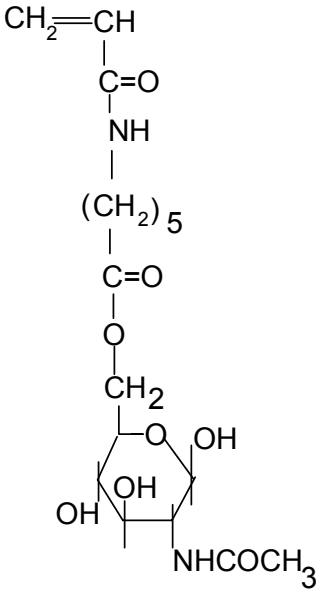


Fig. 3.5: Schematic representation for *N*- Acryloyl- 6-amino caproyl -*N*- acetyl glucosamine (Ac. 6ACA.NAG)

3.1.4 Synthesis of homopolymers of Acryloyl *N*-Acetyl Glucosamine (P.Ac.NAG)

7.27 x 10^{-3} M of Acryloyl-*N* Acetyl Glucosamine (Ac.NAG) was dissolved in 25 ml distilled water in a round bottom flask and stirred continuously to obtain a clear solution. 4 mg. Ammonium per sulphate as initiator and TEMED (1%) as an accelerator were added to the reaction mixture. The reaction was carried out at 40 ^{0} C. for 24 hrs. under continuous nitrogen purging. The solution was concentrated and precipitated in

acetone, reprecipitated in acetone to eliminate unreacted monomers and dried under vacuum.

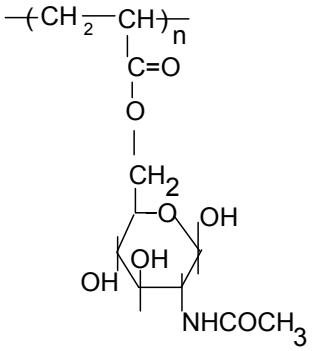


Fig. 3.6 Schematic representation for Poly (Acryloyl NAG)

3.1.5 Vapor Pressure Osmometer: (VPO)

Molecular weights of polymers were measured using Vapor Pressure Osmometer –K 7000 (VPO). The number of moles of substance dissolved in a solution influences the measurement value in osmometry. The molecular mass can be determined if the sample concentrations are known.

The number average molecular weight of the sample is determined by comparing the response of the instrument in terms of millvolts as a function of polymer concentration in terms of k values.

Method of estimation of molecular weight using VPO is as follows:

Solvent used = Dimethyl formoamide (DMF)

$K_{calibrated}$ for Benzil was measured in DMF = 256 kg / mol at 95 ^{O}C.

Temperatures for VPO measuremnets:

a) Cell temperature t_1 = 95 ^{O}C.

b) Head temperature t_2 = 95 ^{O}C.

Gain settings = 32

Time = 4

NIPA-co-Ac.NAG copolymer stock solution (25 mg/ 10 ml) was prepared in spectroscopy grade DMF. Polymer solutions increasing concentrations were injected and maintained in VPO at 95 ^{O} C. Calibration for zero reading was competed and readings for samples were noted.

The molecular mass of the sample was determined from the following eqn:

M [g/ mol] = K_{calib} / $K_{measured}$

Number of Injection	C in gm / kg	MV	MV/C in kg/g
1	20	0.156	128
2	35	0.312	112
3	72	0.625	131
4	165	1.25	132
5	326	2.5	130

Table 3.1 Calculations for molecular weight for NIPA –co-Ac.NAG (86/14)

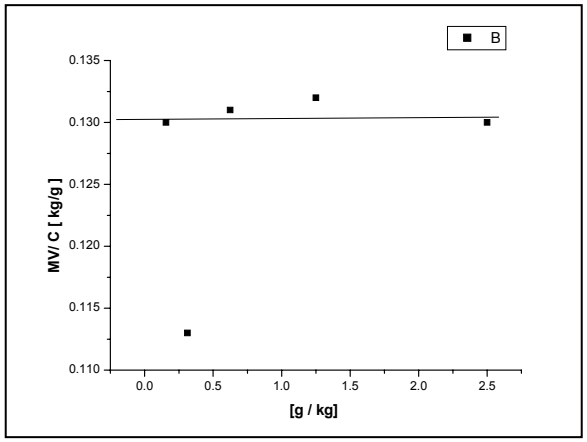


Fig. 3.7: Graph for NIPA- co -Ac.NAG (86:14)

Molecular mass of the sample:

M [g/ mol] = K_{calib} / K_{meas}

K_{calib} = 256

K_{meas} from the graph = 0.130

M = 1969

3.1.6 Synthesis of hydroxyl terminated oligomers of Acryloyl *N*-Acetyl Glucosamine (P. Ac. NAG.ME)

7.27 x 10^{-3} M of Acryloyl-*N*-acetyl Glucosamine (Ac.NAG) was dissolved in 25 ml distilled water in a round bottom flask and stirred continuously to obtain a clear solution. 2.98 x 10^{-3} M of Mecaptoethanol was added as a chain transfer agent. Ammonium per sulphate as initiator and TEMED as an accelerator were added to the reaction mixture. The reaction was carried out at 40 ^{0} C. for 24 hrs. under continuous

nitrogen purging. The solution was precipitated in acetone, reprecipitated in acetone to eliminate unreacted monomers and dried under vacuum. Polymers of various molecular weights were then synthesized using varying ratios of initiator and chain transfer agents.

The data in Table**:** 4.1 of Chapter 4. show that the number average molecular weights of hydroxyl terminated oligomers of P. Ac. NAG as determined by Vapor Phase Osmometer (VPO) range from 400 to 2808.

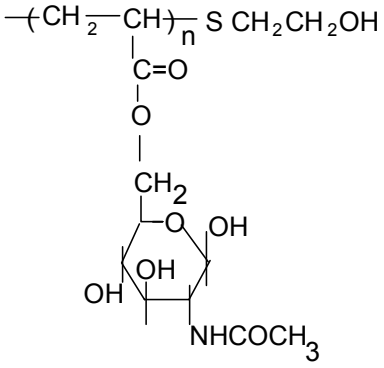


Fig. 3.8: Schematic representation of hydroxy terminated poly Acryloyl NAG

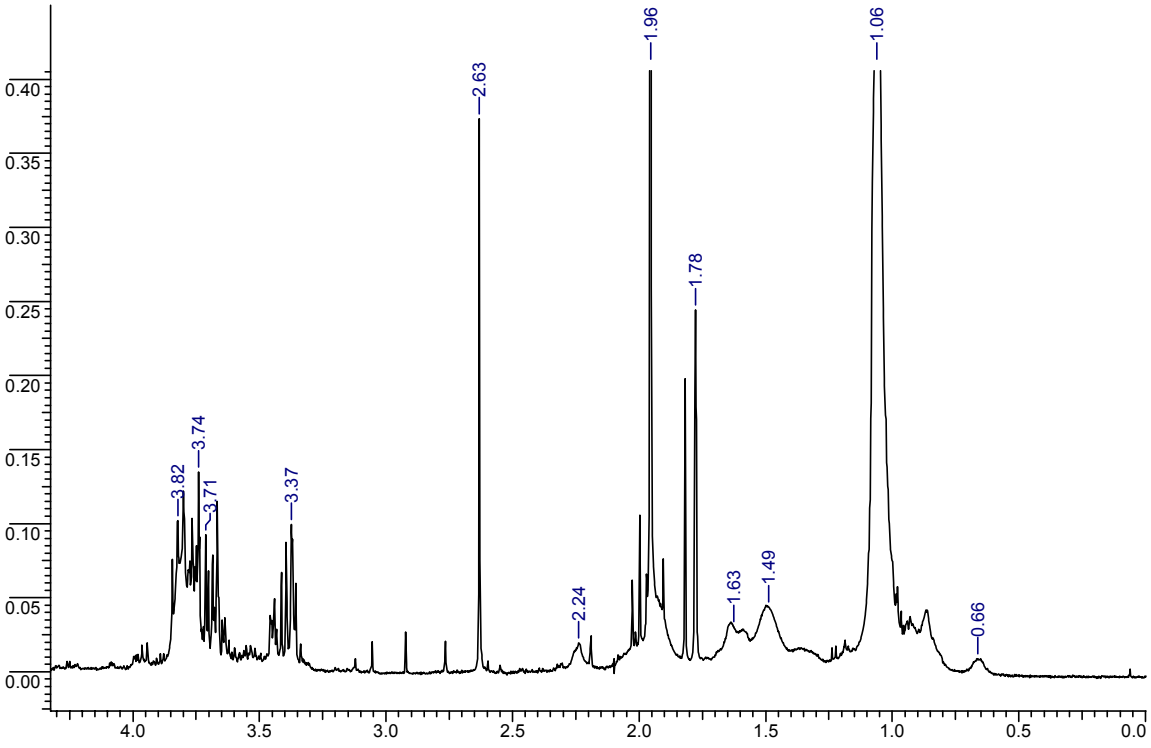


Fig. 3.9: ^{1} H NMR of poly (Acryloyl NAG)

^{1} H NMR (D_2O)

1.3 δ to 1.75 δ 3 H (backbone), 1.86 δ ($NHCOCH_3$),2.6 δ 2H (-O-C$\underline{H_2}$-), 3.25 δ 3.82 6H (NAG).

3.1.7 Oligomers of Methacryloyl *N*-Acetyl Glucosamine (P.M.Ac.NAG)

7.00 x 10^{-3} M of Methacryloyl-*N* Acetyl Glucosamine was dissolved in 25 ml distilled water in a round bottom flask and stirred continuously to obtain a clear solution. 2.2 x 10^{-4} M of Mecaptoethanol was added as a chain transfer agent. Ammonium per sulphate as initiator and TEMED as an accelerator were added to the reaction mixture. The reaction was carried out at 40 ^{0} C. for 24 hrs. under continuous nitrogen purging. The solution was concentrated and precipitated in acetone, reprecipitated in acetone to eliminate unreacted monomers and dried under vacuum. Methacryloyl NAG polymers of various molecular weights were synthesized using varying ratios of initiator and chain transfer agents.

Molecular weights of hydroxy terminated oligomers of poly Methacryloyl N-Acetyl Glucosamine were in the range 1100 to 3000 (Table: 4.2 chapter 4).

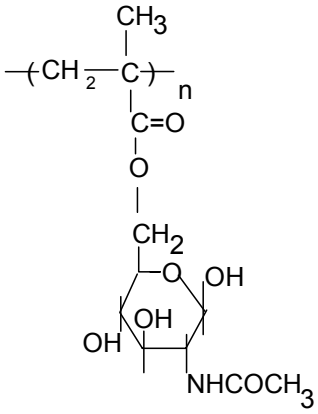


Fig. 3.10: Schematic representation of poly (Methacryloyl NAG)

3.1.8 Synthesis of difunctional polymers of Acryloyl *N*-Acetyl Glucosamine (Ac. NAG) of varying mol. wts.

7.0 x 10^{-3} M of Acryloyl-*N* Acetyl Glucosamine (Ac. NAG) was dissolved in 25 ml distilled water in a round bottom flask and stirred continuously to obtain a clear solution. 4 mg of 3,3 Azobis (3-Cyanovaleric Acid) was added as a initiator. The reaction was carried out at 60 ^{0} C. for 24 hrs. under continuous nitrogen purging. The solution was

concentrated and precipitated in acetone, reprecipitated in acetone to eliminate unreacted monomers and dried under vacuum.

3.2.0 Synthesis of [Acryloyl NAG]-6-(-*N* acryloyl-amino) caproate (Macromer)

Ac.6-Amino Caproic Acid (0.122 gm., 6.6 x 10^{-4} M) and P. Ac. *N*-Acetyl Glucosamine (2 gm, 6.6 x 10^{-4} M) were taken in a 100 ml flask, DMF (25 ml) was added and stirred continuously to obtain a clear mixture. Di Cyclohexyl Carbodiimide (0.136 gm, 6.6 x 10^{-4} M) was first dissolved in DMF (5 ml) and added dropwise to the mixture, stirred continuously for 24 hrs. at room temperature. DCU was filtered off and the macromer was precipitated in acetone, and vacuum dried.

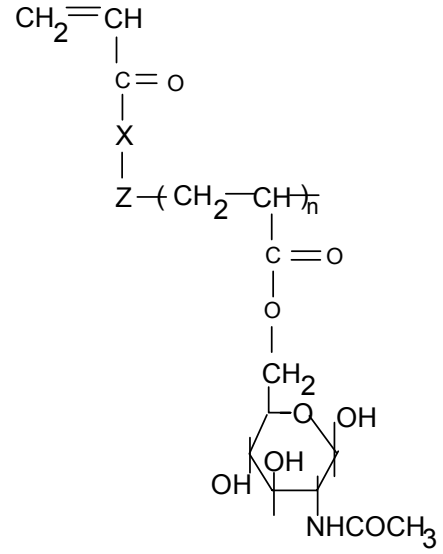


Fig. 3.11: Schematic representation [Acryloyl NAG]-6-(-*N* acryloyl-amino) caproate (Macromer)

where,

X is based on 6-Amino Caproic Acid (6-ACA)

Z is chain terminating agent such as Mercapto Ethanol and Mercapto Propanol, Mercapto Ethylamine HCl.

3.2.1 Synthesis of copolymers:

Synthesis *N*-isopropyl acrylamide and Acryloyl *N*-Acetyl Glucosamine

3.5 x 10^{-2} M of *N*-isopropyl acrylamide and 3.6 x 10^{-3} M of Acryloyl *N*-Acetyl Glucosamine were dissolved in 25 ml of water-jacketed reactor. Polymerization was carried out at 40 ^{0} C under nitrogen purging by addition of 1% (w/w) of ammonium per sulfate as initiator and 1% v/w of TEMED (accelerator) for 4 hours. The copolymer was

precipitated in diethyl ether. Polymer was reprecipitated with diethyl ether and dried under vacuum at room temperature. Molecular weights, NAG content and LCST values are summarized in chapter 4 (Table 4.3).

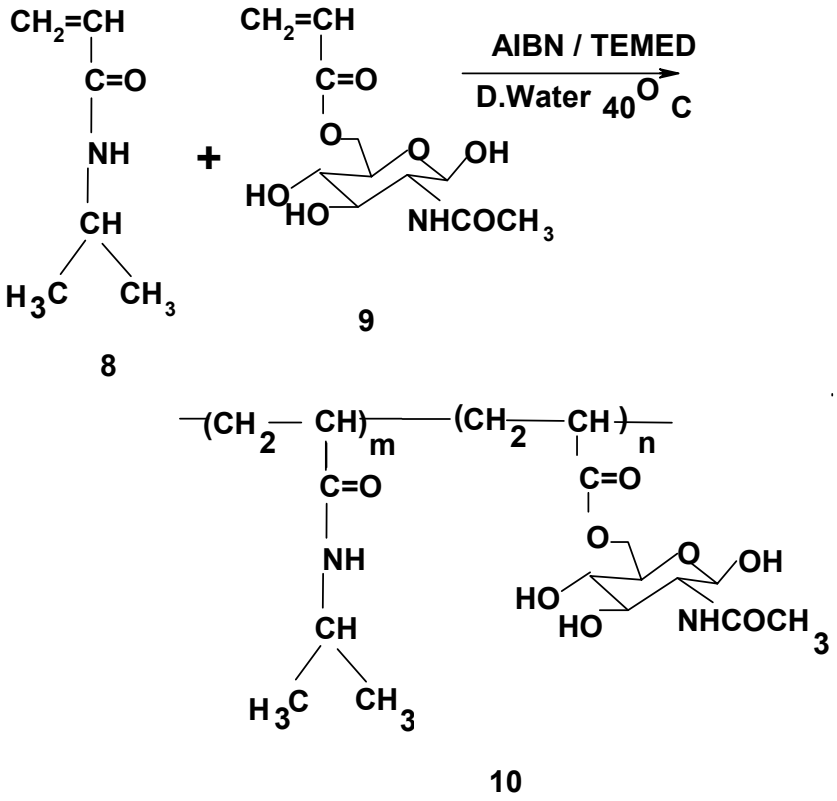


Fig. 3.12: Schematic representation of NIPA–co–Ac.NAG

Where,

8= *N*-isopropyl acrylamide (NIPA)

9= Acryloyl NAG

10= NIPA-co-Ac.NAG

m = 1 to 5000

n = 1 to 5000

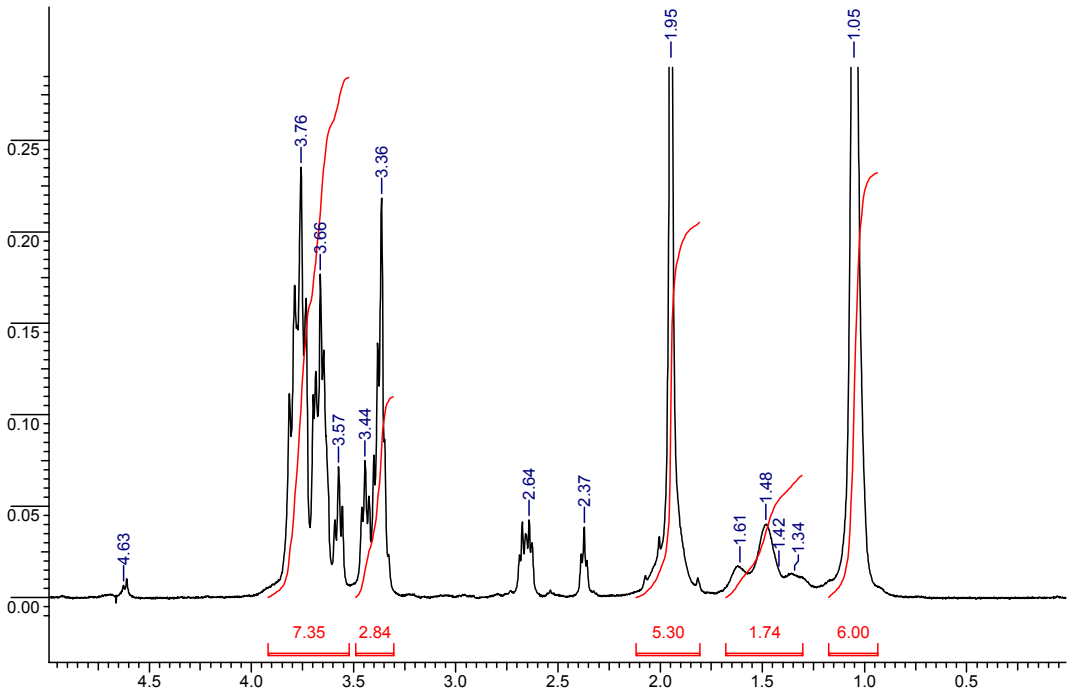


Fig. 3.13: ^{1} H NMR of NIPA-co-Ac.NAG

^{1} H NMR (D2O)

1.05 δ 6 (H), 1.34 δ to 1.61 (b) 3H, 1.95 δ (H) (NHCO-CH_3), 3.25 δ to 4 δ (m) 6H (NAG) + 1H (NIPA).

3.2.2 Estimation of monomer reactivity ratios:

Copolymers of *N*-isopropylacrylamide and Acryloyl *N*-acetyl glucosamine

NIPA (8.83 x 10^{-3} M) and Ac. NAG (8.83 x 10^{-3} M) were dissolved in distilled water (10 ml). The solution was nitrogen purged for 15 minutes and Ammonium per sulphate (1.0 %) and TEMED as accelerator (15 μL) were added and the tubes were sealed. The copolymerization was carried out at 30 ^{0} C for a period of 5 minutes. Each polymer was precipitated in diethyl ether and reprecipitated in diethyl ether. Polymer was redissolved in distilled water and reprecipitated in diethyl ether to ensure complete removal of monomers. The copolymer conversion obtained was lesser than 10 %.

The method of estimation and reactivity ratios are described in Chapter 4 in section 4.2.5.

3.2.3 Synthesis of copolymers of *N*-isopropyl acrylamide and Methacryloyl *N*-Acetyl Glucosamine (Meth.NAG)

3.5 x 10^{-2} M of *N*-isopropyl acrylamide and 3.6 x 10^{-3} M of Methacryloyl *N*-Acetyl Glucosamine were dissolved in 25 ml of water jacketed reactor. Polymerization

was carried out at 40 ^{0} C under nitrogen purging by addition of 1% (w/w) of ammonium per sulfate as a initiator and 1% v/w of TEMED (accelerator) for 4 hours. The copolymer was precipitated in diethyl ether. Polymer was reprecipitated with diethyl ether and dried under vacuum at room temperature. Molecular weights, NAG content and LCST is described in Table 4.4 in chapter 4.

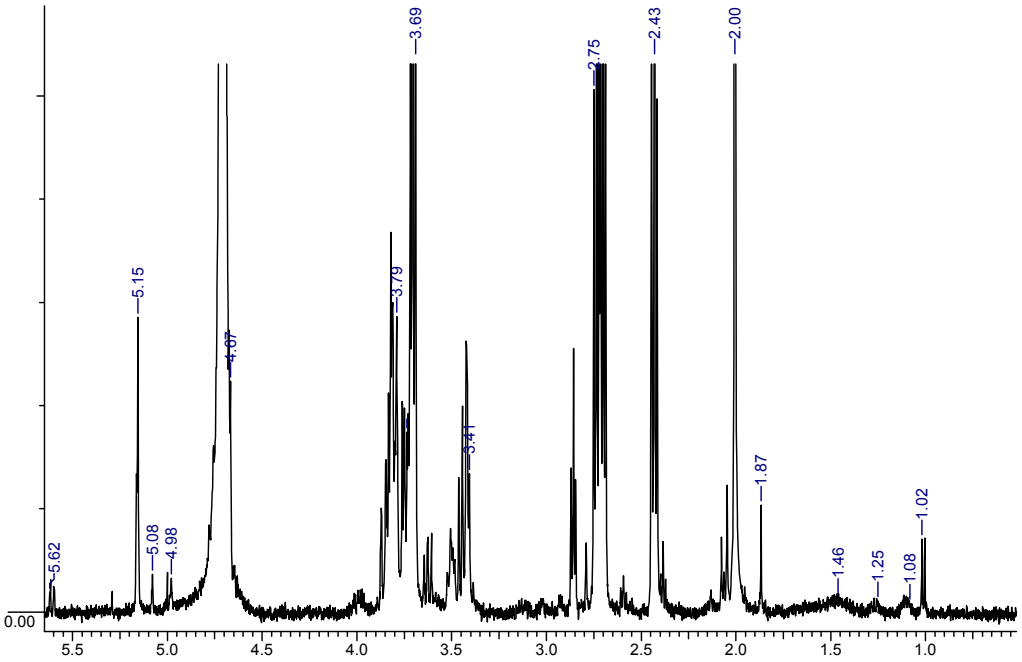


Fig. 3.14: ^{1}H NMR for Methacryloyl NAG

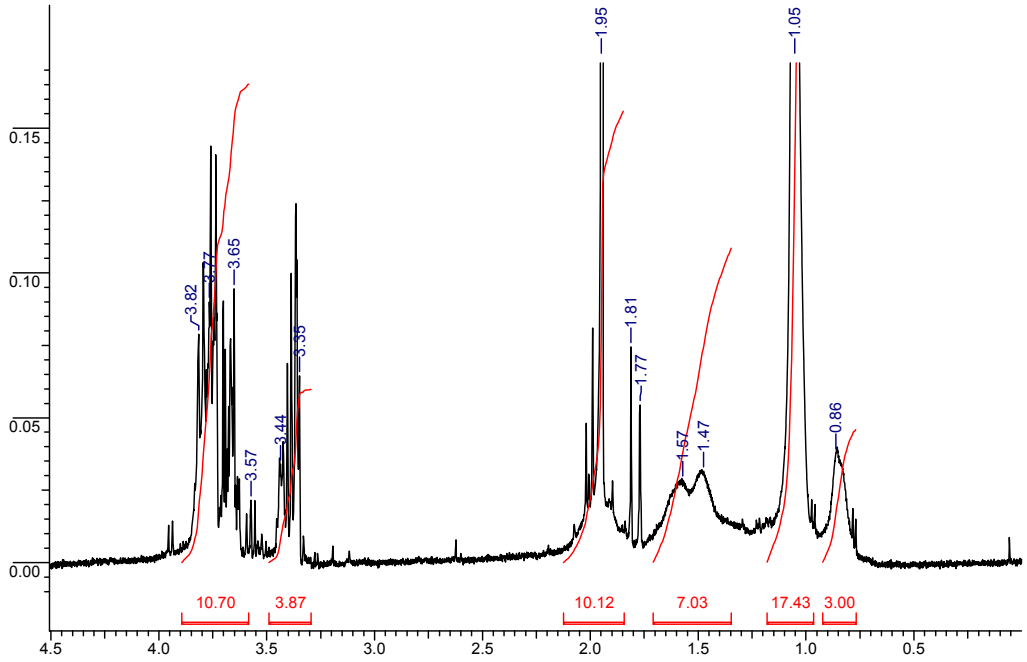


Fig. 3.15: NMR for NIPA-co-Methacryloyl NAG

^{1} H NMR (D_2O)

0.86 δ 3 (H) CH_3 (Methac.NAG), 1.05 δ 6H (NIPA), 1.47 δ to 1.57 (b) 3H, 1.96 δ (H) (NHCO-CH_3), 3.35 δ 3.82 (m) 6H (NAG) + 1H (NIPA).

