Galactose Modified Polyvinylamine, a New Class of Water Soluble Polymers

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By

Hajir Mokhtari, B. Sc.

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AUTHOR: Hajir Mokhtari, B. Sc. (University of Tehran)

SUPERVISOR: Dr. Robert. H. Pelton

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Abstract

Synthetic carbohydrate carrying polymers have gained substantial attention recently due to their biocompatibility and their wide range of applications such as targeted drug delivery systems, gene therapy and cell-specific biomedical materials. The overall objective of this research is to develop novel carbohydrate bearing polymers through modification of polyvinylamine (PVAm) backbone with galactose groups and to discover potential applications for this new category of glycopolymers.

PVAm-g-galactose (PVAm-GAL) with various molecular weights and grafting extents were prepared and characterized by nuclear magnetic resonance (NMR) and potentiometric and conductometric titration. Bonding of PVAm-GAL with phenylboronic acid modified PVAm (PVAm-PBA) and phenylboronate modified surfaces were studied on a quartz crystal microbalance with dissipation (QCM-D). Multilayer assembly of alternating layers of PVAm-GAL and PVA-PBA was formed on the silica sensor.

Interaction of PVAm-GAL with RCA_{120} lectin, a galactose specific protein, was studied on a silica sensor using QCM-D. Galactose binding proteins are overexpressed in hepatocyte and have been widely exploited for targeting the liver tissue with the help of galactosylated polymeric carriers. RCA_{120} lectin shows spontaneous adsorption on galactose rich surfaces obtained by the adsorption of PVAm-GAL on silica sensors. Association constant of the interaction was calculated $K_a = 6.266 \times 10^6\ M^{-1}$. Effect of pretreatment with bovine serum albumin (BSA) was also examined.

Cationic polymers can form polyelectrolyte complexes (PECs) with negatively charged DNA, resulting in formation of nano-sized complexes for gene delivery purposes. PECs based on PVAm-GAL and different DNA samples were prepared and their physicochemical properties were investigated using dynamic light scattering (DLS) and electrophoretic mobility measurements. Furthermore, PVAm-GAL was studied as coating for Ca-alginate beads which are widely used for cell encapsulation purposes.

PVAm-GAL can strengthen the capsule's surface and increase the physicochemical stability of the beads against chemical degradations. PVAm-GAL coated alginate beads successfully survived treatment with sodium citrate and high ionic strength solution.

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1. Introduction and Literature Review

1.1 Glycopolymers

Carbohydrates are among the most abundant, easily available and inexpensive biomolecules in nature. They are known to play a significant role in a wide range of complex biological processes. The interaction of carbohydrates with proteins and other biological entities is the key interaction involved in mediating cellular recognition processes, adhesion, cancer metastasis and infection of pathogens (Lis & Sharon, 1998).

The term glycopolymers in a broad sense refers to both natural and artificial carbohydrate-containing polymers, as well as synthetically modified natural sugar based polymers (Ladmiral, Melia, & Haddleton, 2004). Synthetic polymers with pendant carbohydrate groups, have gained a lot of attention in the field of polymer chemistry over the years due to their biocompatibility and bioactivity. Glycopolymers are considered to be valuable materials for a variety of applications such as cell specific biomedical materials, drug delivery systems and biological recognition substances (Okada, 2001).

1.1.1 Major classes of glycopolymers

Various types of synthetic carbohydrate carrying polymers have been described in the literature. Linear glycopolymers, comb-like glycopolymers, glycodendrimers, cross-linking hydrogels and spherical glycopolymers in the form of micelles, vesicles and micro/nano particles represent some of the major classes (Pieters, 2009) (Wang, Dordick, & Linhardt, 2002)