3.2.4 Synthesis of copolymers of *N*-isopropyl acrylamide (NIPA) and Poly [acryloyl NAG]-6-(-*N* acryloyl amino) caproate (Macromer)

NIPA (8.8 x 10^{-3} M) and Poly [acryloyl NAG]-6-(-*N* acryloyl amino) caproate (Macromer) (6.07 x 10^{-4} M) were dissolved in 25 ml of distilled water in a jacketed reactor. The resulting mixture was nitrogen purged and 1-% ammonium per sulphate was added as a initiator and 1 % TEMED was used as an accelerator. The copolymerization was carried at 30 ^{0} C for 4 hrs. Copolymer was precipitated in diethyl ethyl and reprecipitated to remove unreacted and vacuum dried the polymer at room temperature.

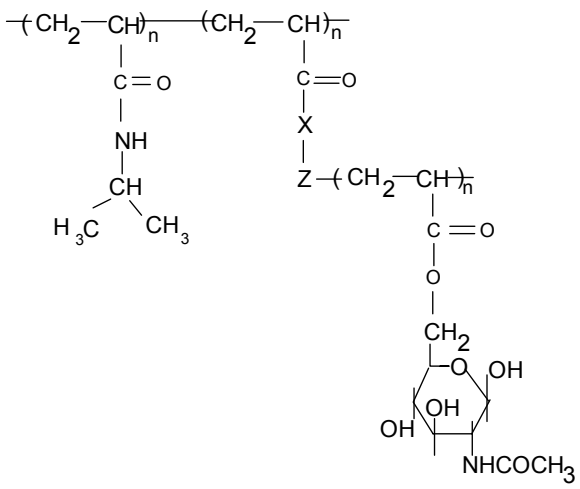


Fig. 3.16 Schematic representation of copolymers of NIPA and Macromer

where,

X is based on 6-Amino Caproic Acid (6-ACA)

Z is chain terminating agent such as Mercapto Ethanol and Mercapto Propanol, Mercapto Ethylamine HCl.

3.2.5 Reactivity ratios for copolymers of *N*-isopropyl acrylamide and Poly [acryloyl NAG]-6-(*N*-acryloyl amino) caproate (Macromer)

NIPA (8.8 x 10^{-3} M) and Poly [acryloyl NAG]-6-(N- acryloyl amino) caproate (Macromer) (6.07 x 10^{-4} M) were dissolved in 25 ml of distilled water in a jacketed reactor. The resulting mixture was nitrogen purged and 1-% ammonium per sulphate was added as initiator and 1 % TEMED was used as an accelerator. The copolymerization was carried out at 30 ^{0} C for 7 minutes. Copolymer was precipitated in diethyl ether and reprecipitated to remove unreacted monomers and vacuum dried the polymer at room temperature. The copolymer conversion obtained was lesser than 10 %.

The method of estimation and reactivity ratios are described in section 4.2.5 and the ratios are shown in Table 4.11

AB Block copolymers:

3.3.0 Synthesis of carboxyl terminated P(NIPA)

2.2 gm. NIPA (0.0194 M) and 776 µM (8 µl) mercaptopropionic acid as a chain transfer agent was dissolved in iso-butyl alcohol (25 ml) in a round bottom flask. The resulting solution was stirred under continuous nitrogen purging. Polymerization was carried out at 60 ^{0} C for 24 hrs using 2,2'-azobisbutyronitrile (AIBN) as initiator. The

AIBN concentration was varied so as to obtain polymers of different molecular weights containing carboxyl end groups. The solution thus obtained was cooled at room temperature and precipitated into diethyl ether. The polymer was dried under vacuum for 24 hrs. The number average molecular weight of the polymer was determined by end group analysis method and VPO.

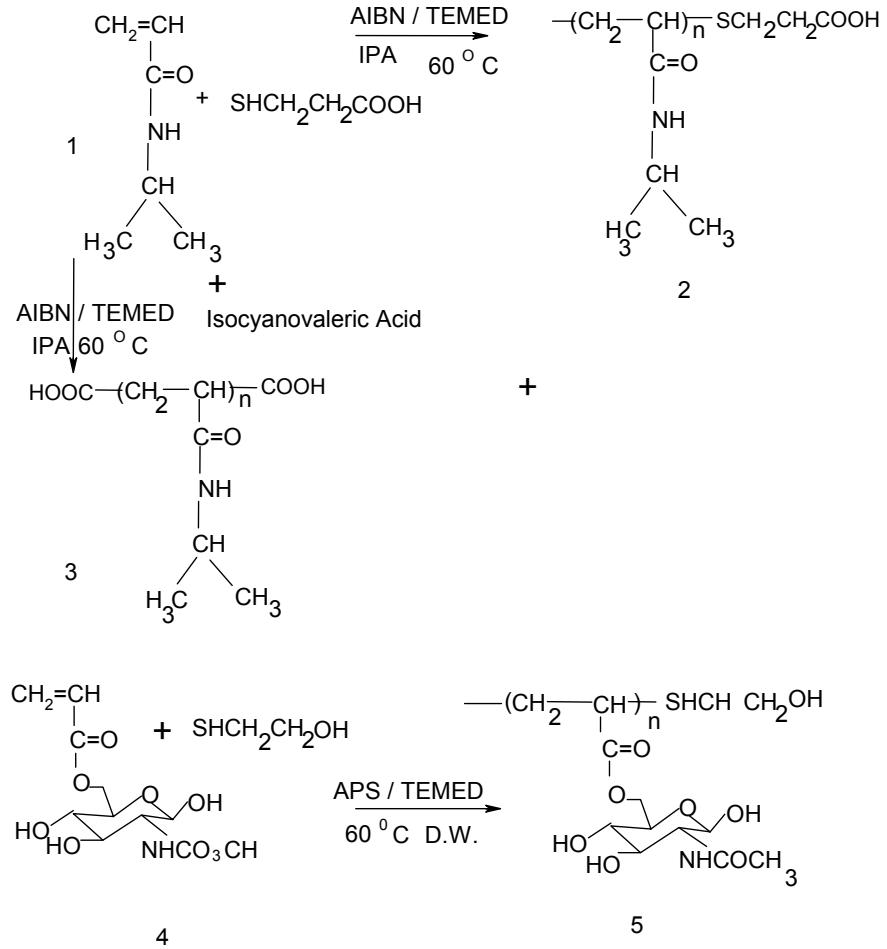
Table: 4.13 shows molecular weights of carboxyl terminated PNIPA which were used for obtaining block copolymers by reacting with hydroxyl terminated P. Ac. NAG.

3.3.1 Synthesis of AB block copolymers:

Carboxyl terminated P (NIPA) and of hydroxyl terminated P.(AcNAG) were polymerized by free radical polymerization using mercaptopropionic acid and mercaptoethanol as a chain transfer agent respectively.

The carboxyl terminated 2.0 gms (7.14×10^{-5} M) of P(NIPA) was dissolved in 25 ml DMF and 46 mg (7.14×10^{-5} M) of P (Ac-NAG) bearing hydroxyl terminal was added and stirred to obtain a solution. 1M of dicyclohexyl carbodiimide (DCC) was added at room temperature and the content stirred for 24 hrs. DCU was filtered off and the polymer was precipitated in diethyl ether and reprecipitated in diethyl ether. Polymer was vacuum dried. AB block copolymers containing P(NIPA) and P(Ac. NAG) segments of varying mol. wt. were synthesized .

Composition of NAG in block copolymers of PNIPA and NAG content varied from 0.64 to 26.5 % mole (Table 4.13 chapter 4).



Where,

1. = N-Isopropylacrylamide (NIPA)
2. = P. NIPA mercaptopropionic acid
3. = Carboxy terminated P.NIPA
4. = Ac.NAG
5. = P.Ac.NAG mercaptoethanol

Fig. 3.17: Schematic representation of functional polymers of NIPA and Ac.NAG

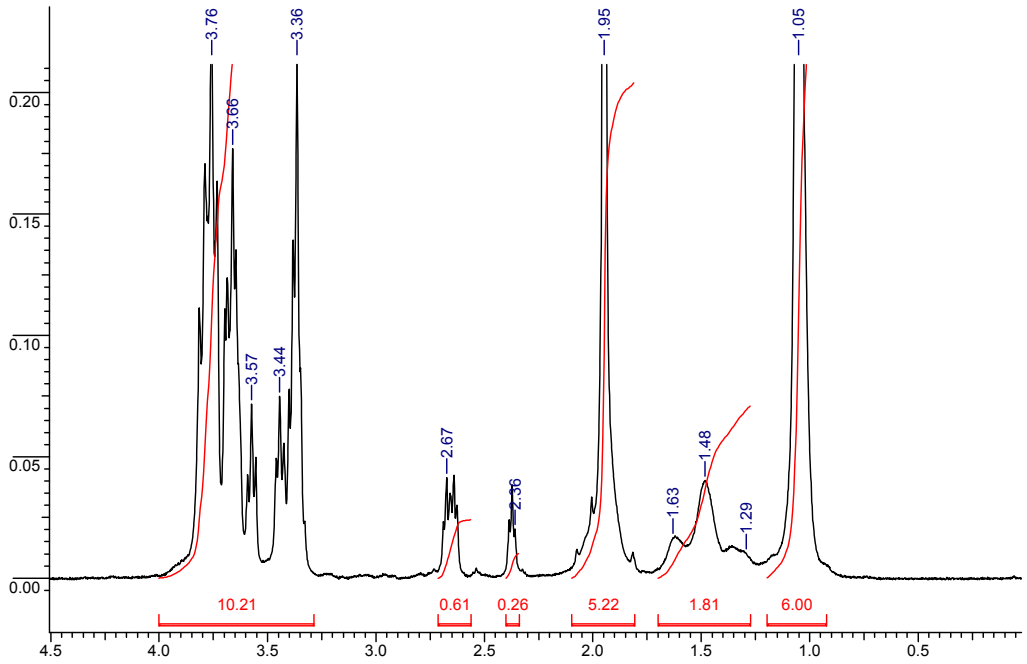


Fig. 3.18: NMR for AB copolymer

^{1} H NMR (D_2O)

1.05 δ 6 (H), 1.29 δ to 1.63 (b) 3H, 1.96 δ (H) (NHCO-CH_3), 3.36 δ 3.76 (m) 6H (NAG) + 1H (NIPA).

B-A-B polymers:

3.3.2 Synthesis of di carboxyl terminated P.(NIPA) bearing terminal carboxyl groups

2.4 g (2.11 x 10^{-2} M) NIPA was dissolved in 25 ml of isobutyl alcohol in a two neck round bottom flask. This was stirred under continuous nitrogen purging and polymerization was initiated by the addition of 2 mg 4-azobis isocyanovaleric acid. The initiator concentration was kept low in order to obtain high molecular weight polymer. The reaction was carried at 60 ^{O} C for 12 hrs. The mass was cooled to room temperature and the polymer was precipitated in diethyl ether. Polymer obtained was vacuum dried at room temperature.

Molecular weights of P (NIPA) comprising terminal COOH groups vary between 36,000 to 90,000 (Table 4.14).

3.3.3 Synthesis of B-A-B block polymer

2.0 gms (2.22 x 10 -^{5}M) of dicarboxyl terminated NIPA and 28 mg of (2.22 x 10 -^{5}M) oligomer were dissolved in 25 ml dimethylformamide (DMF) in a round bottom

flask. The clear solution was obtained by stirring and 45 mg dicyclohexyl carbodiimide (DCC) was added. The reaction was carried out at room temperature for 48 hrs. DCU was filtered off and polymer was precipitated in diethyl ether. Polymer was reprecipitated in diethyl ether and vacuum dried at room temperature.

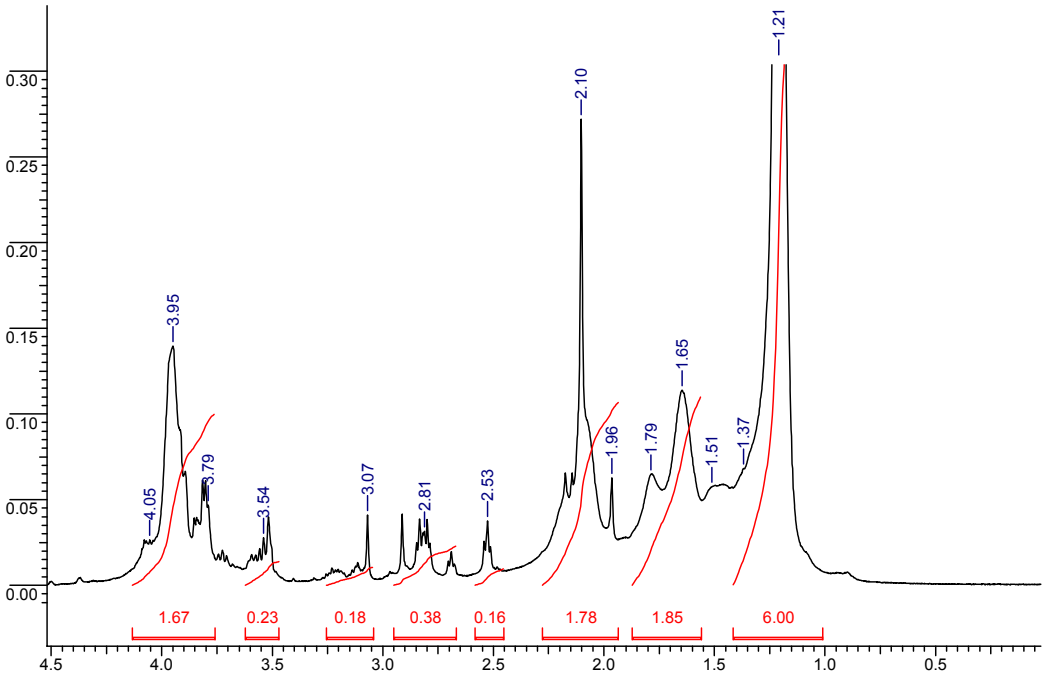


Fig. 3.19: NMR of BAB copolymer

^{1}H NMR (D_2O)

1.21 δ 6 (H), 1.51 δ to 1.79 (b) 3H, 2.10 δ (H) ($NHCOCH_3$), 3.54 δ 4.05 (m) 6H (NAG) + 1H (NIPA).

From Fig 3.17

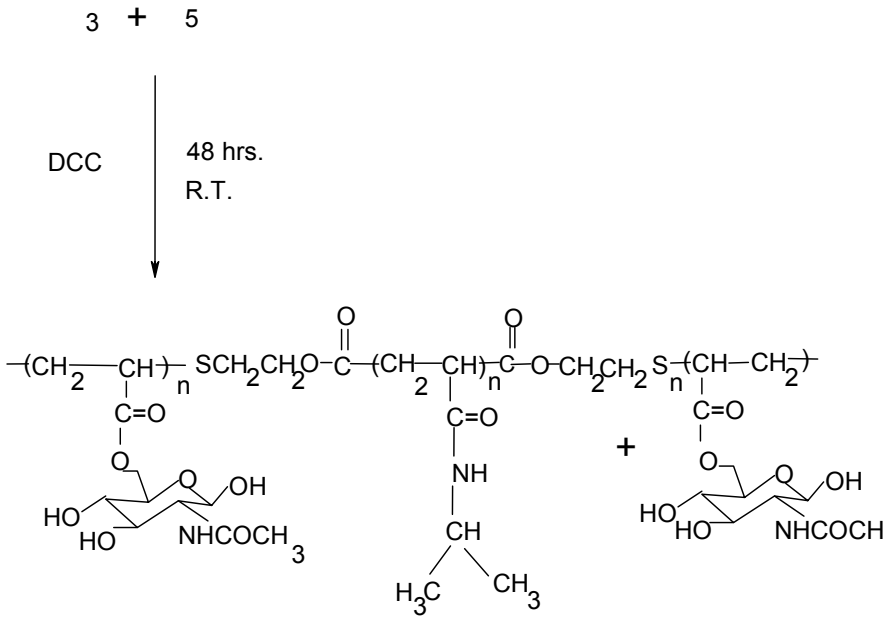


Fig. 3.20: Schematic representation of BAB copolymers

3.3.4 Imprinted polymers:

Preparation of lysozyme imprinted polyvalent copolymers: Variation of degree of inhibition

50 mg of poly (Acryloyl *N*–Acetyl Glucosamine)-6-(-*N*- acryloyl amino caproate) (Macromer) was dissolved in a two neck round bottom flask and 10 ml double distilled water was added. To the resulting solution 10 mg of lysozyme was added and solution was incubated for 15 min at room temperature to form lysozyme- poly (acryloyl *N*–Acetyl Glucosamine)-6-(-N- acryloyl amino caproate) complex. The comonomer 200 mg *N*-isopropylacrylamide (NIPA) and initiator ammonium persulfate 10 mg were added to above aqueous solution. The solution was purged with nitrogen gas for 15 min and 40 µl. tetramethylene ethylenediamine was added. The reaction was carried out at 37 ^{O}C for 4 hrs. The imprinted polyvalent copolymer was precipitated in diethyl ether and vacuum dried.

Imprinted polymers were also prepared by varying the extent of lysozyme inhibition from 20 % to 100 % by the addition of the corresponding amounts of the macromer. In this case the ratio of macromer to NIPA was maintained constant. (Table 4.15 in chapter 4).

3.3.5 Preparation of lysozyme imprinted polyvalent copolymers: Variation of ligand concentration

50 mg of poly (acryloyl *N*–Acetyl Glucosamine)- 6-(-*N*- acryloyl amino) caproate was dissolved in a two neck round bottom flask and 10 ml double distilled water was added. To the resulting solution 10 mg of lysozyme was added and solution was incubated for 15 min at room temperature to form lysozyme- poly (acryloyl *N*–Acetyl Glucosamine)- 6-(-*N*- acryloyl amino) caproate complex. The comonomer 200 mg N-isopropylacrylamide (NIPA) and initiator ammonium persulfate 10 mg were added to above aqueous solution containing lysozyme- poly (acryloyl *N*–Acetyl Glucosamine)-6-(-*N*-acryloyl amino) caproate (Macromer) complex. The solution was purged with nitrogen gas for 15 min and 40 µl. tetramethylene ethylenediamine was added. The reaction was carried out at 37 ^{O} C for 4 hrs. The imprinted polyvalent copolymer was precipitated in diethyl ether and vacuum dried.

The comonomer ratio of NIPA and macromer was varied from 1:4 to 4:1.

3.4.0 Estimation of relative inhibition of lysozyme by NAG ligands

Micrococcus lysodeikticus is a substrate for the enzyme lysozyme. Relative binding was estimated for stock solutions of NAG containing monomers, macromer, copolymers and imprinted polymers by using a procedure reported by Neuberger and Wilson (1967). 1.5 % w/v stock solutions of NAG was prepared in 6.6x 10^{-3} M phosphate buffer pH 6.2 containing 1.54 x 10^{-2} M sodium chloride and 8.0 x 10^{-3} M sodium azide. One milliliter of stock solution containing different ligand concentrations was mixed with 1.6 ml of 78 µg/ml of *Micrococcus lysodeikticus* in a 3-ml capacity glass cuvette. The mixture was incubated for 5 minutes at 20 ^{o} C. To this mixture 0.1 ml of lysozyme (27 µg/ml) was added and mixed thoroughly. The absorbance at 450 nm (Δ_{450}) was recorded for 30 seconds. A blank reading without the polymer ligand was noted and the change in the absorbance per second was calculated [Neuberger et al (1967)].

3.4.1 Estimation of association constant (k_a) between lysozyme and NAG conjugates

Micrococcus lysodeikticus is a substrate for the enzyme lysozyme. Saccharides are relatively similar to lysozyme substrate which are capable of inhibiting the lytic activity of enzyme. Addition of appropriate molar concentrations of each leads to blue shift of about 10 mµ in the fluorescence emissions maximum of the enzyme. The change in the

substrate type changes the fluorescence intensity of the lysozyme and depends upon the efficient binding of the ligand to the active sites of the enzyme. From the fluorescence emissions data the association constants (k_a) between lysozyme and the oligomers, macromer, copolymer and block copolymers containing NAG were estimated. The association constant quantitifies the binding efficiency to the binding subsites of the enzyme.

Fluorescence spectra of lysozyme were recorded on a Perkin Elmer LS-50 B luminescence spectrophotometer. Excitation frequency was 285 nm. Solutions of lysozyme and polyvalent copolymers containing *N*-Acetyl Glucosamine were prepared in 6.6 x 10^{-2} M phosphate buffer pH 6.2, containing 1.54 x 10^{-2} M sodium chloride and 8.8 x 10^{-2} M sodium azide. 0.1 milliliter of lysozyme solution (80 μg /ml) was mixed with solution containing different ligand concentration in a 2 ml capacity 10 mm square quartz cells maintained at 18 ^{o} C. Phosphate buffer was added to make the volume to 2 ml. The fluorescence intensities of the solutions were measured relative to the solutions containing enzymes and buffer mixtures of the identical concentrations as reference. The relative fluorescence intensity of lysozyme saturated with solution containing different ligand concentration, F_{∞}, was extrapolated from the experimental values by plotting 1/ (F_O-F) against 1/[S] where F is the measured fluorescence of a solution containing enzyme with given substrate concentration [S] and F_O is the fluorescence of a solution containing enzyme alone [Chipman et al (1967)]. The highest concentration of polymer substrate used was such that enzyme was saturated more than 85 %.

3.4.2 Estimation of NAG content of the copolymer by NMR

NMR spectroscopy was used for the structural characterization for all the polymers and to calculate percentage incorporation of NAG content. The data were recorded on a Brukers'MSL-300 operating at 300 MHz and 500 MHz. for proton. The integration values were compared for the protons of NAG anomer and isopropyl groups in PNIPA to obtain mole % incorporation as shown below:

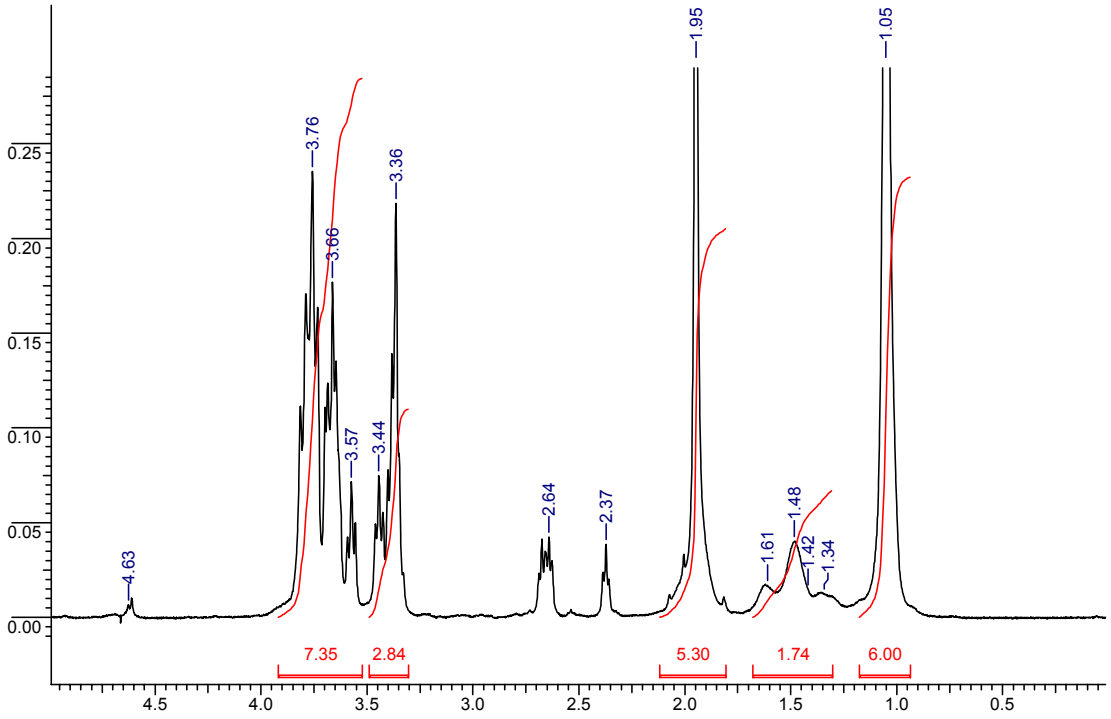


Fig. 3.21: Estimation of NAG content of the copolymer by NMR

4.0.0 Results and discussion:

Molecular associations involved in host–guest interactions are of great importance in both, biology and biotechnology. Recently, the need of mimicking biological interactions in therapeutics has been realized, however few studies so far have focused on macromolecular host-guest interactions.

Ligand–substrate interactions quantitified in terms of efficacy can be estimated using various methods, depending on the nature of the interacting molecules. However, the most crucial aspect in such interactions is that the interaction must be specific and binding of the ligand to the host must be reversible.

The receptor site must have physical characteristics, which would favor ligand binding. The specificity of a binding site for a particular ligand can vary, depending on the structure of a ligand. For example, NAG ligands bind lysozyme and each of these ligands incorporated in different architecture will vary binding activity.

This chapter presents the results of inhibition of lysozyme by monomer, oligomer, macromer, copolymer and imprinted polymers containing NAG. These are discussed in terms of binding constants between the two. Further details such as the precise nature of binding have been investigated by competitive binding studies using Biebrich Scarlet.

4.1.0 Prior efforts:

The recognition of oligosaccharides by proteins represents the basis of many biologically important events [Varki et al (1993)]. Individual protein–carbohydrate interactions are generally weak and the dissociation constant k_D can be as low as 10^{-3} to 10^{-4} M^{-1} [Lee et al (1995)]. Further the specificity is poor. Both could be enhanced through polyvalent binding, which is characterized by the simultaneous contact of multiple ligands between one biological entity and multiple receptors on another.

Despite the critical role of polyvalency in protein-carbohydrate interactions, relatively little is known about such binding mechanisms. In the following paragraphs we present some of the recent findings in this area. Kiessling et al (1996) delineated the possible role of multivalent interactions in carbohydrate mediated cell or virus binding, the targeting of conjugates to particular cell types, the immobilization of specific cell

types, and for inducing cell responses through selective binding and / or aggregation of cell surface receptors.

Although polyvalency readily accounts for increased affinity, the molecular basis for enhanced specificity is not well understood. [Roseman et al 2001)]. Roseman et al studied the specificity of the cysteine–rich domain of the mannose / GalNAc-4-SO_4 receptor using monovalent and polyvalent forms of the trisaccharide GalNAc-beta 1,4 GlcNAc-beta 1, 2 Man-alpha (GGnM) sulfated at either the C4 (S4GGnM) or C3 (S3GGnM) hydroxyl of the terminal GalNAc. Monovalent S4GGnM and S3GGnM displayed affinity values (ki) of 25.8 μM and 16.2 μM, respectively. Polyvalent conjugates of GalNAc-4-So_4 and GalNAc-3-SO_4 bearing trisaccahrides exhibit ki values 0.013 μM and 0.170 μM, respectively. Thus 2000 and 95 fold enhancement in affinity was observed for the polyvalent forms of 4 sulfated and 3 sulphated trisaccharides reflecting a difference in the impact of conformational entropy. A large fraction of $S0_4$-3-GalNAC structures exists in the form that is not favorable for binding to the Cys-rich domain. This reduces the effective concentration of SO_4-3-GalNAc as compared with SO_4-4-GalNAc under the same conditions and results in lower association.

Dimick et al (1999) reported cluster glycoside effects on concanavalin A. It was suggested that the inhibition of protein carbohydrate interaction to provide a powerful therapeutic strategy for the treatment of myriad human diseases. However, applications of such approaches have been frustrated by low affinity between carbohydrate ligands and corresponding protein receptors. In the search for high affinity ligands for lectin, a variety of polyvalent saccharide ligands were prepared. The cluster glycoside effect derived from multivalency in oligosaccharide ligands helped overcome the weak binding interactions. Dendrimers containing benzene 1,3,5,-tricarboxylic acid trimethyl ester were prepared to enhance interactions with concanavalin A. Affinity as observed by agglutination assay was enhanced by 30 folds.

As described earlier, a somewhat similar but less complex situation is presented in the lysozyme–catalyzed hydrolysis of oligomeric saccharides consisting of β-(1→ 4) linked 2-acetamido-2 deoxyglucose.

In the wake of these model studies, it was thought worthwhile to design and develop polyvalent ligands with controlled structure containing NAG for enhanced

affinities with lysozyme. The design of polyvalent NAG can best be undertaken on the basis of the knowledge of the active sites in lysozyme. In the following section a brief overview of the structure of lysozyme and polyvalent ligands designed, is presented.

4.1.1 Lysozyme:

Lysozyme molecule is roughly ellipsoidal (45 by 30 by 30 Å) with a deep cleft running up one side. The polar side chains of lysozyme are distributed on the surface in contact with water, while the interior of the molecule contains nonpolar hydrophobic residues. The cleft is also partially lined with hydrophobic residues [Chipman et al (1969)].

The active site of lysozyme consist of sites designated A-F which can accommodate six pyranose rings of *N*-Acetyl glucosamine (NAG / GlcNAc). The hydrolysis of oligomers of NAG by lysozyme as well as binding between oligomers of NAG and various subsites has been extensively reported.

NAG exists in two anomeric forms α and β, which readily interconvert in water. Dahlquist and Raftery (1968) demonstrated binding of two anomeric forms of NAG by NMR spectroscopy. Orientation of the α-NAG is different from that of β NAG when bound to the same site on lysozyme.

The β anomer binds to subsite C of the enzyme from the reducing terminal NAG unit of trimer. Its binding results in a change of a protein conformation. Binding of the α anomer of NAG has been observed in the same subsite, but its orientation in the subsite is different from that of the β anomer. It is difficult to observe complexes of longer saccharides than the trimer by X ray crystallography probably because it is more difficult for a large saccharide to diffuse into the crystal. Saccharides containing longer chains are easily cleaved by lysozyme and in particular crystalline form of the lysozyme studied by Johnson and Phillips (1965) (tetragonal). The lower part of the enzyme cleft is partially blocked by a portion of a neighboring enzyme molecule.

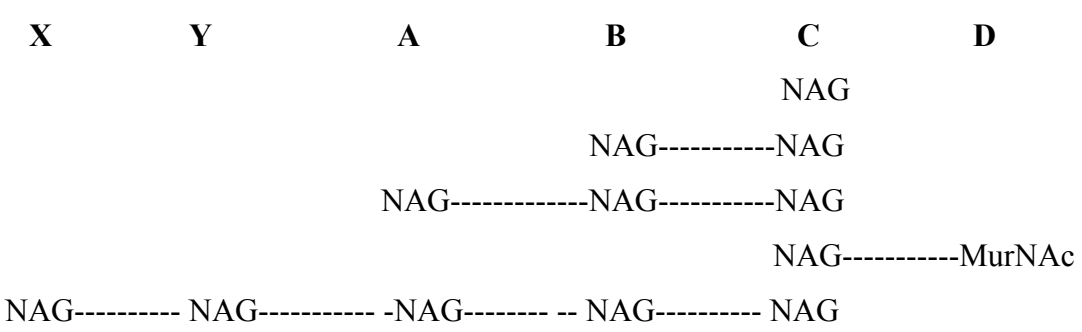
However, Johnson and Phillips (1965) were able to construct a model for a polysaccharide by assuming that the structure of lysozyme–oligosaccharide complex is super imposable on lysozyme $(NAG)_3$ complex as observed by X ray crystallography. The presence of three additional subsites, designated D, E and F, was inferred by fitting molecular models of chitin oligosaccharide substrates to the three dimensional model of

the enzyme and thus lysozyme- $(NAG)_6$ complex model was obtained. The construction of this model has led to a number of interesting inferences enlisted below:

1. The six sugar residues fill the entire length of the lysozyme cleft and it is not easy to fit a larger oligosaccharide such that the additional sugar residues make contact with the enzyme. This means that the sugar residue above subsite A or below subsite F has no significant interactions with the enzyme.
2. Because of steric interactions of the enzyme with CH2OH group on carbon – 6 (C-6) of the sugar unit in subsite D, the pyranose ring residue must be distorted from the normal chair conformation.
3. Since cleavage of the cell wall saccharide occurs only between the *N*-acetyl muramic acid and NAG residues, the linkage affected by the enzyme is either between sugar residues B and C, or between D and E. Sugar residues bound at B and C form part of the lysozyme–$(NAG)_3$ complex, which is stable for weeks. In addition there are no reactive groups on the enzyme in the neighborhood of this linkage. Phillips has therefore suggested that the oligosaccharide linkage cleaved by the lysozyme is between sugar residues D and E. This means that the observed, stable complex of $(NAG)_3$ is a non productive one, which does not lead to bond cleavage.

The most reactive groups in the region of subsites D and E of the enzyme are the carboxyl groups of glutamic acid (Glu) at position 35 and aspartic acid (Asp) at position 52, which are disposed on either side of the β $(1\rightarrow 4)$ linkage. A mechanism has been proposed in which the concerted action of Glu 35 and Asp 52 is involved in catalysis, facilitated by the distortion of the sugar ring occupying subsite D [Voet and Voet (1994)].

Arrangement of saccharides in subsites with lysozyme



NAG----------NAG---------- NAG---------- NAG----------NAG---------- NAG

4.1.2 Studies with chitosan:

Chitosan is prepared by de *N*-acetylation of chitin. Chitosan is a linear, binary heteropolysaccharide which contains 2–acetoamido-2-deoxy-*β*-D-Glucose (GlcNAc; A-unit) and 2-amino-2-deoxy-*β*-D-glucose (GlcN; D unit) residues in various proportions, connected through (1→ 4) glycosidic linkages.

Lysozyme hydrolyses *β* (1→ 4) linkages between A units in chitin and partially *N*-acetylated chitosan. Amongst sixteen possible tetrad sequences only two i.e. the sequences –A-A-A-A and –A-A-A-D- are preferentially cleaved at the middle glycosidic bond by lysozyme [Varum et al (1996)].

Binding between lysozyme and purified oligosaccharides has been extensively studied in the past, especially hexamer sequences containing A-units in de-*N*-acetylated chitosan interact with lysozyme.

Kristiansen et al (1998) reported quantitative studies on the non–productive binding of lysozyme to partially *N*-acetylated chitosan. As lysozyme recognizes six consecutive sugar units, chitosan fractions were prepared, characterized by their degree of polymerization and fraction of A units. The average value of the dissociation constant (k_D) for different sequences that may bind to lysozyme, as well as the number of chitosan units covered by lysozyme upon binding was estimated. k_D decreased with increasing fractions of A-units at pH 3 and 4.5. Contributions from different hexamer sequences of the chitosan were considered.

The binding between lysozyme and chitosan was exploited by Gupta et al (1996) using latter as an affinity macroligand for the recovery of lysozyme and lectin. Binding of proteins with partially deacetylated chitin having sufficient NAG residues was achieved at pH 4.0. Since chitosan is pH sensitive, the co-precipitation of lectin and lysozyme was carried out at pH 8. However, the association constant (k_a) for lysozyme with the cell wall tetrasaccharide is known to be maximum at pH 5.4 as compared to the lower pH (Chipman et al (1967).

It was thought worthwhile to utilize NAG units present in chitin and chitosan by chemical modification to obtain thermoprecipitating affinity macroligand for the recovery of lysozyme**.** It may be noted that NAG units in chitin and partially *N*-acetylated chitosan

are connected through glycosidic β (1→ 4) linkages and lysozyme can hydrolyze the same.

In the first instance we conjugated NAG with acrylic / methacrylic acid and then polymerized the same. Thus the linkage between various NAG units was very different from that present in chitosan. We wished to investigate if the binding between lysozyme and the ligands synthesized by us was as strong as the oligomers of NAG reported in the literature. Further, we wished to confirm, that these ligands were resistant to hydrolysis by lysozyme.

Yet another area, which exploits the protein-carbohydrate interactions, is therapeutics. Inhibition of pathogens using carbohydrates is being investigated as alternative to therapeutics as these agents are not expected to exhibit adverse effects and not lead to drug resistance for the pathogens on long term use. A large number of ligand-substrate pairs involving binding have been recently reviewed. One of the extensively investigated systems involving polyvalent interactions is the binding between hemagglutinin and sialic acid moieties present on the target cell surface.

Sigal et al (1994) studied the importance of polyvalent interactions of polyacrylamide bearing pendent α-sialoside groups that strongly inhibit agglutination of erythrocytes by influenza virus. The strong inhibition reflects enhanced binding through cooperative polyvalent interactions. The objective was to demonstrate the strong binding that reflects the interaction of multiple copies of hemagglutinin on the viral surface simultaneously with multiple sialic acid groups on the cell surface. The polyvalent inhibitors are effective in preventing the attachment of influenza virus to red blood cells at very low concentrations of inhibitors.

It was concluded from the study that the enhanced efficiency of polyvalent inhibition in preventing viral attachment is due to contribution of three factors–two favorable and one unfavorable. i) There is an increase in the affinity, relative to a polymer having only one (or a few) moities of the ligand, due to the binding of multiple sialic acid groups per inhibitor molecule. ii) High molecular weight inhibitors prevent the virus from coming close enough to a cell. iii) an unfavorable factor resulting from the fact that a single ligand attached to a polymer binds less firmly to the receptor than a ligand in a low molecular weight form.

The authors further demonstrated that 1) the effective dissociation constants of the polymeric inhibitors for the viral surface determined by ELISA assay are roughly equal to the measured values of k_i, 2) the differences in the ability of sialic acid containing polymers prepared by copolymerization or by modification of a preformed polymer to inhibit haemagglutination correlate with the relative affinities of the two types of polymers for the viral surface and, 3) the ability of polymers containing sialic acid to inhibit agglutination increases with increasing molecular weight.

Similarly**,** Spaltenstein et al (1991) reported polyvalent derivatives of sialic acid that are much more effective than monomeric units in inhibiting haemagglutination. The efficacy for polyvalent derivatives is increased because of the strategy adapted to design of compounds that inhibit binding of influenza virus to the surface of red blood cells. Ligands based on the polyvalent polymeric inhibitors are able to compete with the polyvalent virus-cell interaction more effectively. It is possible to synthesize such inhibitors by copolymerization reactions using readily synthesized monomers. It was concluded that the strategy can be applied to a generation of a wide range of tight–binding agents for the receptors or ligands of which multiple copies are present on the surface of the target cell, microorganism or virus.

Polyacrylamides bearing pendent α-sialoside groups strongly inhibit agglutination of erythrocytes by influenza virus. These polymeric inhibitors inhibit the binding to the virus of a polyclonal antibody generated against the viral surface and are therefore, capable of steric stabilization for the viral surface.

Mochalova et al (1994) synthesized glycylamido benzylsialoside with poly (acrylic acid-co–acrylamides) and dextrans. The polymeric ligands were evaluated for their ability to bind to influenza A and B virus strains in cell culture.

More recently, Mandeville (2001) reported the advantages of polyvalent interactions and their application in medicine. The fucoside sialic acid moities were linked to polymer for the treatment of rotavirus. Such moieties inhibited rotavirus infection in mammals and humans.

In the wake of these prior findings we wished to investigate whether our systems exhibit polyvalency and if methods can be developed to enhance binding with the interacting receptors so that these can be adapted for therapeutic applications as well.

4.1.3 Strategy planned:

Total sites present on the lysozyme are designated A-F and NAG is ligand for interactions with these active sites.

Therefore, the strategy was to:

1) Synthesize polymerizable monomers containing NAG. Monomer NAG was modified to form acryloyl and methacryloyl conjugates. While acrylate monomers were selected as the representative ones, the later experiments for evaluation of efficacy demonstrated large difference and made the study more interesting.

2) Check how many repeat units of NAG are really needed for the enhanced binding with lysozyme.

3) Establish the copolymer structure. Copolymers of NIPA and Ac.NAG were expected to be random, however LCST remained unchanged although Ac.NAG incorporation was varied.

4) Synthesize block copolymers containing NAG using chain transfer agents and compare the results with those reported with block copolymers reported in the past. For instance Yamada et al (1999) suggested the controlled synthesis of amphiphilic block copolymers with pendent NAG residues by living cationic polymerization method. However, the method of synthesis involves stringent reaction conditions.

We have synthesized block copolymers of types i) AB ii) BAB copolymers using 4-azobis cyanovaleric acid and chain transfer agents [Goethals (1989)].

5) Obtain favorable dispositions for NAG with lysozyme.

We designed and synthesized imprinted polymers using lysozyme as a template in aqueous medium at 25 ^{O} C. Recently, Yu et al (2002) reported imprinted polymers using kallikrein as a template in molecular scale reaction vessels. The process utilizes cavities either within a synthetic polymer or within the active site of an enzyme (direct molding), as molecular scale reaction vessels.

Experimental methods for the synthesis of monomers, macromer, copolymers and imprinted polymers are described in Chapter: 3.0.0

Evaluation of NAG ligand prepared by different approaches was carried out in three stages:

A) Association constant (k_a):

The efficacy of NAG ligands in terms of association constant was evaluated. The process for evaluating k_a is described in section 3.4.1. The NAG incorporated in various architectures of polymers displayed different efficacies.

B) Inhibition concentration (I_{50}):

We have also evaluated the inhibition concentration of the ligands with lysozyme and establish the role of polyvalency and steric stabilization and compared the same with the results obtained by earlier investigators. The method of estimating I_{50} is described in section 3.4.0.

C) Competitive binding:

After estimating the efficacy we further investigated where the binding between NAG and lysozyme active sites takes place. The evaluation was carried out using Biebrich scarlet dye as the competitive binding agent for site F. The competitive binding and results thereof are described in section 4.3.3.

4.1.4 Choice of experimental methods:

Association constant (k_a)

The binding in terms of association constants (k_a) can be estimated using various methods such as fluorescence, UV and NMR spectroscopy. The association constants (k_a) for lysozyme-saccharide complexes containing NAG have been estimated using various spectroscopy methods [Dahlquist et al (1966)].

Fluorescence emission spectrum of lysozyme is due to the indole moieties of the six-tryptophan groups present in the enzyme [Leherer (1966)]. Since the fluorescence is maximum for an indole group, shifts are generally observed to shorter wavelengths as its environment becomes less polar [Teale (1960)]. Shinitzky et al (1966) concluded that the observed blue shift in the fluorescence spectrum of lysozyme on interaction with certain saccharides results form the fact that one or more tryptophan residues are in a more hydrophobic environment than in the free enzyme. The saccharides being hydrophilic, a change in conformation of the enzyme on binding is responsible for the spectral shift.

Chipman (1967) estimated the binding of oligosaccharides containing *N*-acetylglucosamine and *N*-acetylmuramic acid with lysozyme using fluorescence emission spectroscopy. The cell wall tetrasaccharide GlcNAc-MurNAc-GlcNAc-MurNAc and

disacchrides GlcNAc-MurNAc were isolated from *M. lysodeikticus* cell walls. Fluorescence measurements were made with Aminco-Keir spectrophotometer. The illuminating wavelength was 285 mμ. The fluorescence emission intensity of lysozyme at 370 mμ, demonstrated maximum change on binding. Fluorescence emission intensity for GlcNAc and its oligomers with lysozyme was measured at 325 mμ.

The fluorescence intensities of solutions relative to those of the reference solution containing only lysozyme and buffer were determined. The absorbance at 285 mμ and the fluorescence at the appropriate wavelength of solutions of the saccharide alone were measured. The relative fluorescence intensity of lysozyme saturated with saccharide, F_∞ was extrapolated from the experimental data by plotting $1/ (F_o-F)$ against $1/[S]$ where, F was the measured fluorescence of solution containing the enzyme and a known NAG concentration [S] and F_o was the fluorescence of solution of enzyme alone. The slope of unity indicated the formation of one to one complex.

The maximum concentration of NAG monomer used was equivalent to the 85 % saturation. Typical fluorescence plot for the binding of the cell wall tetrasaccharide in GlcNAc-MurNAc to lysozyme was plotted. A typical illustrative plot for NAG made in this work is shown below:

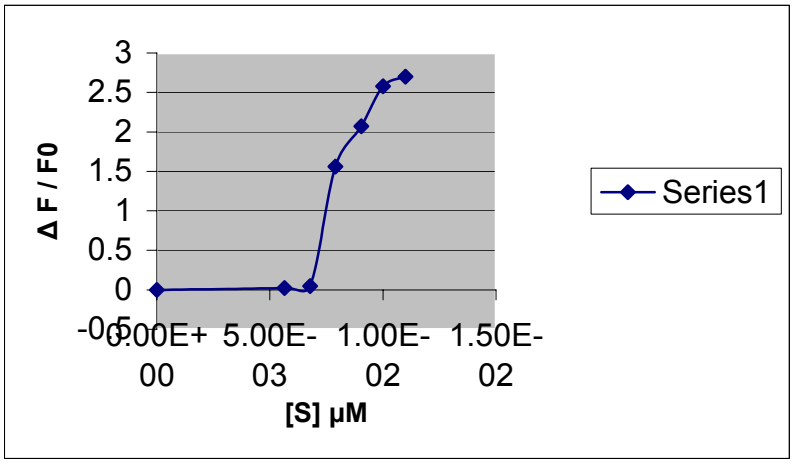


Fig. 4.1 Plot of Δ F/ F_o against [S] for NAG

where, Δ F is the fluorescence intensity at its maximum, F_o is the fluorescence intensity of lysozyme alone and [S] is the concentration of lysozyme–and NAG.