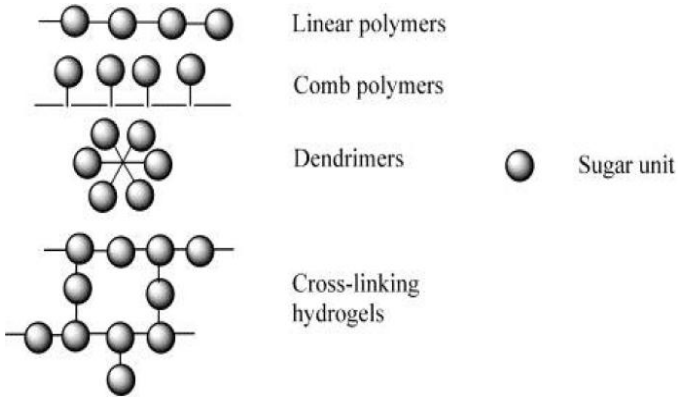

Figure 1.1 Major types of synthetic glycopolymers (Wang et al., 2002)

1.1.2 Strategies for synthesis of glycopolymers

Three major synthetic methods are usually used for preparing carbohydrate carrying polymers: chemical, enzymatic and chemo-enzymatic. Chemical methods which will be discussed in this report include either synthesizing the glycopolymer from a carbohydrate containing monomer through a polymerization technique or by post glycosylation of a polymer backbone.

Various strategies have been employed for the chemical synthesis of glycopolymers. Kiessling et al. used a ring opening metathesis polymerization (ROMP) to synthesize glycopolymers that can efficiently interact with both lectins (Pontrello, Allen, Underbakke, & Kiessling, 2005) and cells (Kiessling & Cairo, 2002). Controlled radical polymerization specially transition-metal-mediated living radical polymerization (ATRP) (Matyjaszewski & Xia, 2001) and radical addition-fragmentation chain transfer (RAFT) polymerization are leading strategies for tailor-made glycopolymers offering control over the desired macromolecular features such as architecture and chain length. Other methods such as free radical polymerization, ring opening polymerization (ROP) and ionic polymerization have also been reported for synthesis of glycopolymers (Ladmiral et al., 2004).

1.1.2.1 Post functionalization of polymers using sugar moieties

Post functionalization approach is convenient to produce various types of glycopolymers by attaching different sugar moieties to pre-formed polymer scaffolds. Post-polymerization reaction on a presynthesized polymer backbone in order to introduce carbohydrate groups tends not to lead to 100% glycosylation, resulting in inhomogeneous sequences. When complete functionalization is not required this method can be applied. This approach is an attractive option for glycopolymer synthesis since it avoids the complex reactions and purification requirements involved when dealing with monomer synthesis (Gibson & Cameron, 2007). There have been many attempts to functionalized preformed polymer with amino saccharides using amide linkage between the polymer back bone and the carbohydrate. Compared to other functional groups, amine groups show good nucleophilicity which provides selectivity without the need for protecting groups. Polymers bearing active carbonyl groups such as carboxylic acid, N-hydroxysuccinimide (NHS) esters and anhydrides have been used in reaction with aminosachcharides (Ting, Chen, & Stenzel, 2010). Poly(acryloyl chloride) was used for synthesis of polyacrylamide compound bearing glucose (PAAm-glucose) and galactose (PAAm-galactose) pendant groups and were tested for their attachment to different cells (Bahulekar et al., 1998). Auzely-Velty et al. prepared a copolymer of N-vinylpyrrolidone and maled anhydride which was subsequently functionalized with a D- galactopyranosyl containing molecule. This resulted in a glycopolymer with good inhibitory reaction when tested with RCA_{120} lectin (Auzély-Velty, Cristea, & Rinaudo, 2002). An attractive polymer for post-modification with carbohydrates is poly(vinyl alcohol) (PVA). Sachez-Chaves and coworkers synthesized functionalized PVA with monosuccinate groups. This polymer was further functionalized with 2-amino-2-deoxy-D-glucose to synthesized a desired glycopolymer with selective recognition for ConA lectin (Sanchez-chaves, Arranz, & Cortazar, 1998).

1.2 Lectins

Lectins are a class of proteins that can bind carbohydrates reversibly and with high specificity. The word "lectin" comes from the Latin word "legere" which means to select or choose (Drickamer, Street, York, & Taylor, 1993; N Sharon & Lis, 1989). These proteins can be found in most organisms ranging from viruses and bacteria to plants and animals and play a crucial role in many biological events such as cell adhesion and cell-cell recognition. Sharon and Lis have highlighted the important role of lectins in biological recognition events (Lis & Sharon, 1998).

The ability to bind carbohydrates is not confined to lectins. Other well-known examples of such interactions are enzymes that bind substrates and inhibitors, and antibodies that bind antigens. However, what distinguishes lectins from carbohydrate-specific antibodies is their non-immune origin. Another marked difference is that antibodies are structurally similar, while lectins are diverse. As compared to enzymes, lectins are devoid of catalytic activity, which means that they do not modify carbohydrate surfaces through binding (C. Lee & Lee, 1995).

1.2.1 Classification of lectins

Hundreds of lectins have been identified and isolated over the years. While all lectin have certain biological properties in common, they are very diverse in terms of structure and size. There are many ways of classifying lectins; they are usually categorized based on the source they are derived from. Lectins occur in all classes and families of organisms; therefore they are commonly divided into animal lectins, plant lectins, lectins from microorganisms, and lectins from viruses (Nathan Sharon & Lis, 2003).

The major animal lectins include C-type (need Ca^{2+} ions for binding), S-type (also known as galectins which are sulfhydryl-dependent), P-type (phosphomannosyl receptors), and I-type (mainly includes siglecs which recognize sialic acids) (Kilpatrick,

2002). Plant lectins are mainly made up of the legumes family and cereal lectins. Concanavalin A (ConA) extracted from jack bean is the most abundant plant lectin from the legume family (Ting et al., 2010). Microorganisms and bacteria produce surface lectins which can be in the form of hair like appendages known as fimbriae. Viruses possess surface lectins which serve for their attachment to host cells (Nathan Sharon & Lis, 2003)

Lectins can also be classified based on their carbohydrate specificity. Broadly speaking, lectins can be divided into those that bind monosaccharides as well as oligosaccharides, and those that recognize oligosaccharides only. Based on the specificity of lectins, they are classified into five groups according to the monosaccharide for which they exhibit the highest affinity: mannose, galactose/N-acetylgalactosamine, N-acetylglucosamine, fucose, and N-acetylneuraminic acid (Lis & Sharon, 1998)

Other methods of classification of lectins is reported in the literature as well, such as classification based on the three-dimensional structure of their binding interaction with carbohydrates (Weis & Drickamer, 1996a)

1.2.2 Carbohydrate-Lectin interaction

In contrast to enzymes which often have buried binding sites, the binding sites on the lectins are normally shallow. Moreover, since very little conformational changes occur upon binding of carbohydrates to lectins, it is assumed that the biding sites to be pre-formed (Weis & Drickamer, 1996b).

Lectins bind their specific carbohydrates through a network of hydrogen bonds, hydrophobic interactions, van der Waals' interactions, and in some cases metal ion coordination (Lis & Sharon, 1991; Rini, 1995). Structural studies have led to the conclusion that hydrogen bonding plays a fundamental role in stabilizing carbohydrate-

lectin complexes. Most polar groups of the carbohydrate such as OH- and NH- groups are found engaged in a network of multiple hydrogen bonds with corresponding acidic groups of the lectins, which is thought to cause an increase in both the total number and the energy of the carbohydrate-lectin interaction (Lemieux, 1989). Water-mediated hydrogen bonding interactions are also visible lectin-carbohydrate interaction. Water with its small size and ability to act as both a hydrogen-bond donor and acceptor, acts as a molecular 'mortar'. Tightly bridging water acts is thought of as structural water, essentially an extension of the lectin surface (Toone, 1994a) . Even though carbohydrates are primarily polar molecules, due to steric disposition of hydroxyl groups, hydrophobic patches are created on the carbohydrate surface that can interact with hydrophobic patches of the lectin. Metal ions such as Ca^{2+} and Mg^{2+} can be found near the carbohydrate binding sites of the lectins but are not always directly involved in the binding. However, they assist in the positioning of amino acid residues of the lectin for better interaction with the carbohydrates (Agrawal & Goldstein, 1968).