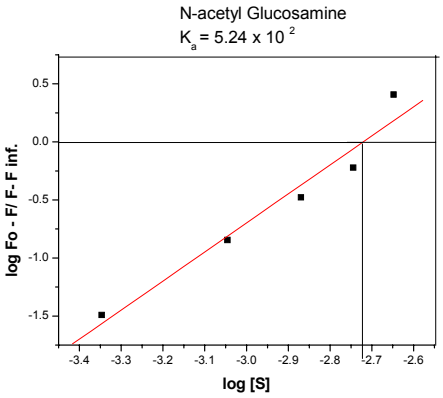


Fig. 4.2: Fluorescence plot for lysozyme-NAG at pH 6.4 and 25 ^{O} C

The interactions of wheat germ agglutinin (WGA) and block copolymers containing saccharides were estimated in terms of association constants by Yamada et al (1999) using fluorescence spectroscopy. Binding properties of the *N*-acetyl glucosamine (GlcNAc) substituted block copolymers with wheat germ agglutinin (WGA) lectin, which specifically recognizes GlcNAc and its β–(1-4) linked oligomers, were discussed. Upon excitation at 290 nm, a maximum in emission spectrum was observed at 350 nm. When the lectin was saturated with GlcNAc carrying block copolymers, the maximum fluorescence intensity was enhanced by 42 % and the emission maximum was shifted to 340 nm. This behaviour is a characteristic of tryptophan residues whose environment becomes less polar than the original one due to the binding of WGA with GlcNAc residues. Thus, it was confirmed that the change in the spectrum results from interactions of WGA with GlcNAc residues in the block copolymers. The plot of $\Delta F / F_o$ vs [S] exhibits a sigmoid shape, which is characteristic of a positive cooperative binding process. It was noteworthy that all the polymers containing GlcNAc displayed more than hundred folds enhancement in association constant relative to the monovalent Ac.GlcNAc itself. Moreover, the GlcNAc- carrying polymers exhibited a Hill coefficient of ca. 2 or above, while monomeric GlcNAc gave a value of unity. The enhanced binding affinity of the GlcNAc containing polymers for WGA is attributed to the polyvalent interactions.

Micrococcus lysodeikticus is a substrate for the enzyme lysozyme. Saccharides are relatively similar to lysozyme substrate, which are capable of inhibiting the lytic activity of enzyme. When appropriate molar concentrations of the two are used a blue shift of about 10 mμ in the fluorescence emissions maximum of the enzyme is seen. The

change in the substrate type changes the fluorescence intensity of lysozyme and depends upon the extent of binding of ligand to the active sites of the enzyme. Using fluorescence emissions data reported in the past by Chipman (1967) and Yamada (1999), we determined the association constants (k_a) for lysozyme complex with monomers, oligomers, macromer, copolymers and imprinted polymers containing NAG.

Fluorescence spectra of lysozyme were recorded on a Perkin Elmer LS-50 B luminescence spectrophotometer. Excitation frequency was 285 nm. Solutions of lysozyme and *N*-Acetyl Glucosamine ligands were prepared in 0.066 M phosphate buffer pH 6.2, containing 0.0154 M sodium chloride and 0.008 M sodium azide. 0.1 milliliter of lysozyme solution (80 μg /ml) was mixed with solution containing different ligand concentration in 2 ml capacity 10 mm square quartz cells maintained at 18 ^{o} C. Phosphate buffer was added to make the volume to 2 ml. The fluorescence intensities of the solutions were measured relative to the solutions containing enzymes and buffer mixtures of the identical concentrations as reference. The relative fluorescence intensity of lysozyme saturated with solution containing different ligand concentration, F_{∞}, was extrapolated from the experimental values by plotting 1/ (F_O-F) against 1/[S] where F is the measured fluorescence of a solution containing enzyme with given substrate concentration [S] and F_O is the fluorescence of a solution containing enzyme alone [Chipman et al (1967)]. The highest concentration of polymer substrate used was such that enzyme was saturated more than 85 %.

4.1.5 Lysozyme inhibition:

Relative binding was estimated for solutions containing saccharides using a procedure reported by Neuberger and Wilson (1967).

N-Acetyl Glucosamine was prepared in 6.6 x 10 $^{-3}$ M phosphate buffer pH 6.2 containing 0.0154 M sodium chloride and 8.0 x 10 $^{-3}$ M sodium azide. One milliliter of stock solution containing different copolymer concentrations was mixed with 1.6 ml of 78 μg/ml of Micrococcus lysodeikticus in a 3-ml capacity glass cuvette. The mixture was incubated for 5 minutes at 20 ^{o} C. To this mixture 0.1 ml of lysozyme (27 μg/ml) was added and mixed thoroughly. The relative absorbance at 450 nm (Δ_{450}) was recorded for 30 seconds. Then difference of initial absorbance containing due to substrate-lysozyme complex alone and the

absorbance after addition of ligand was noted. The relative inhibition per second was measured and % inhibition concentration in mM was calculated.

4.2.0 Choice of ligand:

Synthesis of polyvalent ligands based on NAG:

The binding between lysozyme and saccharide oligomers derived from chitosan increases as the degree of oligomerization increase up to four units, but the oligomers are hydrolyzed by lysozyme. We prepared Acryloyl NAG (Ac.NAG) and Methacryloyl NAG (Meth.Ac.NAG) as the monomeric ligands for enhanced binding with lysozyme. Monomers containing NAG reported here are water soluble, stable and not hydrolyzed by lysozyme. Moreover, the monomers can be further oligomerized and copolymerized to tailor polymer structures for enhanced interactions.

4.2.1 Monomers comprising *N*-Acetyl glucosamine (NAG):

Monomers comprising NAG were synthesized as reported in section 3.1.1. Acryloyl chloride was covalently conjugated to NAG to obtain polymerizable monomer.

The efficacy of NAG in terms of association constant (k_a) and inhibition concentration (I_{50}) is estimated using fluorescence and UV spectroscopy methods respectively. The method for estimation of binding constants and inhibition concentration for monomers containing NAG is described earlier. The molecular weights, association constants and inhibition concentration for NAG monomers are summarized in Table no. 4.1 and Table 4.2.

Table 4.1: Association constant (k_a) & inhibition concentration of lysozyme by NAG conjugates

	Mol.Wt. VPO	k_a M^{-1}	H	I_{50} μM	I_{max}	I_{max} μM
NAG	221	5.24×10^2	2.5	74000	55.29	92500
Ac .NAG	275	7.07×10^4	1.48	14810	50.00	14810
Ac. 6-ACA. NAG	404	1.97×10^5	1.03	35.00	52.50	36.0
P Ac. NAG	638	5.30×10^5	1.3	2.60	89.30	4.30
P Ac. NAG	1315	2.51×10^5	2.0	1.60	73.43	4.20
P Ac. NAG	2631	4.40×10^5	2.37	1.40	73.00	2.10
P Ac. NAG	2808	4.80×10^5	3.1	2.90	69.08	3.70
Ac. 6 ACA. PAc. NAG	823	5.62×10^5	1.60	2.60	94.10	3.60

Binding curve based on Hill model results in a linear graph, where the slope is termed as the Hill Coefficient (H). H is defined as the ability of ligand binding at one site on a macromolecule to influence ligand binding at a different site on the same macromolecule [Forsen (1995)]. Cooperativity is discussed with respect to multisubunit proteins, and is regarded as a quantitative measure, although it is not directly related to how a binding ligand at one site affects the ligand affinity of another sites. A more direct treatment of cooperativity is based on free energy couplings of binding events at different sites.

The association constant for monomer NAG cannot be compared with association constants obtained by the earlier investigators as the molar concentrations and the pH under which the association constants were estimated are different [Chipman et al (1969)]. Dahlquist et al (1966) reported dissociation constant for NAG to be 4-6 x 10^{-2} M. The H value obtained for NAG is an artifact and we cannot rationalize the higher value (2.5) obtained. The inhibition concentration was estimated using the method described earlier. It was not possible to obtain I_{50} values using 1 % (w/v) of NAG, therefore we used higher concentration of the NAG higher to obtain the I_{50} value.

When NAG was conjugated with acrylate monomer either in Acryloyl or methacryloyl form the association constants remained identical (Table 4.1 and 4.2).

Table 4.2: Lysozyme binding & inhibition by methacryloyl NAG and oligomers

	Mol.Wt. by VPO	k_a M^{-1}	H	I_{50} μM	I_{max}	I_{max} μM
M.Ac.NAG	289	6.9 x 10^4	1.30	18100	71.89	21200
P.M.AcNAG	1125	1.0 x 10^5	1.49	3.7	62.00	4.2
P.M.AcNAG	1600	3.9 x 10^5	1.02	2.0	80.95	3.1
P.M.AcNAG	2857	6.3 x 10^5	1.36	1.3	85.23	1.7

The inhibition concentration for Ac.NAG as well as Methacryloyl NAG dropped by five times compared to NAG. The monomers containing NAG have an advantage as they can be further oligomerized or copolymerized to suitable molecular weights.

A further aspect of the investigation was to prepare monomer conjugates comprising a spacer arm and compare the efficacy to NAG. By incorporating the spacer arm, accessibility for binding with active sites of the enzyme is expected to increase.

The spacer arm is inserted between the vinyl group and the NAG ligand. Polymerizable monomer was covalently conjugated to NAG through a spacer arm to form monomer-spacer-ligand conjugate. Spacer 6-Amino Caproic acid (6-ACA) was incorporated to the ligand *N*-Acetyl glucosamine as reported in section 3.1.3.

The association constant increased by almost three folds over Ac.NAG. Increase of association constant is attributed due enhanced hydrophobic effect by spacer arm and to the greater flexibility for binding to lysozyme. The inhibition concentration decreased by more than four hundred folds over to Ac.NAG. Similar results were obtained by Vaidya et al (1999). Series of copolymers containing spacers were synthesized, the value of k_i decreased from 275 x 10^{-6} M [Poly (NIPA-co-Ac.PABA)] to 25 x 10^{-6} [poly (NIPA-co-Ac.6ACA.PABA)] as the spacer chain length increased from 0-5 carbon atoms. Lowering of the k_i values due to the spacers has been attributed due to the spacer effect as well as by the change of microenvironment in the vicinity of the ligand [Pecs (1991)].

As lysozyme consists of six sites designated as A-F, which can bind to saccharides, we thought worthwhile to synthesize oligomers of Ac.NAG and methacryloyl NAG. Low molecular weight oligomers of Ac.NAG and Methacryloyl NAG were prepared by using chain transfer agents such as mercaptoethanol (ME) by varying mole ratio (Takai et al (1993). Oligomers thus synthesized contain hydroxyl end group and can be further conjugated to functionalized theromosensitive polymers containing carboxyl end group. The method of synthesis of the reactive NAG oligomers is reported in Section 3.1.6. The molecular weights of oligomers were determined by VPO and are reported in Table 4.1 and 4.2.

It is important to note that the linkage formed by oligomerization of Ac.NAG and Mathacryloyl NAG is different from that of the linkage found in chitin and chitosan. Therefore, it is expected that these oligomers will not be hydrolyzed by lysozyme.

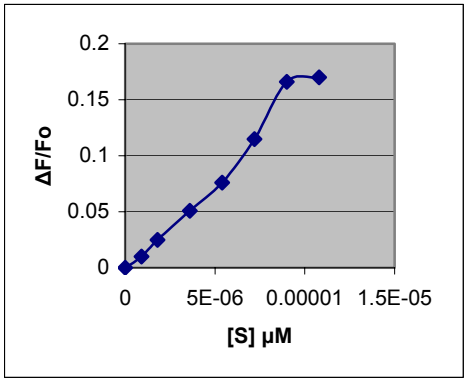


Fig. 4.3: Plot of Δ F/ F_o against [S] for P.Ac.NAG 2808

The plot is sigmoid shape and is a characteristic of a positive cooperative binding process [Yamada et al (1999)].

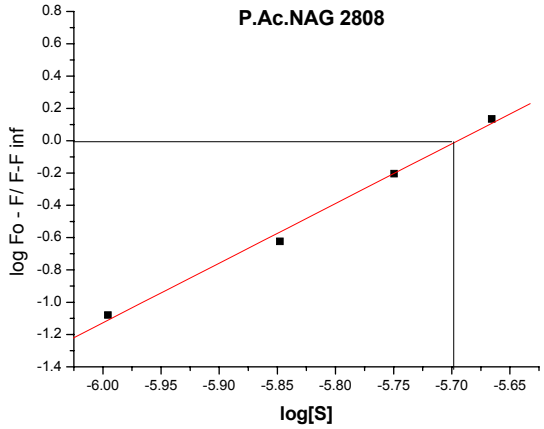


Fig 4.4: Fluorescence plot for lysozyme-oligomer containing NAG of molecular weight 2808 at pH 6.4 and 25 ^{O} C.

Oligomers exhibited increase in three to seven times of magnitude in terms of association constant over to Ac.NAG. Oligomers having varying molecular weight from 638 to 2808 demonstrated same order of association constant. However, the H increased with increase in molecular weight. The same trend was reported by Yamada et al (1999) for block copolymers containing NAG prepared by living cationic polymerization method.

In case of oligomers prepared from Methacryloyl NAG, the association constants were of the same order as for oligomers prepared using Acryloyl NAG. This indicates

that there is no difference in efficacy for oligomers prepared either from acryloyl or methacryloyl monomers.

However, with increase in molecular weights the H value did not increased as rapidly as in case of oligomers prepared from Acryloyl NAG. At this point of time we do not know the reason of difference in H value for the two different series of oligomers except to say that variation of H with molecular weights is specific to a family of polymers.

The inhibition concentrations decreased more than twenty thousand times to that of monomer Ac.NAG. With increase in the molecular weights of the oligomers the I_{50} values were further decreased. Similar trend is exhibited by the oligomers of Methacryloyl NAG. The I_{max} value increased to almost 90 %.

The results indicate the advantage of the polyvalent ligands in binding over monovalent ones. Our results are consistent with those reported by Kristiansen et al (1998) who reported that active sites of lysozyme can accommodate three NAG units and the binding occurs with site C for saccharides containing NAG obtained from chitin and chitosan. Similar findings have also been reported for other systems. For instance Sigal et al (1995) reported sialic acid ligands incorporated in polyacrylamide inhibit virus binding to RBCs. Oligomer exhibit a cluster effect for lysozyme active sites as demonstrated by Dimick et al. (1999) for concanavalin A.

4.2.2 Poly(acryloyl *N*–Acetyl Glucosamine)-6-(-*N*-acryloyl amino) caproate (Macromer):

Oligomers containing polyvalent NAG exhibited greater association constant and lower inhibition concentration than Ac.NAG. It can be concluded that the interactions increase with polyvalency. Earlier, the role of spacer was demonstrated in where there was only one NAG unit. It was thought worthwhile to use simultaneously spacer as well polyvalency effect in a ligand. Moreover, the resulting graft oligomers can be further copolymerized with suitable monomers.

Macromer was synthesized using oligomer of molecular weight 638 exhibiting higher association constant and lower inhibition concentration by conjugation with Acyloyl 6–aminocaproic acid (6-ACA). The method of synthesis of macromer is described in section 3.2.0.

The advantage of synthesizing macromer is that it contains NAG triads and a spacer arm and can be further copolymerized with *N*-isopropyl acrylamide (NIPA) to form thermoprecipitating polymers for recovery of lysozyme and also present multiple copies of the trimer for binding with the substrate. The molecular weight of resulting macromer was 823 and consisted of pendent polyvalent NAG moieties for enhanced interactions with the active sites of the lysozyme.

Association constant (k_a) is of the same order of magnitude orders of magnitude as compared to Ac.NAG oligomers. The relative inhibition concentration for lysozyme is also of the same order as the oligomer. The I_{max} increased from 55.29 to 94.10. I_{max} value reported here for macromer containing polyvalent NAG is maximum ever reported to our knowledge. The results described so far for monomers, oligomers and macromer indicate the difference in monovalent and polyvalent interactions with lysozyme in terms of enhanced association constant and decreased inhibition concentration. However, these do not provide an insight about the specific active sites on the lysozyme occupied. Therefore, competitive binding experiments were carried out using Biebrich Scarlet dye. The results of these experiments are described later in section 4.3.3.

4.2.3 Copolymers bearing NAG:

After having established the effect of the synthetic monomers, oligomers and macromer containing NAG in terms of efficacy of inhibition with lysozyme, thermoprecipitating copolymers were synthesized by copolymerization with NIPA. Advantage of designing copolymers is that the multiple NAG ligands can be incorporated on the polymer backbone to elucidate the polyvalency effect. In addition these copolymers could be used for the recovery of lysozyme by affinity precipitation. The role of copolymers in steric stabilization is also evaluated.

The copolymers of NIPA and Ac.NAG were prepared by conventional free radical polymerization method and are described in section 3.2.1. The molecular weight, mole % NAG, association constant, inhibition concentration and LCST are summarized in Table 4.3.

Molecular weights of copolymers were determined by vapor pressure Osmometer and the method is reported in section 3.1.5. The copolymer composition was estimated by comparing integration values of NAG using ^{1}H NMR. NAG content (% moles) in

copolymers of NIPA and Ac.NAG was estimated by comparing integration of isopropyl groups and NAG signals of corresponding protons by using NMR. Mole % composition NAG in copolymers, LCST, k_a and I_{50} values are summarized in Table 4.3.

Surprisingly, copolymers of NIPA and Ac.NAG prepared by free radical polymerization exhibit identical LCST (34.8 ^{O} C) in spite of variation in comonomer compositions. We believe at this stage that this is due the formation of block structures in copolymers of NIPA and Ac.NAG.

The efficacy of the copolymers in terms of association constant and inhibition concentration was estimated as reported for the monomers in the earlier section.

Table 4.3. Lysozyme binding & inhibition by copolymers of NIPA and Ac.NAG

Mole % NAG by NMR	Mol wt. VPO	k_aM^{-1}	H	I_{50} μM	I_{max}	I_{max} μM	LCST ^{O}C
14	3200	2.03 x 10^5	1.38	2.1	64.95	2.8	34.8
18	1954	1.97 x 10^5	1.27	3.4	52.13	3.6	34.8
34	2830	2.47 x 10^5	1.46	5.6	58.00	6.5	34.8
42	5953	2.05 x 10^5	1.97	5.7	72.50	8.4	34.8
54	2560	1.99 x 10^5	1.18	7.9	68.33	10.9	34.8
78	2784	3.38 x 10^5	2.42	9.9	79.40	15.7	34.8

LCST of the thermoprecipitating polymer usually decreases with increase in the hydrophobicity of the comonomer and increases with increasing hydrophilic content of the comonomer [Hoffman et al (1995)].

It was possible to incorporate NAG in increasing molar concentrations to achieve polyvalency. Molecular weights of copolymers were low enough to interact with the active sites of the lysozyme. The association constant (k_a M^{-1}) is almost identical inspite of increase in NAG content. The k_a values are of same order of magnitude as the oligomers.

Inhibition concentrations in term of I_{50} did not show significant variation with increasing NAG in the copolymer. The inhibition concentration values increased slightly compared to the oligomers. This is due to steric stabilization effect similar to that observed by Spaltenstein et al (1991).

The values of binding constants for the copolymers of NIPA and acryloyl NAG are comparable to the corresponding values of the oligomers of acryloyl as well as methacryloyl NAG. This suggests that the acryloyl NAG units in the copolymers are present as blocks. In contrast the association constants for copolymers of NIPA and methacryloyl NAG are lower than corresponding values for the oligomers (see next section) because methacryloyl NAG units are randomly distributed along the polymer chain. This is further supported by the observation that the LCST of the copolymer of NIPA and Ac.NAG is independent of the copolymer composition whereas that for the copolymers containing methacryloyl NAG increases with the comonomers content and eventually exceeds 70 ^{O} C. Further, increase in the NAG content has very little effect on the binding constant as well as inhibition concentration, probably because Ac.NAG blocks are now not involved in binding due to crowding effect. It was therefore thought worthwhile to synthesize di block and tri block copolymers.

4.2.4 Copolymers of NIPA and Meth.Ac.NAG:

We also designed and synthesized copolymers of NIPA and Methacryloyl NAG to compare the efficacy of the same with copolymers of NIPA and Ac.NAG.

The method of synthesis is reported in section 3.2.3. The molecular weights, association constant (k_a M^{-1}), inhibition concentration and values LCST for copolymers of NIPA and Methacryloyl NAG (M.Ac.NAG) are summarized in Table 4.4.

Table 4.4: Lysozyme binding & inhibition for copolymers of NIPA and Meth.Ac.NAG

Mole % NAG by NMR	Mol. wt. VPO	k_a M^{-1}	H	I_{50} μM	I_{max}	I_{max} μM	LCST ^{O} C
25	3625	8.91 x 10^{3}	1.80	2.00	63	4.80	70>
32	2648	1.00 x 10^{4}	1.99	16.5	51	16.5	70>
38	2430	8.12 x 10^{4}	1.32	5.80	63	7.20	70>
42	2230	7.80 x 10^{4}	2.00	8.00	50	8.80	70>
54	2463	8.00 x 10^{4}	2.20	8.50	61	9.40	70>

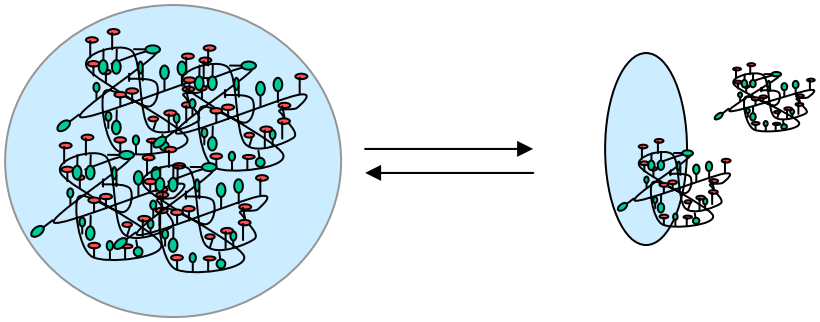
The association constants obtained for copolymers of NIPA and Methacryloyl NAG are lower than the oligomers of Methacryloyl NAG.

The associations constant for the copolymers are lower by one order of magnitude *vis a vis* oligomers. These copolymers did not demonstrate LCST till 70 ^{O} C. This observation lead to the conclusion that comonomers of NIPA and Methacryloyl NAG might lead to random copolymers.

The relative inhibition values for lysozyme in terms of I_{50} are much higher compared to the oligomers of Mathacryloyl NAG indicating the random arrangement of the oligomers in the polymer.

It can be concluded that the random copolymers are not as efficient as to oligomers and copolymers prepared using NIPA and Ac.NAG.

At this point of time we wanted to investigate the unusual LCST behaviour in copolymers of NIPA and Ac.NAG. Therefore, we estimated the reactivity ratios of NIPA and Ac.NAG and NIPA and Methacryloyl NAG.



Below LCST **Above LCST**

Fig. 4.5: Coil to globule phase transition

Copolymers were prepared in varying molar ratios:

1. NIPA co Ac.NAG
2. NIPA co Meth.Ac. NAG

4.2.5 Copolymer Synthesis: Reactivity ratios and copolymer structure

Monomers have different tendencies to undergo copolymerization. Some monomers are more reactive in copolymerization than indicated by their rates of homopolymerization, whereas the other monomers are less reactive. The chemical reactivity of the propagating chain in a copolymerization depends on the identity of the last monomer unit. Copolymerization of the two monomers leads to two types of propagating species – Monomers M_1 and M_2 each can add either to a propagating chain

ending in M_1* or to one ending in M_2*. These can be represented by M_1* and M_2* where the asterisk represents a radical, a carbocation, or a carbanion as the propagating species depending on the particular case. Four propagation reactions are possible:

$$--- M_1^* + M_1 \xrightarrow{K_{11}} -- M_1M_1^* \quad (4.1)$$

$$---M_1^* + M_2 \xrightarrow{K_{12}} --M_1 M_2^* \quad (4.2)$$

$$---M_2^* + M_1 \xrightarrow{K_{21}} -- M_2 M_1^* \quad (4.3)$$

$$---M_2^* + M_2 \xrightarrow{K_{22}} -- M_2M_2^* \quad (4.4)$$

where,

k_{11} is the rate constant for a propagating chain ending in M_1* adding to monomer M_1,

k_{12} is the rate constant for a propagating chain ending in M_1* adding to monomer M_2, and so on. The propagation of a reactive centre by addition to the same monomer is termed a homo propagation or self propagation and propagation of a reactive centre by addition of the other monomer is referred to as cross propagation or a crossover reaction. All propagation reactions are assumed to be irreversible [Odian (1991)].