1.2.3 Kinetics of Carbohydrate-Lectin interactions

Binding of carbohydrates to lectins is relatively weak and does not result in the formation of covalent bonds. Since the reaction is reversible the following equilibrium can be represented:

Lectin + Sugar ⇔ Lectin-Sugar complex ($L + S \Leftrightarrow LS$)

$$K_a = \frac{[LS]}{[L]\times[S]} \qquad \text{(Equation 1.1)}$$

Where K_a is the association constant or the binding constant for the Lectin-carbohydrate complex. The reaction can be examined by physicochemical methods used for similar protein-ligand complexes, mainly by spectrophotometry, spectrofluroimetry, calorimetry (Dam & Brewer, 2002), nuclear magnetic resonance (Poveda & Jiménez-Barbero, 1998), surface plasmon resonance (Haseley, Talaga, Kamerling, & Vliegenthart,

1999), atomic force microscopy, quartz crystal microbalance (Lekka, Lebed, & Kulik, 2007), or affinity chromatography (Hirabayashi, Arata, & Kasai, 2003). These methods provide a way of calculating the association constant, the number of combining sites, as well as other thermodynamic and kinetic parameters of the interaction.

1.2.4 Thermodynamics of Carbohydrate-Lectin interactions

The process of binding of a ligand to a lectin involves the encounter of solvated carbohydrate and a solvated binding site. Water molecules at the combining site of lectins are in a special perturbed state of higher energy than of bulk water and when the ligand comes in contact to a binding site, water can be displaced and binding occurs. Release of this water provides driving for carbohydrate complexation (Clarke et al., 2001; Lemieux, 1996). The differences between the hydrogen bond energies of the solute-solvent interactions (i.e. lectin-water and carbohydrate-water complexes) on the one side, and those of the solute-solute interactions (i.e. lectin and the carbohydrate) on the other, is a crucial contributing factor in determine the binding energy. Other contributing factors are changes in lectin conformation, van der Waals bonds, hydrophobic interactions, as well as entropic effects (Ernst, Hart, & Sina, 2000; Lemieux, 1996; Toone, 1994b).

Entropy of the interaction is affected by translational, rotational and conformational effects in addition to solvatation effect. The rotational and conformational contributions are unfavorable to entropy as there is a loss in the degree of freedom upon docking of carbohydrate in the binding site of the lectin as the lectin-carbohydrate complexes are formed. On the contrary, the entropy of solvatation is favorable to the association. As mentioned earlier, lectin and carbohydrate are hydrated prior to binding and there is release of water molecules from the binding sites as the two come in contact to each other. However the magnitude of this favorable entropy effect is too low for the lectin-carbohydrate interaction to be considered entropy driven.

According to calorimetric data the lectin-carbohydrate interactions are enthalpy driven. Solvent reorganization has a considerable contribution to the enthalpy of complexation. Thus, even though there is an unfavorable loss in entropy due to changes in rotational degree of freedom upon binding, it is compensated by a desired change in enthalpy resulting from the removal of water that is at a higher energy state and formation of new hydrogen bonds between the ligand and receptor (Freichels, Jérôme, & Jérôme, 2011; Nathan Sharon & Lis, 2003).

1.3 Glycopolymer-lectin interaction

1.3.1 Multivalency

The 'valency' of a molecule is defined as the number of separate connections of the same kind that it can form with other molecules through ligand-receptor binding (Mammen, Choi, & Whitesides, 1998). Monovalent carbohydrate ligands usually have low association constants to lectins ($10^3 to\ 10^4\ M^{-1}$) (Nathan Sharon & Lis, 2007; Toone, 1994a). For effective biological activity, stronger and more selective binding is required. Multivalent interactions are prevalent in biology such as adhesion of viruses and bacteria to cell surface and binding of cell to other cells. Compared to monovalent interaction, multivalency can lead to significant increase in the strength of the interaction. Natural and synthetic multivalent ligands present multiple copies of a binding ligand that increases the binding constant. This phenomenon has become known as the "cluster glycoside effect" or the "glycocluster effect" (Lundquist & Toone, 2002). Lee and Lee were among the first to introduce this effect by demonstrated that simple cluster glycosides exhibited significantly higher binding affinities to the asialoglycoprotein receptor when compared to the monomeric ligand (Y. C. Lee & Lee, 1995). Since then, there has been significant interest in developing synthetic multivalent carbohydrate ligand. As glycopolymers are typically multivalent by definition, due to the large number of repeating carbohydrate units along the polymer, they provide simple methodologies for accessing the cluster glycoside effect.

1.3.2 Mechanism of multivalent binding to receptors

Several mechanisms have been suggested to be responsible for the increased affinity of multivalent ligands for their receptors. The possible binding mechanisms according to Kiessling et al. (Kiessling, Gestwicki, & Strong, 2006) are depicted in Figure 1.2 and will be described shortly here:

a) Chelate effect (chelation): this mechanism which occurs with proteins having closely separated carbohydrate binding sites (oligomeric receptors) is believed to have the highest contribution to the enhanced binding of multivalent ligands. The multivalent ligand must be able to bridge the distance between the adjacent receptor sites. The chelate effect is usually used for small molecules such as metals and ions, binding to multivalent hosts.
b) Subsite binding: Some proteins have binding site in addition to the primary binding site. The primary binding to the receptor promotes secondary binding to other sites.
c) Receptor clustering: multivalent binding results in collecting of receptors which alters the signaling properties of the receptors. This can be facilitated by two-dimensional diffusion of receptors within a fluid membrane bilayer.
d) Statistical/ proximity effect: local concentration of binding species is higher for the multivalent ligands which promotes rebinding. This is because of the slower off-rate of binding due to the close proximity of other ligands that can take the place of the first ligand after it releases.
e) Steric stabilization: when a large multivalent ligand binds to the receptor it inhibits other ligands from binding to the surface due to steric hindrance of its size and hydration shell around it.
f) Polyelectrolyte effect: as the lectin binds to the carbohydrate, the entropically favorable release of counter ions in the bulk solution acts as a driving force for

association of the two macromolecules. This is an important mechanism in systems such as protein-DNA association.