Methods for calculating reactivity ratios can be classified as linear least square (LLS) and non-linear least square (NLLS) methods. It is accepted that LLS method such as those proposed by Fineman and Ross can be applied to experimental data at sufficiently low conversions, because the calculation is based on the conventional copolymerization equation, whereas the Fineman-Ross method for estimation of reactivity ratios extended by Kelen Tudos can be applied to medium, high conversions experimental data without significant errors [Mao et al (1993)].

Table 4.5 indicates reactivity ratios for NIPA and Ac.NAG prepared in distilled water at temperature below LCST (25 ^{O}C.). Copolymerization conversion was controlled below 10 %.

All copolymers were water soluble and NMR was taken in D_2O. The composition of the copolymer was determined by analyzing the relative integration of protons

corresponding to the number of protons of each monomer as shown in NMR spectra as below:

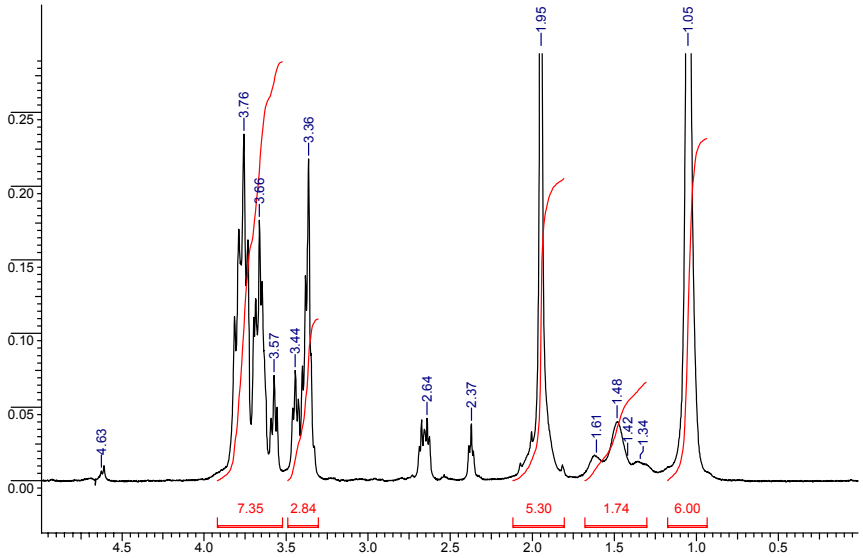


Fig. 4.6: ^{1} H NMR integration for protons in copolymers of NIPA and Ac.NAG

From the monomer feed ratio and the resultant copolymer compositions, the reactivity ratios of NIPA and Ac.NAG were evaluated by Kelen-Tudos (K-T) method.

Using the Fineman –Ross equation:

$$f(F-1)/F = r_1 (f^2/F) - r_2 \qquad 4.5$$

where,

Subscript 1 is NIPA and subscript 2 is Ac.NAG, and $F = F_1 / F_2$ and $f = f_1 / f_2$.

f_1 and f_2 = Mole fraction of monomer in feed

F_1 and F_2 = Mole fractions of monomer in copolymer

The linear plot of $f(F-1)/F$ Vs f^2/F is shown in Fig. 4.6.

The monomer reactivity ratios were calculated using the method of K-T according to equation,

$$\eta = [\, r_1 + (r_2/\alpha)\,\zeta\,] - [\, r_2/\alpha\,], \qquad 4.6$$

where,

$\alpha = \sqrt{H_{max} \times H_{min}}$

G and H are derived from Fineman- Ross parameters (See Table 4.6)

η and ζ = Kelen-Tudos parameter are functions of molar ratios of the monomers in the copolymer and in the feed,

Thus a plot of f / (F-1) against f^2 / F is linear with slope r_1 and r_2 intercept. A plot shown in figure 4.7 leads to r_1= 1.12 and r_2 =1.88.

Table 4.5: Copolymerization of NIPA and Ac.NAG for the determination of the reactivity ratios

NIPA (f_1)	Ac.NAG (f_2)	F_1	F_2
0.215	0.780	0.107	0.893
0.291	0.708	0.140	0.860
0.489	0.510	0.240	0.755
0.622	0.377	0.320	0.680
0.787	0.212	0.580	0.420

Table 4.6: Kelen-Tudos parameters for the copolymerization of NIPA and Ac.NAG in water

$f= f_1/f_2$	$F=F_1/F_2$	$G=f(F-1)/F$	$H=f^2/F$	$\alpha+ H$	$\eta =G/(\alpha+ H)$	$\zeta=H/ \alpha+ H)$
0.275	0.119	-2.02	0.635	3.13	-0.64	0.20
0.411	0.162	-2.11	1.04	3.54	-0.59	0.29
0.960	0.324	-1.99	2.84	5.34	-0.37	0.53
1.645	0.470	-1.85	5.75	8.25	-0.22	0.69
3.701	1.380	1.02	9.92	12.4	0.08	0.79

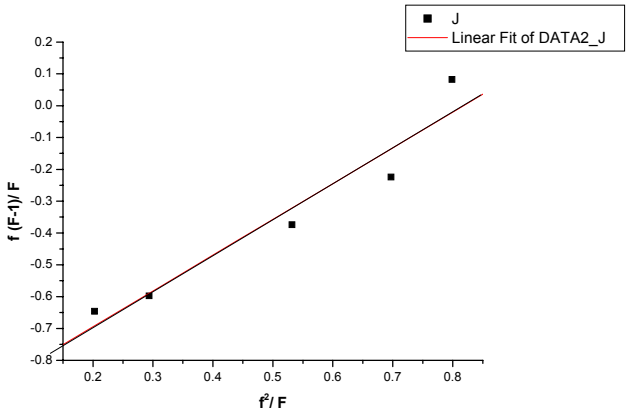


Fig 4.7: Kelen- Tudos plot for reactivity ratio determination for NIPA-co–Ac.NAG

The r_1 and r_2 values for NIPA and Ac.NAG are 1.12 and 1.80. When both r_1 and r_2 are greater than unity i.e r_1 and r_2 >1, there is a tendency to form block copolymers in

which there will be blocks of both monomers in the chain. In such copolymerization the monomer has a tendency on the part of the radical to add to the same monomer thereby forming the blocks. The formation of blocks is supported by LCST values as they are identical (34 ^{o} C) for all comonomer compositions.

4.2.6 Reactivity ratios for copolymerization of NIPA and Methacryloyl NAG

Similarly, reactivity ratios were estimated for copolymers of NIPA and Methacryloyl NAG and the values are represented in table 4.7.

Table 4.7: Copolymerization of NIPA and Methacryloyl NAG for the determination of the reactivity ratios

NIPA (f_1)	MethAc.NAG (f_2)	F_1	F_2
0.209	0.793	0.462	0.537
0.369	0.630	0.575	0.420
0.610	0.389	0.677	0.320
0.770	0.221	0.680	0.319

Table 4.8 : Kelen-Tudos parameters for the copolymerization of NIPA and Methacryloyl. NAG in water

$f= f_1/f_2$	$F=F_1/F_2$	$G=f(F-1)/F$	$H=f^2/F$	$\alpha+ H$	$\eta =G/(\alpha+ H)$	$\zeta=H/ \alpha+ H)$
0.26356	0.86034	-0.04279	0.08074	0.758	-0.05699	0.10754
0.58571	1.36905	0.15789	0.25058	0.92	0.17151	0.2722
1.56812	2.11563	0.82691	1.16231	1.84	0.4513	0.63434
3.48416	2.13166	1.84968	5.6948	6.37	0.29061	0.89473

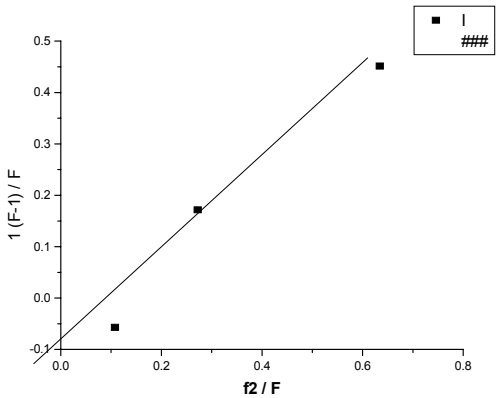


Fig 4.8: Kelen-Tudos plot for reactivity ratio determination for NIPA-co–Meth.Ac.NAG

Copolymers of NIPA and Methacryloyl NAG form random copolymers as indicated by reactivity values ($r_1 < 0.47$ and $r_2 < 0.067$). In this case monomer preferably adds to the other radical. Yet r_1 is not low enough to lead to alternating copolymers.

This is further supported by the fact that the copolymers of NIPA and Methacryloyl NAG do not exhibit LCST even at temperature 70 ^{O} C indicating formation of true random copolymers.

4.2.7 Reactivity ratios for copolymers of NIPA–co-Macromer containing P.Ac.NAG 638

The system NIPA and Macromer exhibits formation of random copolymers as indicated by the values of reactivity ratios.

Table 4.9: Reactivity ratio for copolymers of NIPA and macromer

NIPA (f_1)	Ac.NAG (f_2)	F_1	F_2
0.215	0.784	0.18	0.81
0.381	0.618	0.35	0.65
0.410	0.620	0.38	0.62
0.622	0.380	0.62	0.38

Table 4.10: Kelen-Tudos parameters for copolymers of NIPA and macromer

$f= f_1/f_2$	$F=F_1/F_2$	$G=f(F-1)/F$	$H=f^2/F$	$\alpha+ H$	$\eta =G/(\alpha+ H)$	$\zeta=H/ \alpha+ H)$
0.2744	0.222	-0.962	0.339	0922	-1.041	0.36
0.6165	0.538	-0.528	0.7058	1.288	-0.41	0.547
0.6612	0.6129	-0.4176	0.7135	1.29	-0.322	0.550
1.63	1.63	0.6315	1.630	2.21	0.285	0.736

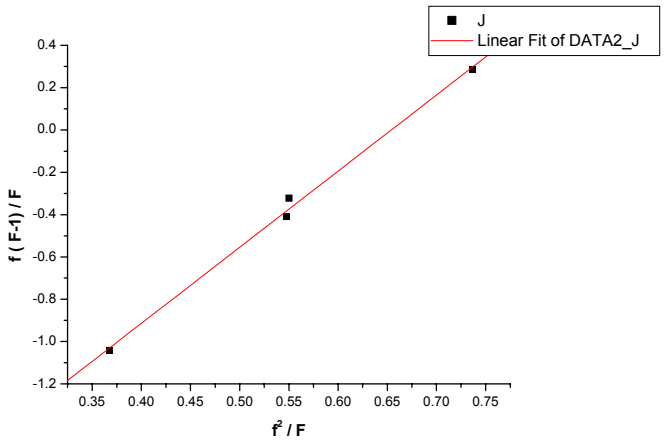


Fig 4.9. Kelen-Tudos plot for the free radical copolymerization of NIPA–co-Macromer containing P.Ac.NAG 638

Table 4.11: Reactivity Ratios for copolymers by Kelen-Tudos parameters

	r_1 (M_1)	r_2 (M_2)
NIPA (M_1) co Ac. NAG (M_2)	1.12	1.880
NIPA (M_1) co Meth.Ac. NAG (M_2)	0.47	0.067
NIPA (M_1) co Macromer (M_2)	3.08	0.743

4.2.8 NIPA-co-Macromer / graft copolymer:

The results of association constant and inhibition concentration for lysozyme indicate that the block copolymers are more efficient than the random copolymers in the inhibition of lysozyme. The differences in the monomer reactivity ratios for the systems NIPA / Ac.NAG and NIPA / M.Ac.NAG indicate that the monomer reactivity ratios are highly susceptible to the minor variations in the comonomer structure and cannot be readily predicted. In order to ensure that the copolymer contains repeat units of NAG in juxtaposition we used the macromer technique for copolymerization (see section 3.2.5**).**

Using this method it is possible to control the spacing, steric accessibility, number of ligand molecules in the polymer solubility and physical structure of the polymeric conjugates. The macromer approach results in the polymers containing repeat NAG units, regardless of the copolymerization behaviour of the two.

Table 4.12: Lysozyme binding & inhibition by copolymers of NIPA & Macromer

Moles % NAG by NMR	Mol.Wt NIPA	$k_a M^{-1}$	H	I_{50} μM	I_{max}	I_{max} μM	LCST ^{o}C
40	4108	5.08×10^5	1.14	0.120	89.65	0.11	34.8
36	4010	5.01×10^5	2.23	0.117	88.12	0.80	34.5
33	3573	3.80×10^5	2.23	0.133	90.00	0.33	34.6
17	3852	2.45×10^5	1.80	0.343	81.00	0.34	34.3

The significant observation for copolymers of NIPA and Macromer is that the association constants (k_a) have remained practically the same as the oligomers but the inhibition concentration (I_{50}) has decreased tremendously for the thermoprecipitating copolymers containing affinity ligands *vis* a *vis* their monomeric / oligomeric counterparts.

Although the arrangement of the NAG is random, these copolymers exhibit better efficacy than random copolymers of NIPA and Mathacryloyl NAG. The order of magnitude in terms association constants has increased by one fold to that of random copolymers of NIPA and Methacryloyl NAG. The result is attributed to the prearranged polyvalent blocks of NAG in juxtaposition.

The Hill constant (H) has increased to 2.23 demonstrating the polyvalent binding and cooperativity with the active sites of lysozyme. The I_{max} values increased upto 90 % indicating higher inhibition of the substrate.

Although copolymers of NIPA and macromer are prepared by conventional free radical polymerization method and the monomer reactivity ratios indicate the formation of random copolymers. the resulting copolymers will contain polyvalent NAG units. Moreover, the enhanced binding is due to contribution of steric stabilization by polymer backbone along with the bound ligands to lysozyme.

4.3.0 Block copolymers containing NAG:

It is not always possible to obtain the desired sequence of ligands during copolymerization using free radical technique since this is governed by the reactivity ratios of monomers involved. However, in block copolymerization methods sequential

arrangement of monomers can be obtained. The chain length and molecular weights of the blocks can be varied independently during synthesis.

The block copolymers were synthesized to obtain well defined polymeric structures containing polyvalent NAG units. Moreover, the resulting block copolymers being temperature sensitive can be used for the recovery of lysozyme.

Similarly, the triblock copolymers are designed by synthesizing difunctional homopolymers of the poly *N*-isopropylacrylamide using 4-azobis cyanovaleric acid [Goethals (1989)] and chain transfer agents such as mercapto propionic acid. The reactive oligomers containing NAG are prepared using mercapto ethanol [Takai et al (1993)] and are further coupled with poly *N*-isopropylacrylamide containing terminal functional groups using coupling agents. The resulting BAB block copolymers contain low NAG incorporation. It was expected that the polyvalent NAG units would bind effectively and simultaneously to the active sites of the lysozyme. In addition, the central segment of polymer will provide steric exclusion.

We therefore designed and synthesized AB and BAB block copolymers of varying chain lengths consisting of polyvalent NAG ligands for enhanced binding.

Recently, Yamada, et al (1999) reported controlled synthesis of amphiphilic block copolymers bearing pendent *N*–Acetyl–D-Glucosamine residues by living cationic polymerization and the interaction of the resulting diblock copolymers with lectins. The methodology of synthesizing homopolymers and the block copolymers containing *N*–Acetyl–D-Glucosamine residues demonstrates significant increase in binding affinity for lectin than to monomer counterpart. It is reported that the block copolymers exhibit greater efficacy than homopolymers in binding affinity for WGA. In addition to this, irrespective of the DP_n of the GlcNAC-carrying hydrophilic segment, the incorporation of the hydrophobic poly isobutyl vinyl ether (PIBVE) sub chain of n = 10 brings about the most effective binding with an increase in k_a by one order of magnitude relative to those of the homopolymers. The applicability of the method for obtaining block copolymers is however, limited by the need for low temperature and stringent polymerization conditions.

We report the conjugation of PNIPA polymers of varying chain length with PAc.NAG to obtain AB block copolymers. Synthesis of block copolymers is reported in

section 3.3.1. Block copolymers containing poly NAG and poly NIPA have not been reported in the past. The LCST of the block copolymers was 34.5 ^{O} C (Table: 4.13). The molecular weights of each segment in block copolymers were varied in order to establish the affect of independent chain lengths in resulting block copolymer. Blocks of PNIPA were prepared using varying mole ratio of 4-azobis-cyanovaleric acid and conjugated to blocks of reactive oligomers containing NAG prepared using chain transfer agent.

Table 4.13: Lysozyme binding & inhibition by A-B block copolymers P.(NIPA)-co-P. (Ac.NAG)

Mole % of NAG	Mole A	Moles B	Mol. Wt. A	Mol. Wt .B	ka M^{-1}	H	I_{50} µM	% I_{max}	I_{max} µM	LCST O C
8.04	26.51	2.32	3000	638	5.01 x 10^{5}	2.50	2.41	67.25	3.50	34.5
4.19	53.02	2.32	6000	638	6.30 x 10^{5}	3.60	0.76	68.00	1.50	34.5
3.17	70.67	2.32	8000	638	1.00 x 10^{6}	3.30	0.75	70.02	1.40	34.5
11.91	70.67	9.56	8000	2631	2.05 x 10^{5}	2.05	3.49	71.17	4.90	34.5
0.64	123.7	0.80	14000	221	1.70 x 10^{5}	2.14	-	-	-	32.5
1.15	123.7	1.45	14000	400	1.69 x 10^{5}	1.26	-	-	-	34.4
1.84	123.7	2.32	14000	638	1.38 x 10^{6}	1.00	2.60	94.30	0.06	34.5
3.72	123.7	4.78	14000	1315	6.40 x 10^{5}	3.70	6.90	74.22	1.50	34.5
7.17	123.7	9.56	14000	2631	1.26 x 10^{6}	3.13	1.59	73.80	2.80	34.5
26.5	26.51	9.56	3000	2631	3.20 x 10^{5}	1.58	3.80	61.83	7.50	34.5
15.59	53.02	9.56	6000	2631	6.30 x 10^{5}	2.05	2.90	69.22	6.90	34.5
9.76	88.37	9.56	10000	2631	8.37 x 10^{5}	2.45	2.10	75.90	3.70	34.5

AB block copolymers exhibit an order of magnitude higher association constant as compared to Ac.NAG oligomers and are summarized in Table 4.13.

In the first are the molecular weight of the segment A was increased and keeping segment B constant the association constants increased. The inhibition concentration was decreased.

In the second case the length of segment A was kept constant and the length of segment B was increased. This led to an increase in the association constant. The same trend was found for the inhibition concentration.

In third case the molecular weight of segment A was increased keeping molecular weight of segment B constant but at a higher value than a case I. The association constants increased and the inhibition concentration decreased. The increase in H value indicates that the binding of the subsequent NAG units on lysozyme site is enhanced as the binding between NAG unit and lysozyme takes place as a result of increase in the molecular weight of segment A. The results are consistent with the observation that the adsorption coefficient of the polymer chain is increased with polymer molecular weight.

It can be noted that oligomer of mol.wt. 638 when conjugated with PNIPA to form block copolymer exhibit higher association constant than the oligomer itself. The additional enhancement in binding in block copolymer is a contribution arising from the PNIPA segments i.e. a steric exclusion effect. The result is identical as demonstrated by Spaltenstein et al (1991) for the polyacrylamide bearing pendent α-sialoside groups for binding to virus.

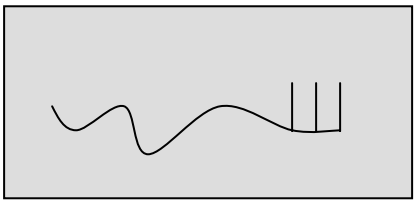


Fig. 4.10: Scheme of AB block copolymers containing trimer NAG

4.3.1 BAB copolymers containing NAG:

In case of AB block copolymers the oligomer conjugated was at only one end and therefore the binding of such polymer is possible only for a single lysozyme. However, this can be enhanced by preparing BAB block copolymers as the latter contains oligomers at both the terminals. The higher binding can result in improved lysozyme recovery.

Functional oligomers were prepared using chain transfer agents as reported in section 3.1.7 and coupled with the PNIPA having di carboxyl end groups prepared using 4-azobis cyanovaleric acid. Length of the P.Ac.NAG block was kept constant (638) since this demonstrated highest association constants and lower inhibition concentration. The LCST remains constant for triblock copolymers due to conjugation of blocks of PNIPA to the oligomer, which can not influence the LCST which is 32.7 ^{O} C (Table 4.14).

Table 4.14: Lysozyme binding & inhibition by B-A-B block copolymers of P.(NIPA)-co-P.(Ac.NAG)

Mole % of NAG	Mol.wt A	Mol.wt B	k_a M^{-1}	H	I_{50} µM	% I_{max}	I_{max} µM	LCST ^{O}C
0.72	36,000	638	3.98×10^{6}	3.01	0.350	50.00	0.35	32.7
0.46	56,000	638	4.36×10^{6}	3.21	0.220	64.11	0.22	32.7
0.29	90,000	638	4.96×10^{6}	3.40	0.085	83.33	0.14	32.7

The results on BAB copolymers are summarized in Table 4.14. These polymers exhibit highest association constant (k_a M^{-1}) amongst all the monomers, oligomers and copolymers investigated. The interesting observation is that the association constant has increased tremendously by an order of magnitude over the A-B copolymers in spite of very low ligand concentration. To evaluate the effect of molecular weight on association constants, the molecular weight of segment A was varied and the molecular weight of segment B was kept constant. With increase in the molecular weight the association constant increased. Also the inhibition concentration decreased substantially. The enhanced efficacy can be attributed to two factors 1) Since the ligands are far apart, the terminal NAG ligands can bind to two lysozyme units simultaneously 2) increase in molecular weight of polymer leads to higher inhibition concentration for lysozyme by steric stabilization. The H values are higher and explain the positive cooperativity and polyvalency. Since BAB polymers contain two ligands at the terminal, which can bind to two different lysozymes. It can be concluded that the BAB copolymers exhibit higher association constant over to random and AB block copolymers.

One of the advantages of block copolymers reported in this investigation is that the methodology is simple and yet efficient. Moreover, ligands can be incorporated at very low concentration and yet demonstrate the higher association constant. This is critical and important for the expensive ligands such as sialic acid. Although, Whitesides et al (1996) reported the effect of polyvalency for polyacrylamides bearing sialic acid, block copolymers could have been more efficient for the inhibition of virus.

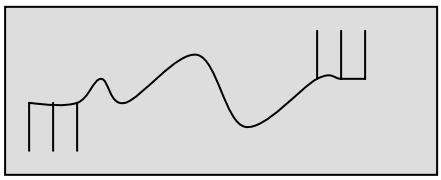


Fig. 4.11: BAB triblock copolymers containing polyvalent NAG

4.3.2 Molecular imprinting (MIP): Selective recognition method for biomolecules

So far we demonstrated the efficacy of the NAG ligands in the form of monomers, oligomers, macromer and copolymers. The monomers containing NAG were oligomerized to obtain the polyvalency effect. We prepared copolymers of NIPA / Ac.NAG and NIPA / Methacryloyl NAG to obtain copolymers. We also prepared copolymers of NIPA and macromer, which contain NAG in juxtaposition. The efficacy of oligomers, macromer and copolymers enhanced over monomer. However, it was thought worthwhile to synthesize block copolymers containing NAG oligomers at the terminal. These copolymers exhibited higher association constant and lower inhibition concentration. One of the objectives of the present investigation was to recover lysozyme and the results are discussed in next section.