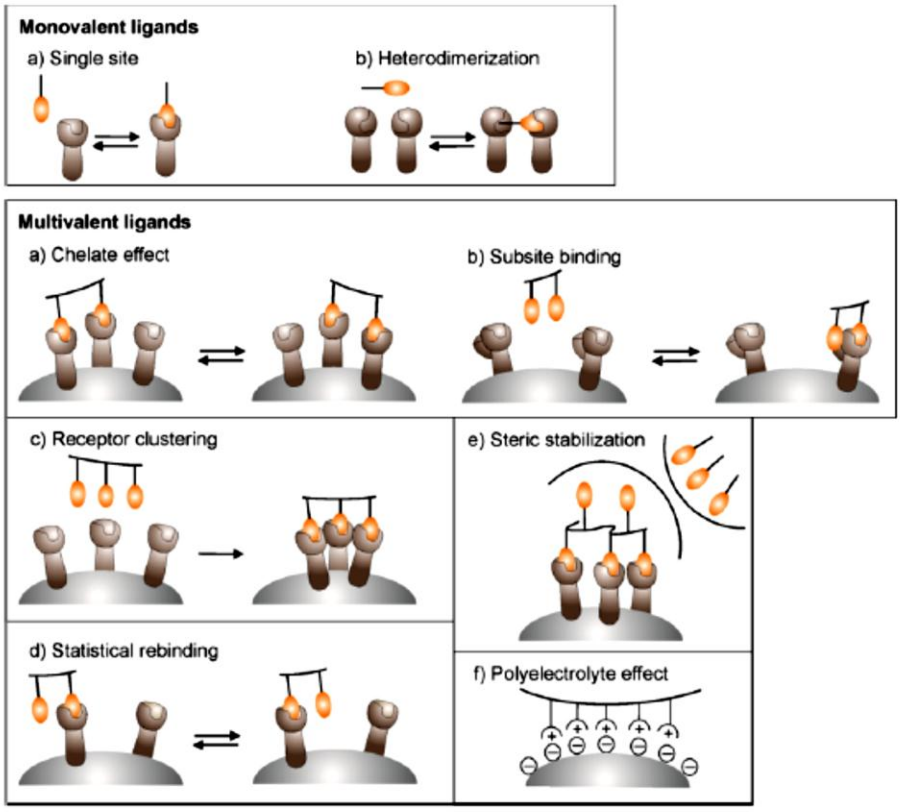

Figure 1.2 Mechanisms of multivalent binding with cell-surface receptors (Kiessling et al., 2006)

The docking of the carbohydrate in the binding sites of the lectin is accompanied by a loss in translational and rotational entropy. This entropy loss happens for every interaction in monovalent ligand-monovalent receptor binding. However, in case of multivalent binding, this entropy loss takes place only for the first binding, and favoring the overall binding, the other ligand-receptor bindings will not suffer this loss. The translational entropy cost is paid with the first ligand-receptor contact and additional binding interactions happen with less entropic cost. To explain the cluster glycoside

effect, effective concentration was suggested as an alternative approach. This model states that during multivalent interactions the first binding forces the other ligands to come closer to the receptor. Therefore, the local concentration of ligand is greater than of that in the bulk solution, favoring the multivalent binding (Gargano, Ngo, Kim, Acheson, & Lees, 2001).

1.3.3 Interaction of lectins with different glycopolymer architectures

1.3.3.1 Linear glycopolymer

Linear synthetic glycopolymers are the most widely examined polymers in terms of their ability to bind to proteins. Lectin studies are normally conducted in aqueous systems due to high water solubility of glycopolymers. High concentration of polymer is usually avoided as it can lead to clustering of polymers due to hydrogen bonding between hydroxyl groups of the pendant carbohydrate moieties or hydrophobic interactions from the polymer back bone (You, Lu, Li, Zhang, & Li, 2003).

Kobayashi et al. studied the interaction of ConA lectin and maltose and maltotriose containing polymers via turbidity assay and UV-Vis spectroscopy. The accessibility of ConA deep binding cavity sites was enhanced by the terminal glucose residue of the pendant carbohydrate chain (Kobayashi, Sumitomo, & Ina, 1985).

Hsegawa et al. compared the lectin binding abilities of rigid helical phenyl acrylamide glycopolymers to flexible ones. The compatibility and spacing of clustering carbohydrate chains were found to be essential for specific multivalent ligand recognition by lectins (Hasegawa, Kondoh, Matsuura, & Kobayashi, 1999).

Linear glucose-based glycopolymers synthesized via ROMP were studied for their binding towards ConA lectin by Kiessling and co-workers. Multivalent effects of the glycopolymers were examined by agglutination inhibition assay (Mortell, Weatherman, & Kiessling, 1996). Due to the lectin and glycopolymer structure present, it

was observed that decreasing the carbohydrate density along the backbone resulted in an increase in the activity towards ConA lectin as shown in Figure 1.3.

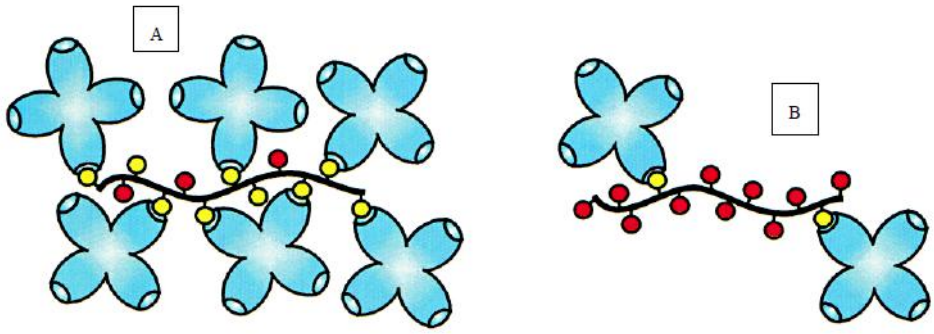

Figure 1.3 (A) High density glycopolymers recruit many receptors to a single molecule, but steric hindrance prevents binding of every ligand. (B) Low density glycopolymers bind fewer receptors per molecule. Increasing spacing between sugar ligands can result in more efficient binding (Ting et al., 2010)