Apart from various methods used to prepare NAG ligands, one of the ideal methods to enhance binding between lysozyme and NAG would be molecular imprinting. As discussed in first chapter, two basic approaches are used for molecular imprinting (1) the pre-organized approach, developed by Wulff and coworkers, where the aggregates in solution prior to polymerisation are maintained by (reversible) covalent bonds, and (2) the self-assembly approach, developed by Mosbach and coworkers, where the pre-arrangement between the print molecule and the functional monomers is formed by non-covalent or metal coordination interactions. However, both imprinting approaches make use of a high percentage of crosslinker. The resulting polymers are of substantial rigidity and completely insoluble. The template-assisted assembly, leads to an artificial recognition matrix.

Imprinting strategies involving, non-covalent interactions between the print molecule and the functional monomers are more versatile. The apparent weakness of this interaction may be overcome by allowing a multitude of interaction points simultaneously.

Karmalkar et al (1996) reported polymers comprising Methacryloyl histidine, Methacrylic acid and 2-hydroxy ethyl methacrylate firstly in the presence of the cobalt and the template molecule and, secondly in the absence of cobalt and the template molecule. No crosslinker was used in either case.

Imprinted polymers are usually prepared in non-polar organic solvents such as chloroform or toluene. Biological recognition mainly occurs in hydrophilic environment and therefore we synthesized copolymers of NIPA and Macromer in aqueous medium.

As discussed in earlier section the presence of sequences of NAG units can be ensured by incorporating. Macromer contain monomer conjugated with a spacer arm and polyvalent NAG ligand. The imprinted copolymers were prepared using NIPA as a comonomer and lysozyme as a template.

We used two approaches for preparation of lysozyme imprinted polyvalent copolymers

1) Variation of degree of inhibition and

2) Variation of ligand concentration

The method of preparation is described in sections 3.3.4 and 3.3.5.

The molecular weights, association constant, inhibition concentration and LCST are described in Table 4.15.

Table 4.15 : Imprinted polymers of NIPA & poly (acryloyl *N*–Acetyl Glucosamine)-6-(-*N*- acryloyl amino) caproate (Macromer) by variation of degree of inhibition

% Inhibition for Imprinting	% Moles by NMR	k_a M^{-1}	H	I_{50} µM	I_{max}	I_{max} µM	LCST ^{O}C
100	30	1.25×10^6	1.45	0.06	96.00	0.3	34.3
80	28	3.57×10^5	1.39	0.11	92.64	1.1	34.3
60	21	8.7×10^5	2.21	0.74	92.00	1.6	34.2
40	24	3.01×10^5	1.00	0.96	88.39	2.4	34.3
20	18	2.17×10^5	1.62	0.18	88.00	1.8	34.2

Table 4.16: Imprinted copolymers of NIPA & poly (acryloyl *N*–Acetyl Glucosamine)-6-(-*N*- acryloyl amino) caproate (Macromer) by variation of ligand concentration

% Inhibition for Imprinting	% Moles by NMR	k_a M^{-1}	H	I_{50} µM	I_{max}	I_{max} µM	LCST ^{O}C
100	69	3.9×10^5	1.79	1.25	60.00	6.94	> 70
100	62	3.38×10^5	2.14	1.25	66.66	3.75	> 70
100	2.4	5.0×10^5	1.04	26.6	61.00	66.6	32.3

Imprinted polymers prepared in the presence of lysozyme exhibit higher association constant than copolymers prepared in the absence of lysozyme.

Moreover, the inhibition concentrations decreased significantly as compared to the copolymers prepared using NIPA and Methacryloyl NAG in the absence of lysozyme. It was interesting to note that as the inhibition of lysozyme during synthesis decreased, the association constant also decreased demonstrating the incomplete imprinting. Moreover, the imprinted polymers prepared by variation of ligand concentration exhibited lower association constants than the polymers prepared using variation of degree of inhibition.

The result proves the efficacy can be increased by using 1) preformed polyvalent ligand exhibiting higher efficacy and 2) imprinted copolymer in the presence of template lysozyme. I_{max} improved to 96 %. LCST was constant demonstrating the formation of NIPA blocks along with the NAG.

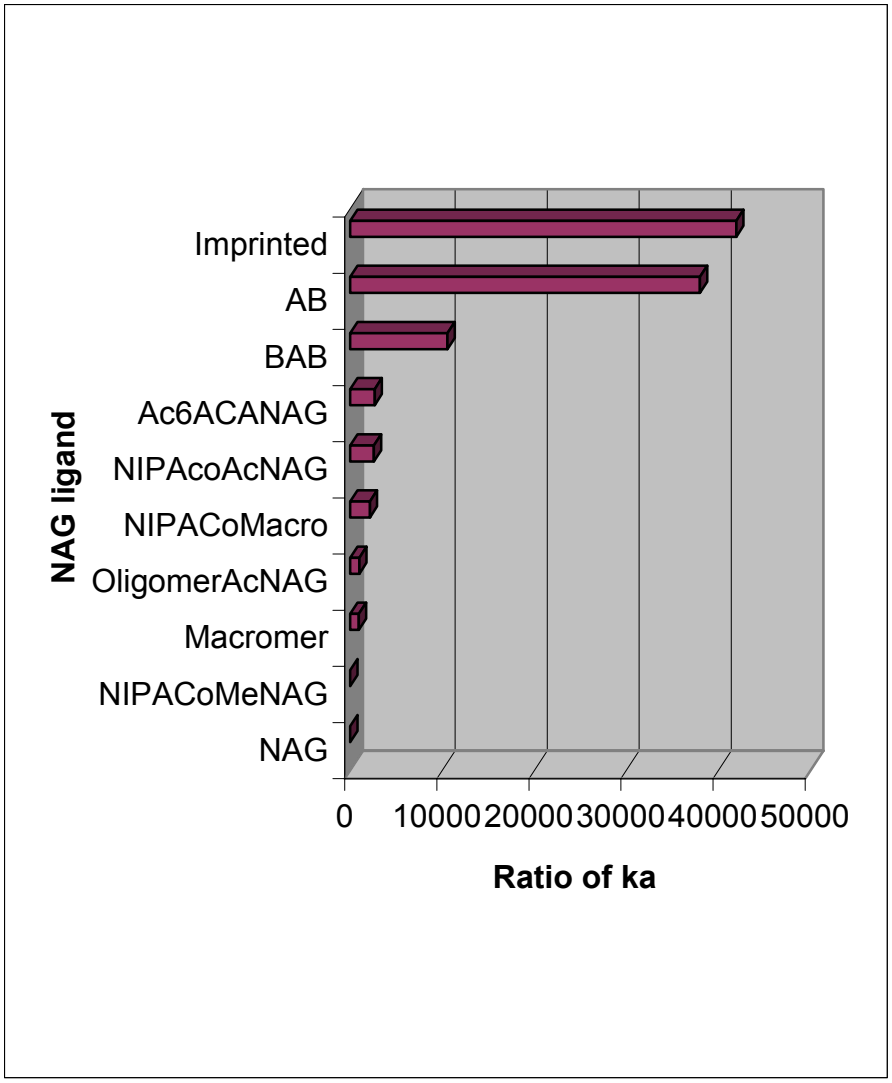


Figure 4.12: Comparative ratios of k_a for ligands to NAG

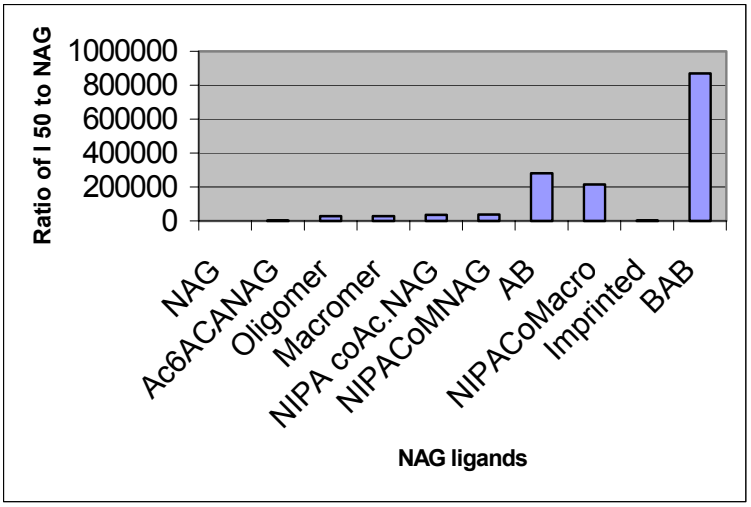


Fig. 4.13: Comparative ratios of I_{50} by NAG ligands

4.3.3 Productive binding by NAG ligands:

The binding between the ligand and the substrate needs to be analyzed not only in term of the magnitude but also the specific sites within the substrates in view of the importance in governing catalytic activity. This aspect was addressed by Holler et al (1975) with reference to binding between chitin oligosaccharides and lysozyme. The frame work used, its application to the polyvalent NAG lysozyme binding and implications are discussed in details below:

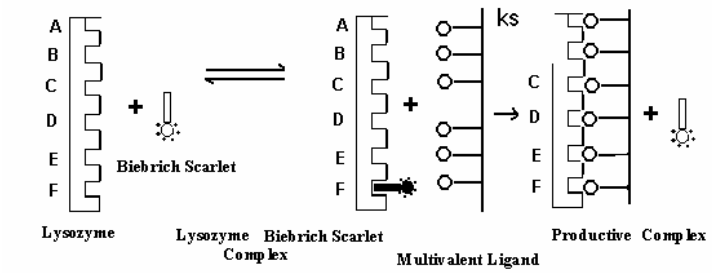


Fig. 4.14: Productive binding by multivalent saccharides

The association constants and inhibition concentration values for the monomers, macromer, oligomers, copolymers and imprinted polymers demonstrate the role of polyvalent ligands in enhanced binding of lysozyme. Lysozyme has six sites designated as A-F, out of which A, B and C site can be occupied by the trimer of NAG. Only

chitohexose can bind to site F as has been demonstrated by Biebrich scarlet dye displacement method. [Holler et al (1975)].

Oligomers, copolymers and imprinted polymers containing NAG demonstrate higher association constants as well as lower inhibition concentrations than values for saccharides containing NAG reported in the literature. The results explain the gross efficacy in terms of association constants and inhibition concentration. However, the results do not highlight the details of binding such as the actual occupied sites by individual ligands on lysozyme.

4.3.4 Rationale of competitive binding:

The competitive binding studies are carried out using Biebrich scarlet dye as it occupies site F on the lysozyme. The ability of the saccharide ligand that can bind to site F on the lysozyme and competitively displace the dye is referred as the productive binding. Holler, et al discussed the variation in assurances various wavelengths resulting from ligand enzyme complexation and the method of estimating productive and non productive lysozyme–chitosaccharides complexes. If Biebrich scarlet–lysozyme complex is compared with Biebrich Scarlet alone, a difference in spectrum due to a red shift from 505 nm to 510 nm is observed. The difference spectrum has a small maximum at 459 nm and an isobestic point at 550 nm. The extinction difference coefficient for the minimum at 495 nm was obtained from the intercept of a Hildebrand–Benesi plot. Addition of lysozyme and Biebrich Scarlet produces a number of changes in the difference spectra.

Biebrich scarlet forms a 1:1 complex upon binding of lysozyme to chitotriose at concentrations approaching to saturation of A-C sites. The enzyme does not affect the dissociation constant of the lysozyme-Biebrich Scarlet complex, but perturbs the absorption spectrum of the inhibitor. The experiments show that Biebrich scarlet is a competitive inhibitor of cell wall hydrolysis and demonstrate that the compound binds to the catalytic part of the active site of lysozyme from which it can be displaced by chitosaccharides, which interact with the site. Some chitosaccharides such as chitohexose can bind to site F and displace the dye from the catalytic site.

Based on Biebrich scarlet–lysozyme interactions three major chitosaccharides complexes are involved a) those involving only sites A-C, b) those involving sites C-D

and, in case of chitohexose it is considered to involve c) total binding sites on the lysozyme. See Fig. 4.14.

We followed the same principle and the experimental methodology as described by Holler et al for evaluating competitive binding of polymers containing polyvalent NAG prepared by various methods.

X ray crystallography studies for the hen egg white lysozyme have demonstrated that the enzyme contain six subsite binding sites, A-F, and three of these subsites, A,B,C have been well characterized by many methods. However, the binding characteristics for sites D,E and F have not been well established [Ikeda (1976)]. Ikeda reported similar experiments to that of Holler et al by circular dichronism (CD) of the dye bound to lysozyme, but the results obtained were not matching to the proposed principles.

Saccharide binding to the upper portion of the active site cleft of the lysozyme molecule is independent of the binding to dye, whereas binding to the lower cleft is competitive.

4.3.5 Binding efficacy of NAG to the lysozyme is described below:

a) Estimation of dissociation constant

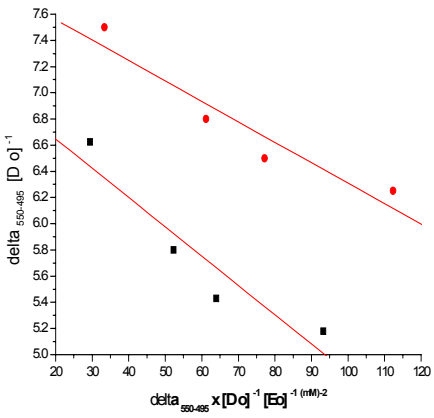


Fig. 4.15: Binding of Biebrich Scarlet to lysozyme and to lysozyme-NAG complex

Difference in a absorbance at 495 nm for a solution containing Biebrich Scarlet and a solution containing an equal concentration of Biebrich Scarlet but varying amounts of lysozyme (Line a) or of a lysozyme–saccharide complex (line b) was measured. The absorbance measurements are in reference of the difference spectra at 550 nm. The data were plotted according to equation 4.7

$$\Delta A = \Delta \varepsilon_M [D_o] - (\Delta A/ [Eo]) K_{ED} \quad 4.7$$

where,

$\Delta A = \Delta A_{550-495}$

$[D_o]$ represent the total molar dye concentration and

$\Delta \varepsilon_M$ = Molar difference $_{550-495}$ obtained from the intercept of the line A

E_0 = moles of enzyme

K_{ED} = dissociation constant for the binding of the dye to the enzyme

The $\Delta A_{550-495}$ values used in the graph were obtained from the experiments in which lysozyme and Biebrich scarlet concentrations were constant and NAG concentration was varied (Fig. 4.15).

In order to obtain $\Delta A_{550-495}$ values, the ΔA *vs.* NAG values, which were obtained at high concentrations of sugar, were extrapolated to zero saccharide concentration (dotted line in Fig 4.17).

The $\Delta A_{550-495}$ values used in figure represent extrapolated values obtained in experiments using varying concentrations of lysozyme.

Dissociation constant (K_{ED}) for binding of the dye to the enzyme is calculated from Fig 4.15 by extrapolation and is 0.039 mM for NAG. The molar difference coefficient, $\Delta \varepsilon_{M\ 55-=495}$, obtained from the intercept of the same line (a) in Fig 4.15 has a value of 7500 mM.

The lines (a) and (b) are parallel within the experimental error indicating that the dissociation constants of the lysozyme-Biebrich scarlet complex is not affected by formation of the lysozyme- NAG complex. The difference in $\Delta\varepsilon_M$ obtained in two experiments represented by curves (a) and (b) in Fig 4.15 is due to the perturbation of the dye spectrum by NAG.

b) Binding of oligomers and copolymers to lysozyme :

Method: In the experiments shown in Fig 4.17 the difference in absorbance measured at 495, 525 and 565 nm. between a solutions containing Biebrich scarlet and solutions containing equal concentrations of Biebrich scarlet, a constant concentration of lysozyme and varying concentrations of oligomers was measured. All absorbance measurements were recorded with reference to the isobestic point of the difference spectra at 550 nm. i.e., a) $\Delta A_{495-550}$, b) $\Delta A_{565-550}$. c) $\Delta A_{525-550.}$

ΔA is dependent on NAG concentration at the maximum at 565 nm, ΔA becomes independent of oligomer concentration higher than 1 mM.

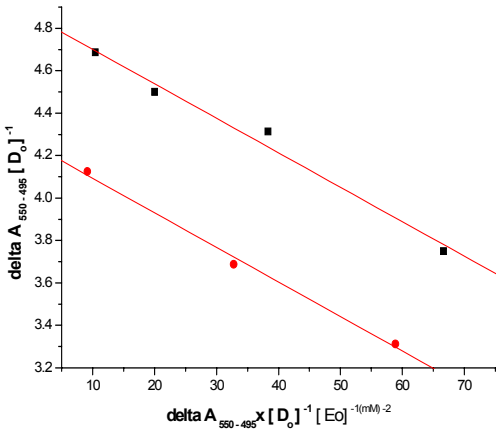


Fig 4.16: Binding of Biebrich Scarlet to lysozyme and to lysozyme-PAc.NAG complex

At all wavelengths, extrapolations of the data obtained at high NAG concentrations to zero NAG concentrations give an intercept on the Y axis at zero NAG concentrations. The k_s values obtained from Fig 4.17 indicate that this is due to a perturbation of the absorption spectrum of the enzyme–Biebrich scarlet complex. A low NAG concentrations sites A-C are filled, but Biebrich scarlet is not displaced.

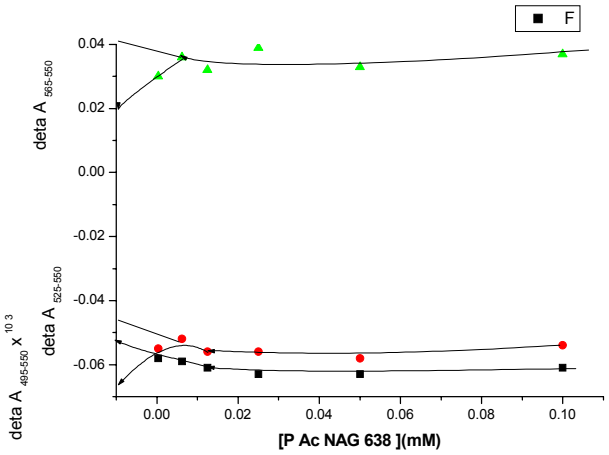


Fig 4.17 for P.Ac.NAG: The difference in absorbance measured at 495, 525 and 565 nm, between a solution containing Biebrich Scarlet and solutions containing an equal

concentration of Biebrich scarlet, a constant concentration of lysozyme and varying concentrations of NAG ligands.

c) Binding of oligomers to the affinity sites in the enzyme:

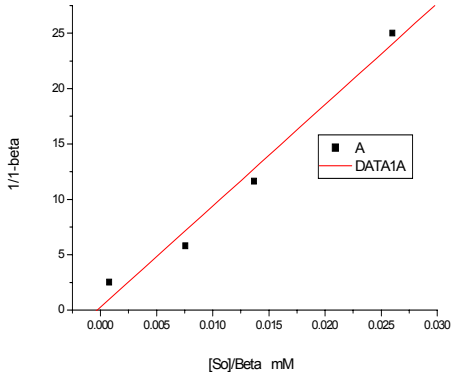


Fig. 4.18: PAcNAG 638: The determination of the dissociation constant of the high affinity lysozyme –NAG complex. The ΔA values obtained at three wavelengths at low NAG concentrations as shown in Fig. 4.17 are plotted according to eqn. 4.11.

The dissociation constants of NAG for 1:1 lysozyme–NAG complex were determined. ΔA value is obtained at particular wavelength from the difference spectrum between lysozyme plus Biebrich Scarlet *vs* Biebrich Scarlet, alone is termed ΔA_o. Equal concentrations of NAG are then added to the cells. The line defined by ΔA values obtained at high NAG concentrations is extrapolated to the ordinate to give the intercept value. ΔA_{SED} is the difference (Fig. 4.18) in the absorbance at a particular wavelength between Biebrich Scarlet and solutions containing the dye and the high affinity 1:1 lysozyme–NAG complex. ΔA_o value for any particular low NAG concentration is referred to as ΔA_{obsd} (In Fig.4.18).

In order to compare results from different experiments, the normalization procedure is used (eq. 4.8 and 4.9) .

$$\Delta_o = \Delta A_{SED} - \Delta A_o \qquad 4.8$$

$$\beta = (\Delta_o - \Delta_{obsd}) / \Delta_o \qquad 4.9$$

If the absorbance changes at low NAG concentration are a measure of the formation the 1:1 lysozyme–NAG complex, the concentration of the complex [SE] is given by (β[Eo]).

The dissociation constant k_u of the complex then is defined by **:**

$$k_u = \frac{([E_o] - [SE])([S_o] - [SE])}{[SE]} = \frac{1-\beta}{\beta}([So] - \beta[E_o]) \quad 4.10$$

k_u can be evaluated from a linear form of eq. 4.10

$$(1-\beta)^{-1} = [So]\,\beta^{-1}\,ku^{-1} - [E_o]\,ku^{-1} \quad 4.11$$

In Fig 4.17 the data shown in Fig. 4.16 are plotted according to eq 4.11.

k_u is the dissociation constant for (oligomer) non catalytic binding at sites A, B, and C. and is 0.19 mM describing the high dissociation constant.

d) Binding of NAG oligomers to the productive biding site of the enzyme :

The change in absorbency of the difference spectra at high concentration of various oligomers is shown in Fig 4.18. The lines are extrapolated to the ordinate at low oligomer concentrations as indicated by the dashed lines in Fig 4.18.

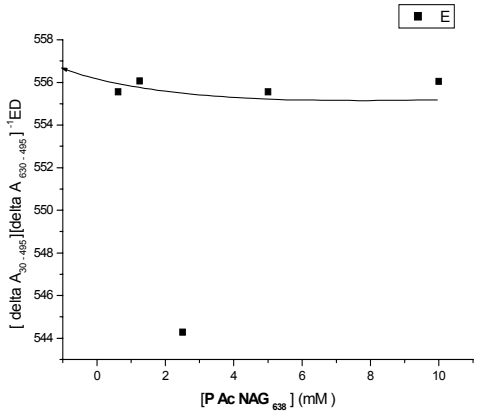


Fig. 4.19: PAc.NAG 638: Difference in absorbance, $\Delta A_{495\text{-}630}$ was recorded as a function of NAG oligomer concentrations. Difference in absorbency was measured at 495 nm between a solution containing Biebrich scarlet and solutions containing equal concentration of Biebrich scarlet, constant concentrations of lysozyme, and varying concentration of NAG.

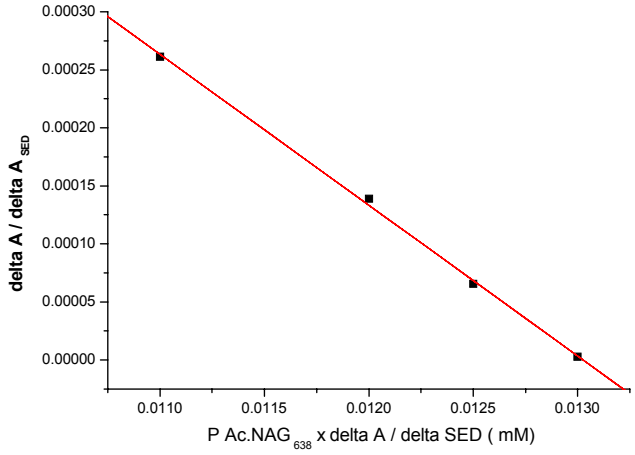
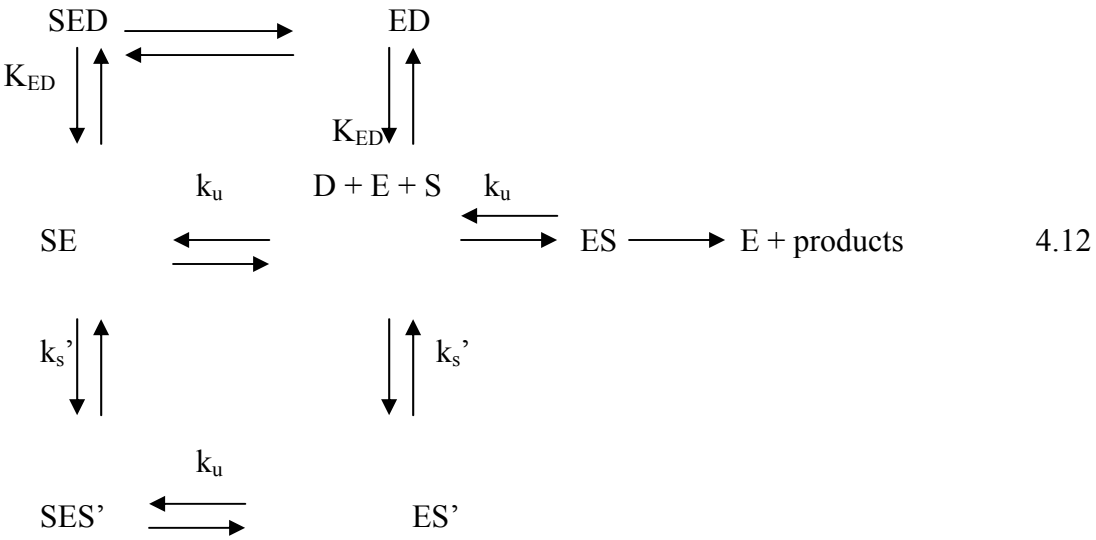


Fig. 4.20: PAc.NAG 638: The data from Fig. 4.17 were plotted to determine the dissociation constant k_s and k_s ' pertaining to this model.

It should be noted that the absorbance difference decreases with increasing oligomer concentrations as a result of displacement of Biebrich scarlet from the catalytic site of the enzyme.

The displacement of Biebrich scarlet by even low concentrations of oligomer is incorporated in the proposed mechanism is shown in figure below:



Oligomers bind to lysozyme, without interaction with Biebrich scarlet, to form complex SE, which is characterized by a dissociation constant k_u (Fig. 4.16, 4.17 and 4.18). This involves the binding site, which does not catalyze the bond breaking step.

Oligomer investigated also binds to the catalytic site to form complex ES', characterized by a dissociation constant k_s'. Oligomers and copolymers can bind to the noncatalytic sites simultaneously to form complex ES, characterized by dissociation constant k_s. ES is considered to be a productive complex in the catalytic reaction. Biebrich scarlet competes with the formation of productive complexes by both cell wall *Micrococcus lysodeikticus* and oligomers and copolymers. And therefore it is assumed that it binds to the catalytic sites of the enzyme.

The formation of ES accounts for the changes in the difference spectra observed at lower concentrations of oligomer, where $[S_o]$, $[E_o]$ and $[D_o]$ represent the initial concentrations of the oligomers, enzyme and dye respectively, the concentration of the lysozyme–Biebrich scarlet complexes in the presence of oligomer, providing $[S_o] > [E_o] > [D_o]$ used to evaluate the displacement of Biebrich Scarlet, that is at high oligomer concentration.

The values of k_s and k_s' can be calculated from the slope and intercept of lines, using the independently determined values and k_u.

$$\frac{1}{Ks} + \frac{1}{ks'} = \frac{[Eo] + K_{ED}}{K_{ED}\, ku} \quad \frac{(1 - \text{intercept})}{\text{intercept}} \qquad 4.13$$

k_s' is the dissociation constant referred to binding to the catalytic portion of the active site of lysozyme

k_s' for NAG is 3.47 mM.

k_s for NAG is the dissociation constant = 0.074 mM

4.3.6 Productive–Nonproductive binding between lysozyme and NAG ligands

The values for competitive binding were obtained for monomer NAG, oligomer, macromer, copolymer and imprinted polymer. The calculations were carried as mentioned in earlier section.

The Biebrich scarlet–lysozyme interactions help us distinguish between three three major NAG-complexes 1) those involving sites A-C 2) those involving sites C-D and 3) the total binding site of the lysozyme A-F.

Table 4.17 Competitive binding of NAG to lysozyme at pH 7.4 and 24 ^{O} C using Biebrich Scarlet dye

	Mol Wt.	Mole% NAG	k_{ED} mM	Slope (mM^{-1})	Intercept	k_u mM	k_s 'mM	k_s mM
NAG	221	-	0.039	0.90	1.63	0.19	3.47	0.074
Ac.6-ACA.NAG	404	-	0.086	0.96	1.72	0.22	0.00059	0.000148
P.Ac. NAG	638	-	0.026	1.94	2.40	0.09	0.000028	0.0000032
PMeth.NAG	2857	-	0.012	1.04	1.54	0.08	0.000891	0.0000406
NIPA-Ac.NAG	1954	34	0.037	0.91	2.76	0.08	0.000628	0.000055
NIPA-M.Ac.NAG	2430	38	0.015	0.06	2.30	0.09	0.000611	0.000072
NIPA –Macromer	3573	33	0.011	0.81	0.33	0.11	0.000019	0.0000018
Imprinting NIPA-Macromer AB	4814	30	0.06	1.30	0.23	0.10	0.000038	0.0000065
BAB	14,638	1.84	0.021	2.23	0.13	0.09	0.000564	0.000135
	90,638	0.029	0.043	1.96	0.43	0.15	0.000093	0.0000086

Table 4.18 Competitive binding of chitosaccharides to lysozyme at pH 7.4 and 24 ^{O} C using Biebrich Scarlet dye (Holler et al (1975)

	Slope mM^{-1}	Intercept	k_u mM	K_s' mM	k_s mM
Chitotriose	0.031	1.0	0.01	15	-
Chitotetrose	0.052	1.0	0.02	9	-
Chitopentose	0.075	0.99	0.02	6	-
Chitohexose	0.084	0.87	0.02	5	0.03

4.3.7 Discussion:

The values of model parameters for chitosaccharides reported by Holler et al are reproduced in Table 4.17. A comparison of the values of k_u, k_s' and k_s for the chitosaccharides *vis* a *vis* ligands investigated by us leads to following conclusions.

The values of k_u for the NAG bearing ligands reported in Table 4.17 are an order of magnitude higher than those for the chitosaccharides (Table 4.18). This mode involves binding sites A-C, which do not catalyze the bond cleavage. This indicates that this mode of binding is less preferred by the ligands investigated by as compared to the chitosaccharides. Further the values for oligomers, copolymers, block copolymers, imprinted polymers are almost identical indicating thereby that binding by this mode is practically independent of the architecture so long as a polyvalent NAG is present. While the complexes formed between lysozyme and NAG bearing ligands investigated by us characterized by the dissociation constant k_u are more unstable k_u (~0.1mM) than those formed by chitooligosaccharides (k_d ~ 0.01 mM), this step is accompanied by a ternary complex which involves binding between another polymeric NAG ligand which binds at sites E & F. The dissociation constant k_s' associated with this step is at least four to five orders of magnitude lower K_s' ($9.00 \times 10^{-4} - 2.00 \times 10^{-5}$ mM) than the lowest value for the chitosaccharides 5 mM. This indicates that the ligands reported by us can more effectively displace Biebrich scarlet in the ternary complexation step as compared to the chitosaccharides. Further the values for the copolymers involving the macromer synthesized using molecular imprinting technique or otherwise are an order of magnitude lower than random and block copolymers involving Acryloyl NAG. This indicates that the incorporation of the spacer has led to in the enhancement of binding between AG and the sites E & F during the ternary complexation step

The third mode of binding wherein the polyvalent NAG ligand directly binds to the sites A-F is referred to as producing binding and is characterized by the dissociation constant k_s. For chitohexose, this value (0.03mM) is of the same order as k_u (0.02 mM), although much smaller that k_s' (5mM). The values of k_s for polyvalent NAGs vary between ($1.3 \times 10^{-4} - 1.8 \times 10^{-5}$ mM). This reflects the ability on the part of these ligands to bind productively at the lysozyme active sites A-F including the catalytically active sites. These values are almost a million folds lower than the dissociation constant for

Biebrich scarlet-Lysozyme complex (≈ 0.15mM) [Holler et al (1975)] thus indicating that the complexes are far more stable than the complex formed with Biebrich scarlet and hence can effectively displace it. It may further be noted that in all cases investigated the values of k_s are lower than k_s' indicating that the productive complex formed is more stable than the ternary complex.

In particular we wish to point out that the copolymers based on the macromer are far more effective than the copolymers based on acryloyl or methacryloyl NAG and that the triblock copolymers (BAB) are far more effective than the diblock copolymers (AB). It may be further noted that while all these polyvalent ligands bind at the catalytically active site of lysozyme, none of those are hydrolyzed by lysozyme since they do not contain 1,4 glycoside linkage.

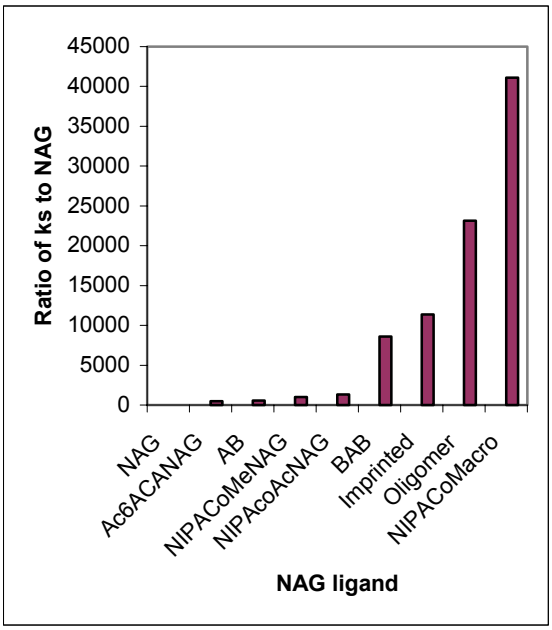


Fig. 4.21: Comparison of k_s by Biebrich scarlet dye method.

4.3.8 Concluding remarks:

Substrate ligand interactions play a critical role in biological processes such as protein – carbohydrate binding and biotechnological processes such as affinity-based separations. It is envisaged that polyvalent ligands will lead to enhanced substrate

binding. We selected NAG-lysozyme as a representative ligand–substrate system and designed various architectures comprising polyvalent NAG to investigate the effect of ligand structure on binding. Having demonstrated that the oligomers of acryloyl and methacryloyl as well macromer comprising NAG lead to enhanced binding, we synthesized copolymers of acryloyl NAG as well as methacryloyl NAG with *N*-isopropylacrylamide as polyvalent ligands. The differences in their binding and inhibition characterstics were attributed to the differences in the monomer sequences in the polymer structure. These wee confirmed by reactivity ratio measurements. Having realized the importance of the sequence length we synthesized di and triblock copolymers comprising oligomers of NAG. The triblock polymers were more efficient than the diblock polymers probably because each end segment of the triblock polymer binds to a different lysozyme molecule and the increasing molecular weight of the central segment leads to steric exclusion during inhibition. Finally, we demonstrated that the synthesis of the copolymers comprising NIPA and the macromer in the presence of lysozyme leads to a preferential conformational disposition of NAG ligands which leads to enhanced binding and lowered I_{50} values during subsequent inhibition experiments. We have thus succeeded in enhancing the substrate ligand interactions by manipulating the polymer architecture. In the next chapter, these polymers will be evaluated for the recovery of lysozyme by affinity precipitation to check if this leads to enhanced recoveries of lysozyme by affinity precipitation.

Recovery of Lysozyme: New polymeric affinity ligands containing NAG

5.1.0 Introduction

Previous chapters have highlighted the importance of designing polyvalent ligands for enhanced binding with lysozyme. The rationale behind tailoring novel stimuli sensitive polymers, especially theromosensitive polymers containing NAG was highlighted and synthesis and characterization of the polymers was described.

The need for effective utilization of affinity synthetic ligands during bioseparations is obvious. Chitin and chitosan are natural polysaccharides containing NAG units that bind to lysozyme. However, these ligands are insoluble in water and undergo hydrolysis by the enzyme. Moreover, natural polymers containing glucose undergo microbial degradation and hence such ligands have limitations in recovery of lysozyme [Tyagi et al (1996)]. Polymeric NAG ligands reported in this investigation have been designed as to overcome these limitations. The enhanced binding between polyvalent NAG and lysozyme as well as the origins thereof have been already discussed. In this chapter, the utility of these polymers for the recovery of lysozyme is examined.

5.1.1 Background of lysozyme recovery:

Lysozyme is an industrially important enzyme and finds applications in wound healing as a bacteriolytic agent [Ruckenstein and Zeng (1997)], baby food additive and as a preservative in cheese making. Lysozyme is widely distributed in animals and plants and is also found in mammalian secretions, tissues, saliva, tears, milk, cervical mucus, leucocytes, kidneys, etc. Hen egg white contains 3.5 % lysozyme and is used routinely as a source for the recovery of lysozyme.

Various methods have been reported in the past to recover lysozyme. Kokai (1988)] demonstrated separation of lysozyme and avidin from egg white. Egg white was contacted with a weak cation exchanger for lysozyme and avidin adsorption and the cation exchanger was eluted with an eluent of low ionic strength to release lysozyme. Homogenized egg white was passed through a Duolite C-464 column and the column was eluted with distilled water, 0.1 M Sodium phosphate containing 0.15 M NaCl for lysozyme recovery.

Durance et al (1988) developed a single column cation exchanger, which allowed simultaneous recovery of lysozyme and avidin from undiluted egg white. A unique

adsorption-elution sequence was developed. This involved accumulation of avidin on the column through several cycles of egg white adsorption and lysozyme elution. Lysozyme recoveries were higher (86 %) than reported for the isoelectric precipitation methods often used in the industry (60-80%). Lysozyme peaks appeared homogeneous on SDS-PAGE. Avidin recovery was also higher than previously reported using ion exchange methods (74-80%).

Chitosan exhibits pH dependent solubility, Tyagi et al (1996) reported use of chitosan as an affinity macroligand for the recovery of lysozyme. The binding of lysozyme and chitosan was carried out at pH 4.0 and the precipitation of the complex was achieved by raising pH to 8.0. However the binding of the lysozyme and NAG is maximum at pH 5.5 [Chipman et al (1967)]. Since the polymers used in this work are temperature sensitive the pH for optimum binding (6.7) was selected.

5.1.2 Experimental procedure:

1 % w / v polymer was dissolved in ten ml of distilled water and mixed with lysozyme solution (27 μg / ml). The mixture was incubated to form a complex at 20 ^{O} C with continuous shaking at 200 rpm for 16 hours. The temperature of the complex was raised above LCST of the polymer resulting in precipitation of polymer-lysozyme complex. The complex was separated by centrifugation above LCST at 10,000 rpm for 10 minutes. The precipitated complex was redissolved in distilled water and the pH was adjusted to 2.0 by dilute acetic acid solution to decouple the complex (Ruckenstein and Zeng (1997). The temperature of dissociated complex was raised above LCST and the precipitated polymer was separated by centrifugation. The clear filtrate was assayed for lysozyme recovery as described below.

The lysozyme activity was measured using the procedure reported by Neuberger and Wilson (1967). Stock solutions of lysozyme of increasing concentrations were prepared in 6.6x 10^{-3} M phosphate buffer (pH 6.2) containing 1.54 x 10^{-2} M sodium chloride and 8.0 x 10^{-3} M sodium azide. 0.1 ml of stock solution containing different lysozyme concentrations was mixed with 1.0 ml of 78 μg/ml of *Micrococcus lysodeikticus* in a 3-ml capacity glass cuvette and one ml of buffer. The mixture was incubated for 5 minutes at 20 ^{o} C and the absorbance at 450 nm (Δ_{450}) was recorded for

30 seconds. The standard plot for lysozyme at varying concentrations is demonstrated below (Fig. 5.1)

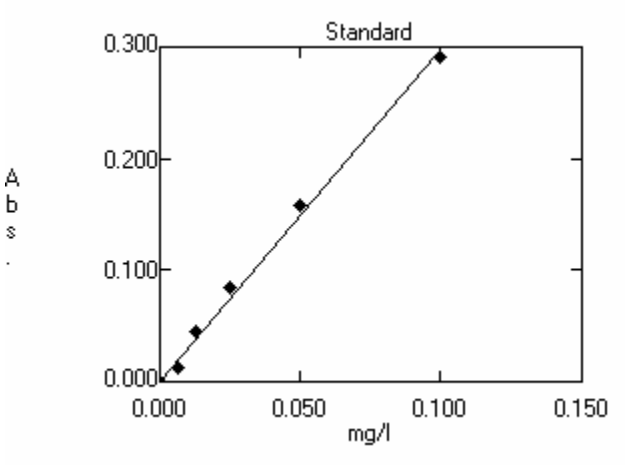


Fig. 5.1: Standard plot for lysozyme

Absorbance was measured for 0.1 ml of standard lysozyme solution using *Micrococcus lysodeikticus* substrate. The ratio of absorbance for recovered lysozyme vs that for standard lysozyme was taken as a measure of lysozyme % recovered

5.1.3 Estimation of lysozyme recovered as a protein:

Protein content was obtained for recovered lysozyme using Lowry's procedure. Folin-Ciocalteu phenol reagent is widely used to determine the concentration of proteins since it was first described [Lowry et al (1951)]. The assay is commonly referred to as the Lowry Method, after its author. The blue color that is produced and analyzed by visible spectroscopy is a result of the following reactions:

Complexation of $Cu2^{+}$ with the peptide bond in an alkaline solution. The reduction of the phosphomolybdate containing Folin-Ciocalteau reagent by the conjugated side chains of the amino acids, tyrosine and tryptophan.

5.1.4 Recovery of lysozyme using polymeric ligands:

The graph was plotted for standard lysozyme using increasing concentrations.(Fig. 5.2). The absorbance for recovered lysozyme using polymers containing NAG was taken as a measure of lysozyme and compared with standard lysozyme using a calibrated graph. Lysozyme recovery in term protein recovered (μM of lysozyme / mM of NAG and mg of lysozyme / mg of polymer) was estimated.

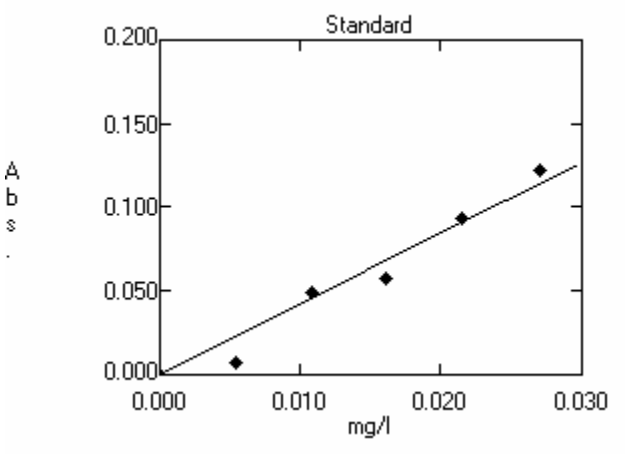


Fig. 5.2 : Lowry's plot for lysozyme

Results:

Table 5.1. Lysozyme recovery by copolymers of NIPA and Ac.NAG

Mole % NAG	Mol. wt. VPO	% lysozyme activity recovered	mg of lysozyme / gm of polymer	μM lysozyme /mM of NAG	LCST ^{O} C
14	3200	57.80	4.590	7.30	34.8
21	2830	73.02	2.990	2.80	34.8
54	2560	77.80	1.150	3.83	34.8
78	2784	89.30	0.005	0.0131	34.8

Copolymer of NIPA and Ac.NAG containing 14 % NAG demonstrated higher recovery in terms of μM of lysozyme / mM of NAG compared to the polymers of higher NAG content. This can be attributed to the crowding effect because of which all NAG units are not accessible for bindng with lysozyme. Crowding effect for NAG ligands for recovery of lysozyme was also demonstrated by Vaidya et al (1999). The recovery in terms of mgs of lysozyme / gm of polymer exhibit identical trend.

5.1.5 Lysozyme recovery by NIPA- co -macromer copolymers

We synthesized copolymers of NIPA and poly (acryloyl *N*–Acetyl Glucosamine)-6-(-*N*-acryloyl amino) caproate (Macromer). The effect of spacer in polyvalent NAG oligomers for association constant and inhibition concentration was established and is reported in section 4.1.

The results of lysozyme recovery for copolymers of NIPA and macromer are reported in Table 5.2.

Table 5.2 : Lysozyme recovery by copolymers of NIPA & Ac.6ACA.PAc.NAG (Macromer)

Mole % NAG	Mol.Wt by VPO	% lysozyme activity recovered	mg of lysozyme / gm of polymer	μM lysozyme /mM of NAG	LCST ^{O}C
36	4010	75.00	73	5.70	34.5
17	3852	68.24	83	1.32	34.3

Copolymer of NIPA and macromer containing 36 % NAG demonstrated higher recovery expressed in terms of μM of lysozyme / mM of NAG than the macromer containing 17 % NAG. Lysozyme recovery by copolymers of NIPA and macromer was greater compared to copolymers of NIPA and Ac.NAG. This is due to the presence of polyvalent NAG units in juxtaposition which leads to enhanced binding. Recovery in terms of mg of lysozyme / gm of polymer is also higher than copolymers of NIPA and AcNAG.

We could not compare the recovery of lysozyme using copolymers of NIPA and Methacryloyl NAG as these copolymers do not exhibit LCST till 70 ^{O} C.

5.2.0 Lysozyme recovery using block copolymers :

Results of Lysozyme recovery are reported in Table 5.3.

It is noteworthy that lysozyme recovery from AB block copolymers is almost identical to that obtained using copolymers of NIPA and macromer. However, NAG content in block copolymer is very low compared to copolymers of NIPA and Ac.NAG. The recovery expressed in terms of μM lysozyme / mM NAG is higher compared to copolymers of NIPA and Ac.NAG but is poor when expressed in terms of lysozyme /

gms of polymer. This is because the ligand utilization is enhanced but the polymer contains NIPA segments which do not contribute to binding.

Table 5.3 : Lysozyme recovery by A-B block copolymers

Mole % NAG	Mol.Wt. A	Mol. Wt .B	% lysozyme activity recovered	mg of lysozyme / gm of polymer	μM lysozyme /mM of NAG	LCST ^{O} C
16.09	6000	2808	77.00	1.60	6.13	34.5
1.84	14000	638	92.85	1.00	56.0	34.5
7.17	14000	2631	81.87	6.60	12.0	34.5
9.76	10000	2631	79.00	113	10.2	34.5

5.2.1 Lysozyme recovery using BAB block copolymers :

AB block copolymers exhibit enhanced binding, but contain only one block of polyvalent NAG. Therefore, it was thought worthwhile to design BAB block copolymers wherein the polymer will contain two blocks of polyvalent NAG which will interact simultaneously with two lysozymes.

Lysozyme recovery for BAB block copolymers of PNIPA and P.Ac.NAG is reported in Table 5.4.

The results indicate that the lysozyme recovery for BAB copolymers expressed in terms of μM lysozyme / mM NAG has significantly enhanced although the NAG content was as low as 0.29 mole %. The enhanced recovery is attributed to the simultaneous binding of terminal NAG ligands to two lysozyme molecules. However, the efficiency in terms of mg of lysozyme / g of polymer is poorer than AB block copolymers. This is because of very large mol weight of the central segment. It thus appears that the polymer composition to be optimized to strike a balance between utilization of ligands and recovery on weight basis.