Polymers with high density ligands can recruit more receptors to a single molecule, but steric hinderance prevents binding of every residue. On the other hand, low density carbohydrate polymers bind fewer toatl receptors per molecule. It was found that incresaing spacing between the sugar residues on the polymers backbone resulted in more efficient binding.

Haddleton et al. introduced galactose and mannose derivatives onto the polymer backbone bearing alkyne functional groups using click chemistry methods. They studied the lectin binding abilities of various polymer structures by changing the carbohydrate content on the polymer. Their results suggested that the average number of ConA lectin bound to the polymer increased with the carbohydrate content in the polymer. There was a maximum number of bound lectins and after a mannose contet of 70% the number of bound lectin remain constant (Ladmiral et al., 2006).

In another study hepatic lectin from human hepatocarcinoma cells were used to study their interaction with galactose modified N-(2-hydroxypropyl)methacrylamide

(HPMA) polymers. The results suggested that glycopolymers with multiple sugar moieties such as tri-saccharides show much higher binding as compared to monosaccharide carrying glycopolymers (David, Kopecková, Kopecek, & Rubinstein, 2002).

Baek and Roy (Baek & Roy, 2001) studied relative lectin binding properties of T-antigen containing glycopolymers and did a comparison between copolymerization of N-acrylolated T-antigen monomer to graft cojugation of aminated T-antigen ligand onto poly(N-acryloxysuccinimde). Binding abilities of glycopolymers with various spacer arms with peanut agglutinin (PNA) were tested. The results suggested that glycopolymers with shorter spacer arms from their polymer backbone showed lower affinity due to lack of accessibilty to PNA lectin binding sites. Miyachi et al, studided the effect of multiple sugar units on the polymer backbone on its cytotoxicity and its binding ability to lectins. It was discovered that by designing multivalent galactose based glycopolymer in a way that another disacchairde was incorporated in the glycopolymer, the lectin binding could be enhanced. The results indicate that galactose-trehaloses (GT) glycopolymers could bind much stronger to Bandeiraea implicifolia ($BSI\text{-}B_4$) lectin (Miyachi et al., 2009).

1.3.3.2 Superamolecular glycopolymers

While linear glycopolymers display desired bioactivity and efficient multivalent effect for binding to proteins, their use for some biomedical application might still be limited. One way to achieve a more functional material for the use in biological systems is designing supramolecular glycopolymeric structures. These complex architectures derived from glycopolymers usually show much greater affininty towrds lectins, since aggregated and three diemsnioned structures tend to give higher surface area for lectins to access their binding ligands. Diverse scaffolds can be used for multivalent interactions including glycodimers and glycotrimers, glycodendrons, glycodendrimers, 3-D cavity containing scaffolds such as glyco-cyclodextrins and glyco-calixarenes, glycopeptides,

glycoliposomes, self assemebeld glyco-micelles, and 2-D self assembled monolayers (SAM).

Similar to linear polymers, a high density of carbohydrate is not ncessearily required to have optimum binding of supramolecular glycopolymers with lectins (Serizawa, Yasunaga, & Akashi, 2001). An additional parameter introduced to the system in self assemebled structures is the change in the conformational of the polymer chains below and above the critical micelle concentration (CMC) which affects the binding to lectins. A more rigid chain conformation as observed by the more brush like conformation of micelles has been suggested as the contributing factor that improves binding . Flexibility of the structure and folding or overlaping of the chain conformation can hide the binding sites from their coressponding ligands which results in reduced recognition and binding. Chaikof et al. (Dong, Faucher, & Chaikof, 2004) synthesized a helical triblock copolymer which was then self assembled into lactose installed polymeric aggregates. By altering the initial copolymer concentration, they were able to arrive at different morphology changes from sphere to lamellae and worm-like micelles. Then these supramolecular glycopolymers were investigated for their interaction with RCA120 lectin using turbidity measurements. The results suggested that the lactose moieties were located at the outer surface of these aggregates.