Table 5.4: Lysozyme recovery by B-A-B block copolymers of P.(NIPA)-co-P. (Ac.NAG)

Mole % NAG	Mol.wt A	Mol.wt B	% lysozyme activity recovered	mg of lysozyme / g of polymer	µM lysozyme /mM of NAG	LCST ^{O}C
0.72	36,000	638	60.20	0.140	50	32.7
0.46	56,000	638	57.00	0.859	7 35	32.7
0.29	90,000	638	54.89	5.160	1120	32.7

The terminal polyvalent NAG units in BAB polymer bind simultaneously to the different lysozyme thereby increasing the recovery. This is not possible in case of random copolymers.

5.2.2 Imprinted polymers:

Table 5.5: Lysozyme recovery for imprinted copolymers of NIPA & Macromer by imprinted method

% Inhibition for Imprinting	% Moles by NMR	Mol.wt	% lysozyme activity recovered	mg of lysozyme / gm of polymer	µM lysozyme /mM of NAG	LCST ^{O}C
100	30	4814	93.40	10	12	34.3
20	18	-	89.83	-	19	34.2

Copolymers of NIPA & poly (acryloyl *N*–Acetyl Glucosamine)-6-(-*N*-acryloyl amino) caproate (Macromer) synthesized by imprinting method containing 30 mole % NAG demonstrate higher recovery over random copolymers of NIPA and Ac.NAG and identical to copolymer of NIPA and macromer.

Lysozyme recovery for copolymers of NIPA and poly (acryloyl *N*–Acetyl Glucosamine)-6-(-N- acryloyl amino) caproate (Macromer) at 100 % inhibition & increasing NIPA dilution is shown in Table 5.6.

Table 5.6: Lysozyme recovery for copolymers of NIPA & Macromer at 100 % inhibition & increasing NIPA dilution

% Moles by NMR	% lysozyme activity recovered	µM lysozyme /mM of NAG	LCST ^{O} C
69	-	-	> 70
62	-	-	> 70
2.4	78.32	125	32.3

Imprinted copolymer containing 2.4 mole % NAG demonstrated higher efficiency than any other random copolymers.

5.2.3 Conclusions:

Copolymers containing NIPA and Ac.NAG were prepared in various molar ratios. However, it was fortuitous to obtain these copolymers as block rather than random coplymer. The copolymers exhibits constant LCST inspite of the variation in the commoner composition. We therefore synthesized truely random copolymers of NIPA and Methacryloyl NAG. As expected the resulting copolymers did not exhibit LCST even at 70 ^{O} C. Therefore, it was not possible to use these copolymers for the recovery of lysozyme.

The recovery of lysozyme for copolymers of NIPA and Ac.NAG was not satisfactory although the polymers contain polyvalent NAG. The utilization of ligands was poorer with increasing NAG content in the polymer because of the crowding effect. However, copolymers prepared using NIPA and Macromer exhibited greater recovery than the copolymers of NIPA and Ac.NAG as the former contained preformed NAG units in the juxtaposition. The enhanced recovery is due to appropriate arrangement of polyvalent NAG units in the polymer.

AB block copolymers containing thermoprecipitating polymer segment A and NAG segment B of varying molecular weights of oligomer and exhibited higher efficiency compared to the random copolymers inspite of low NAG content. These polymers contain one polyvalent NAG triads at the terminal which can freely bind to lysozyme.

BAB block copolymers exhibited highest recoveries of lysozyme from the aqueous solution on ligand utilization basis in spite of very low ligand concentration.

In summary thermoprecipitating polymers of various architectures have been prepared and their efficacy in lysozyme recovery is estimated. One such architecture was molecularly imprinted polymer prepared in presence of lysozyme as a template. The recovery was higher than the copolymers prepared in the absence of lysozyme. However, the recovery was not higher than BAB block copolymers. This could be attributed to crowding of NAG units in random copolymers.

The investigation has led to realization that the binding between ligand and lysozyme can be enhanced by polyvalent binding. It has also highlighted further possibilities of designing affinity precipitating polymers for enhanced binding and recovery.

6.0.0 Summary and conclusions

Present investigation was undertaken to design, synthesize and evaluate thermoprecipitating polymers, which will exhibit enhanced substrate binding. It was anticipated that this will lead to more efficient enzyme recoveries of lysozyme by affinity precipitation. *N*-acetyl glucosamine was selected as the ligand since it is known to bind to lysozyme and oligomers and polymers of NAG have been investigated in the past. (page 90).

Another objective of this investigation was to quantity the influence of molecular architecture on the binding between NAG and lysozyme especially polyvalent interactions.

Towards this end, we conjugated NAG with acrylic as well as methacrylic acid. The monomers, oligomers, macromer and copolymers containing NAG were synthesized and evaluated for their efficiency in the inhibition of lysozyme by the *Micrococcus lysodiekticus* method (page 75). Subsequently di and tri block polymers and copolymers synthesized in the presence of lysozyme as template were also investigated. The binding between NAG and lysozyme was quantified independently by fluorescence spectroscopy measurements (page 75).

1) It was observed that conjugation of NAG with both Acrylic and methacrylic acid leads to enhancement in k_a by two orders of magnitude and I_{50} by five folds (page 90).
2) Oligomers of both acryloyl NAG and methacryloyl NAG were equally effective as reflected in values of k_a and I_{50} (page 90 and 91).
3) Copolymers of Acryloyl NAG and NIPA exhibited binding constants k_a (2.03×10^5 M^{-1}) comparable to oligomers (2.51×10^5 M^{-1}), whereas the copolymers comprising methacryloyl NAG and NIPA exhibited lower k_a values (8.91×10^3 M^{-1}) (page 96 and 97).
4) This is attributed to the presence of NAG blocks in the copolymers of NIPA and Ac.NAG, whereas the NAG in the copolymers of NIPA and methacryloyl NAG, was randomly distributed. (page 97).

5) Measurements of monomer reactivity ratio led to r_1 (NIPA) =1.12 and r_2 (Ac.NAG) = 1.80 while r_1 (NIPA) = 0.47 and r_2 (methac.NAG) =0.067.This confirmed that the copolymers of Ac.NAG contained blocks of AcNAG and NIPA whereas in the copolymer of methacrylic NAG and NIPA, both monomers were randomly distributed. (page 104).
6) This was further validated by observation that the LCST of copolymers containing Ac.NAG was independent of composition whereas the LCST of copolymers containing methacrylic NAG increased with NAG content (page 96 and 97).
7) Realizing that the presence of NAG sequences leads to enhanced binding to lysozyme, a macromer comprising repeat units of Ac.NAG was conjugated to acrylic acid through a spacer viz 6-amino caproic acid. The macromer itself was quite efficient in binding to NAG k_a ($5.62 \times 10^5 M^{-1}$) and also inhibited lysozyme effectively I_{50} 2.60 μM (page 90).
8) This macromer when was copolymerized with NIPA, led to a random copolymer r_1 (NIPA) = 3.08, r_2 (macromer) = 0.743. Yet the copolymer exhibited a higher association constant ($5.08 \times 10^5 M^{-1}$) and inhibited lysozyme more efficiently I_{50} (0.120 μM). This was because although the macromer was randomly distributed, three units of NAG were always in juxtaposition (page 105).
9) Having validated the role of NAG sequences, diblock and triblock copolymers comprising AcNAG and NIPA of increasing sequence length were synthesized and evaluated. It was concluded that beyond an optimum sequence length (Mol. wt. 638) increase in sequence length of NAG units offered no particulate advantage. The same was also true for the length of NIPA segments (page 107).
10) The triblock polymers were far more efficient that the diblock polymers, possibly because the NAG segments at the two ends bind to different lysozyme molecules.
11) Binding between the macromer and lysozyme prior to copolymerization with NIPA leads to a conformationally predisposed copolymer which binds k_a ($1.25 \times 10^6 M^{-1}$) (page 111) to lysozyme more efficiently (I_{50} = 0.06 μM) than the copolymer synthesized in the absence of lysozyme ($k_a = 5.08 \times 10^5 M^{-1}$ and I_{50} = 0.120 μM) (page 105).

12) During template polymerization, when all NAG binds to lysozyme and the macromer is copolymerized with NIPA, at low NAG concentrations (2.4 %) the association constant is low (k_a = 5.0 x 10^5 M^{-1} and I_{50} = 26.6 μM) and also the cooperativity factor (H) (1.04) (Page 112). As a result the I_{50} values are higher. At intermediate NAG content (30 %) the association enhanced, the cooperativity factor (H) is enhanced (1.25) and the I_{50} values are lowest (1.25). With increasing NAG concentration (30 %) the association constant decreased again (k_a = 3.9 x 10^5 M^{-1}), probably due to the crowding effect. Also at such high concentrations of the macromer, the LCST behavior is no more observed and the polymers are no more suitable for affinity precipitation.

13) The competitive binding studies against Biebrich scarlet indicate that all the polyvalent NAG form productive complexes with lysozyme and can displace Biebrich scarlet bound to lysozyme. Further, the complexes were more stable than the ternary complexes as reflected by lower values of k_s (0.000135 to 0.0000032 mM) compared to k_s' (0.000564 mM to 0.000028 mM). (page 123).

14) The random copolymers of NIPA and methacryloyl NAG did not exhibit LCST and hence could not be evaluated. The copolymers of NIPA and acryloyl NAG on the other hand exhibited block structures and hence constant LCST irrespective of composition. In these cases the recovery of lysozyme decreased with increasing ligand content as result of crowding effect.

15) At comparable NAG contents lysozyme recovery (μM of lysozyme / mM of NAG) was higher that copolymers of acryloyl NAG (page 130).

16) Triblock copolymers give higher lysozyme compared to the diblock polymers possibly because each end segments of NAG binds to one lysozyme unit (page 132-133)

17) While di and triblock copolymers exhibited higher recovery on the basis of NAG; the values on a weight basis were lower. Hence the structures need to be optimized to strike a balance between the two.

7.0.0 Direction for further work:

Present investigation was undertaken to design, synthesize and evaluate thermoprecipitating polymers, which will exhibit enhanced substrate binding. It was anticipated that this will lead to more efficient enzyme recoveries of lysozyme by affinity precipitation. *N*-acetyl glucosamine was selected as the ligand since it is known to bind to lysozyme, copolymers of NAG have been investigated.

Significant findings of our investigation are summarized in chapter 6. It is concluded that the polymers containing NAG synthesized by us are far efficient than the natural polymers such as chitin and chitosan. We have demonstrated binding of A-F sites on lysozyme to be essential for productive complex formations. While the objective was to use affinity precipitating polymers for biomolecules, we have studied the recovery of lysozyme.

As the research is never completed the present investigation has led to newer issues. The approach of synthesizing affinity polymers can be applied for other ligands and use for the recovery of biomolecules such as Concanavalin A, trypsin and lectins.

If the work can be continued the same polymers can be utilized for the recovery of Wheat germ agglutinin from various sources. As we have prepared di and triblock copolymers, multiblock copolymers can be synthesized. Similarly, dendrimers can be prepared using NAG as a ligand and studied for the binding with lysozyme and the recovery.

NMR spectroscopy can be used to estimate the binding constants and can be compared with our results obtained by using fluorescence. Powerful tools such as molecular modeling can be used to understand the role of polyvalent NAG in enhanced interactions with the active sites of the lysozyme.

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Chapter 1

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Voet, D.,Voet,J.G., Biochem., I st Ed., John Wiley,381-386,(1994).

Curriculam vitae

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Strengths

- A broad, interdisciplinary scientific background in Pharmacy, Biopolymers, Carbohydrate-Protein Interactions, Molecular Imprinting, Biomolecule recovery by affinity precipitation, allows creative problem solving and productive team interactions.
- A quantitative and analytical approach to synthesis of Biopolymers, Carbohydrates, Enzyme – Substrate interactions using Fluorescence, UV and NMR techniques.
- Effective writing and oral presentation skills result in the clear communication of scientific concepts

Scholastic Record:

Ph.D. University of Bombay

Research Guide	**Dr. M.G. Kulkarni**
Position:	**Senior Research Fellow,**
Duration:	28 th April ,1999 – March, 2003
Institute:	Polymer Science & Engg. Unit, Chemical Engg. Division, National Chemical Laboratory, Pune M.S. (India)

Ph.D. Research *:* "Studies on Affinity Precipitation of Biomolecules Using Stimuli Sensitive Polymers "

- Synthesis and characterisation of Glycopolymers and Stimuli Sensitive Polymers for use in medicine and biotechnology.
- Synthesis of monomers, oligomers, macromers, di / triblock, multiblock copolymers and Molecular Imprinting Polymers (MIP) containing *N*-Acetyl Glucosamine (NAG).

- Protein-Carbohydrate Interactions/ Polyvalent Interactions - NAG with Lysozyme and Wheat Germ Agglutinin (WGA), Binding constants, Dissociation constant and Productive binding.

- Enhanced Interactions for polymers containing NAG with Lysozyme and WGA using UV, Fluorescence and NMR. Also by molecular modeling & Docking., X ray Crystallography studies with specialised Groups.

- Biomolecular recoveries of Lysozyme and WGA by Affinity Precipitation method using heterobifuctional ligands.

Master In Pharmacy (Tech.) **Pharmaceutics, Bombay University**

Duration: 1992 - 1995

Result: Ist Class (62 %)

Bachelor of Pharmacy, Govt. College of Pharmacy, Karad, Shivaji University,

Duration: 1988-1992

Result : Ist Class (67 %)

Research Publications:

- Polyvalent Interactions of Methacryloyl *N*-Acetyl Glucosamine for Medicine and Biotechnology
  Recent advances in polymers and composites, Macro 2002
  Khandare J. J. and Kulkarni M. G.
- Smart Polymers for Enhanced Substrate Binding and Recovery in Biotechnology
  Recent advances in polymers and composites, 253-55, Macro 2000
  Khandare J. J. and Kulkarni M. G.
- Studies on Degradation and Complexation of Drugs Using Pharmacological Bioassay
  Indian Drugs, 31, 12, 579-86,1994

Research Publications (Communicated):

- Polymerizable Monomers and Preparation Thereof for Application in Medicine and Biotechnology, United States Patent *(Filed for granting in July 2002 US Patent Office)*
  Khandare, J. J. and Kulkarni, M. G.
- Oligomers and Preparation Thereof for Application in Medicine and Biotechnology United States Patent *(Filed for granting in July 2002, US Patent Office)*

Khandare, J. J. and Kulkarni, M. G.

- Polymerizable Macromers and Preparation Thereof for Application in Medicine and Biotechnology

  United States Patent *(Filed for granting in July 2002, US Patent Office)*

  Khandare, J. J. and Kulkarni, M. G.

- Copolymers and Preparation Thereof for Application in Medicine and Biotechnology

  United States Patent *(Filed for granting in July 2002, US Patent Office)*

  Khandare, J. J. and Kulkarni, M. G.

- Block Copolymers and Preparation Thereof for Application in Medicine and Biotechnology

  United States Patent *(Filed for granting in 2002, US Patent Office)*

  Khandare, J. J. and Kulkarni, M. G.

- Triblock Copolymers and Preparation Thereof for Application in Medicine and Biotechnology

  United States Patent *(Filed for granting in Aug 2002, US Patent Office)*

  Khandare, J. J. and Kulkarni, M. G.

- Polyvalent Imprinted Polymers and Preparation Thereof for Application in Medicine and Biotechnology

  United States Patent *(Filed for granting in Dec. 2002, US Patent Office)*

  Khandare, J. J. and Kulkarni, M. G.

Indian Patents - Filed 7 Indian Patents

Research Publications (to be communicated):

- Manuscript preparation of 5 research publications based on the research work in international journals is in progress
- Polyvalent Glycopolymers Containing *N*-Acetyl-d-Glucosamine for Enhanced Protein-Carbohydrate Interactions : Binding Constant, Relative Inhibition and Cooperativity Studies using UV, Fluorescence and Molecular Modeling (Manuscript under preparation)

  Khandare, J. J. and Kulkarni, M. G.

- Glycopolymer Architectures of *N*-Acetyl-d-Glucosamine for Enhanced Protein-Carbohydrate Interactions with Lysozyme : Binding Constant, Relative Inhibition and Cooperativity Studies using UV, Fluorescence and Molecular Modeling

  Khandare, J. J. and Kulkarni, M. G. (manuscript under preparation)

- Molecular Imprinting Copolymers and preparation Thereof

  United States Patent (manuscript under preparation)

  Khandare, J. J. and Kulkarni, M. G.

Research Publications Presented in Conference:

- NMR studies on Thermoprecipitating Copolymers of *N*-isopropylacrylamide and Acryloyl / Methacryloyl *N*-Acetyl-D-Glucosamine

  Phalgune U. D, Khandare J. J., Rajmohanan P. R., and Kulkarni M. G.

Presented as a poster in Special Symposisum on recent developments in NMR methodology and National symposium on Magnetic Resonance held at Indian Institute of Science, Bangalore, India on 3-6 Feb.2003.

- NMR studies of Thermo-Precipitating Polymers Containing *N*-Acetyl-D-Glucosamine and Its Interactions With Lysozyme

  Phalgune U. D, Rajmohanan P. R., Khandare J. J., and Kulkarni M. G.

Presented as a poster in the 8 th National Magnetic Resonance symposium held at Central Drug Research Institute, Lucknow in 2002.

- Photodegradation of Drugs d-Tubocurararine HCl and Gallamine Triethiodide-Estimation by Spectroscopy and Bioassay

  Khandare J. J. and Hadkar U. B., Presented as a poster, Indian Pharmaceutical Conference, New Delhi in 1993.

Scholarships:

1. Senior Research Fellow

Council for Scientific and Industrial Research (CSIR), India,

From April 1999 till April 2003

2.GATE- Graduate Aptitude Test in Engg. (Pharmacy Faculty), 1992

From June 1993 to Feb 1994

3. Central Board Secondary Education, New Delhi – 7 yrs

From VI std to XII

Experience: Academic

- June 1997 to April 1999

  Lecturer in Pharmaceutics (Pune University Approved)

  M.I.T., Pune , **Subject for Teaching**– Pharmaceutics, Pharma. and Industrial Microbiology, Industrial Psychology and Dispensing Pharmacy

- **1996 to 1997**
- **Lecturer in Pharmaceutics** (Shivaji University, Approved)

  College of Pharmacy, Solapur, **Subject for Teaching** – Pharmaceutics, Pharmacology Pharma. & Industrial Microbiology, Dispensing Pharmacy and Industrial Management,

Industrial Experience / Training:

- **Ethnor Pharmaceuticals**, Bombay - 1992
- **Johnson and Johnson**, Bombay - 1992
- **Core Parenterals**, Ahmedabad , Trainee Apprentice for 2 months -1993
- **Gran Heal Pharmaceuticals**, Bombay, Research & Development Executive –1998

Hobbies and Interest

- **Articles on Science** (10 Articles) , **Times of India, Education Times,** Pune
- **Articles on Great Scientist** (65 Scientist and Their Inventions), Lokmat, Pune for one and half year.

Research Area :

Protein Carbohydrate Interactions and Affinity Precipitation

Brief outline, objective and scope of Research for Ph.D. from April 1999 to March 2003

Background:

Receptor recognition and binding of ligands to the host receptors are remarkable events by which the numerous interactions are being controlled in the body. Carbohydrates play critical role in various biological processes such as cell recognition, cell adhesion, cell differentiation, inflammation, viral and bacterial infection, tumerigenesis, and metastasis.

Despite the known role of carbohydrates in biology, relatively few investigations are reported on the methods of enhancing these interactions. New biological targets for

carbohydrates such as enzymes, proteins, bacteria and viruses are being identified which, can have numerous applications in therapeutics.

Site-specific interactions in general and protein-carbohydrate interactions in particular are key to enhanced binding. The monovalent interactions are weak whereas, multivalent interactions can lead to effective inhibition even at very low concentration. Most of the protein-carbohydrate interactions reported are of low affinity. If relative density and spatial arrangement of ligands incorporated is optimized, then the binding can be substantially enhanced. The enhanced interaction between polyvalent ligands and specific binding sites of biomolecule can also find applications in affinity separations, drug delivery and biotechnology. To imitate and exploit this mechanism, there is a need to devise simple methodologies for the synthesis of polymeric carbohydrate ligands, which will enhance the substrate ligand interactions.

Scope of Work:

has unique edge over monovalent interactions. Moreover, to the best of our knowledge the approach of synthesizing polyvalent carbohydrates adopted by us has not been reported in the past and is suitable for wide range of carbohydrates. Furthermore, the method of synthesis of polymers containing NAG is simple and these polymers are water soluble and resistant to enzyme hydrolysis .

The active site of lysozyme comprises subsites designated A-F. Specific binding of chitosan sequences to lysozyme begins with binding of the NAG units in the subsite C. Moreover, natural ligands derived from glucose are susceptible to microbial growth. There is a need to synthesize ligands similar to repeat units of chitosan which will not be hydrolyzed by lysozyme. The polymers synthesized by us are more stable than chitin and chitosan.

My research involves thorough characterization for enhanced binding by polyvalent NAG than the monomer, dimers, trimers reported in the past, using Fluorescence Spectroscopy in terms of binding constant (K_b) and relative inhibition concentration using *Micrococcus lysodeikticus* in term of I_{50} and I_{max}. The cooperativity and productive binding between the lysozyme sites A-F and the polyvalent NAG is estimated using dye displacement methods .

The enhanced binding by Polyvalent NAG with lysozyme is being estimated using 500 MHz NMR spectroscopy and Molecular dynamics, by collaboration with specialized groups in National Chemical Laboratory, Pune and Indian Institute of Chemical Tech., Hyderabad.

X ray crystallography studies are being carried on our systems.

Monomers and polymers containing NAG exhibit enhanced hydrolytic stability and water solubility than natural polymers containing NAG such as chitosan. The polymeric ligands containing polyvalent NAG are conjugated to stimuli sensitive polymers and therefore, utilized for the recoveries of biomolecules such as lysozyme and Wheat-Germ Agglutinin (WGA) using affinity precipitation technique.

The polymers comprising carbohydrate conjugates may further be used in the treatment of bacterial or viral infections, and are expected not to cause drug resistance.

They may be also used as anti infective agents both for prevention and treatment of diseases, recovery of the naturally occurring as well as genetically manipulated biomolecules.

My area of research relates to the synthesis of polymerizable monomers, oligomers, macromers, random copolymers, block co polymers (AB, BAB), imprinting polymers containing *N*-acetyl-d-glucosamine (NAG) and their characterization for applications in medicine and biotechnology.

Another objective of the research is to synthesize multivalent / polyvalent saccharides for enhanced substrate interactions with greater affinity and specificity. The binding of cell surface receptors to multivalent carbohydrate molecules exhibit wide variety of biological responses and has unique edge over monovalent interactions. Moreover, to the best of our knowledge the approach of synthesizing polyvalent carbohydrates adopted by us has not been reported in the past and is suitable for wide range of carbohydrates. Furthermore, the method of synthesis of polymers containing NAG is simple and these polymers are water soluble and resistant to enzyme hydrolysis .

The active site of lysozyme comprises subsites designated A-F. Specific binding of chitosan sequences to lysozyme begins with binding of the NAG units in the subsite C. Moreover, natural ligands derived from glucose are susceptible to microbial growth. There is a need to synthesize ligands similar to repeat units of chitosan which will not be hydrolyzed by lysozyme. The polymers synthesized by us are more stable than chitin and chitosan.

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The polymers comprising carbohydrate conjugates may further be used in the treatment of bacterial or viral infections, and are expected not to cause drug resistance.

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