Variations in the architecture of supramolecular structures can be made using different compositons, spacer length between the branching units, spacer lenth between the carbohydrate units, and the size of the dendritic scaffold. Synthesis of heterofunctionalized saccharide shells in glycondenrimers is an attractive area of research since it has been proven that coupling of at least two different saccharide units or one saccharide unit with another water-soluble functional group enhances the binding affinity to lectins (Rotello & Thayumanavan, 2008). The influence of the density of the carbohydrate shell on binding affinities to lectin has been also evaluated (Reuter et al., 1999).

1.4 Carbohydrate-Lectin Binding Assays

Binding of carbohydrates to lectins can be observed using various techniques, ranging from the earliest hemagglutination inhibition assay (HIA) to the quartz crystal microbalance with dissipation (QCM-D) which is a mass adsorption method onto surface of resonating sensors. The basic principle behind lectin binding assays is formation of isolated complexes between the protein and its corresponding carbohydrate**.** A short review about some of these techniques will be discussed in this section.

1.4.1 Hemagglutination inhibition assay

HIA is amongst the earliest techniques of detecting the interaction between viral antigen and their corresponding ligands. Different concentrations of the ligand solution are first placed into the microwells, followed by addition of soluble lectin in order for aggregates to precipitate. The minimum concentration of carbohydrate that inhibits the hemagglutination is reported (Lundquist & Toone, 2002).

1.4.2 Enzyme-linked lectin assay

Enzyme-linked immunosorbent assay (ELISA) and its variant, enzyme-linked lectin assays (ELLA), are other methods of assessing lectin-carbohydrate binding. ELISA uses enzyme-linked reagent usually for detecting specific antibody-antigen reactions, while ELLA which is a similar assay performed on microtiter plates, uses an enzyme-lectin conjugate for semi-automated and quantitative detection of specific carbohydrate end groups in an analogous manner to ELISA (Mccoy, Varani, & Goldstein, 1984).

1.4.3 Isothermal calorimetry

In order to determine thermodynamic and kinetic parameters of the interaction, carbohydrate-lectin binding constants are usually calculated through isothermal titration calorimetry (ITC), quantifying the heat generated from the binding, in other words the enthalpy of the reaction (Freire, Mayorga, & Straume, 1990). A carbohydrate solution of know concentration is added at regular intervals (usually 20 to 50 injections with constant

volume) into a calorimeter that contains the lectin solution with known concentration. Heat of reaction either released (exothermic binding) or absorbed (endothermic binding) is measured over time. The intensity of the each peak produced is proportional to the temperature change (ΔT) with respect to a reference cell. These ΔT's are converted to heat of interaction and their quantities are directly proportional to the extent of binding, which shows a decreasing trend as the number of injections increase. As the system approached saturation, the heat signal diminishes and only dilution heat is monitored.

The binding curve obtained is then compared against binding models for the interaction such as the Langmuir model. The model can provide thermodynamic binding parameters of the interaction, binding association constant (K_a), enthalpy change (ΔH) and stoichiometry (n) (Brown, 2009). Free energy of binding (ΔG) and the entropy of binding (ΔS) can be calculated using the following equations:

$$\Delta G = -RT \ln K_a$$

(Equation 1.2)

$$\Delta G = \Delta H - T\Delta S$$

(Equation 1.3)

To calculate the heat capacity (ΔC_p) using equation 1.4 the experiment is repeated at various temperatures.

$$\Delta C_P = \frac{\delta \Delta H}{\delta T}$$

(Equation 1.4)

This technique does not require any chemical modification or immobilization of the receptors and gives direct and accurate thermodynamic parameters of the binding.

1.4.4 Surface plasmon resonance

Surface plasmon resonance (SPR) is based on interaction of an incident polarized light with the surface plasmon of thin metal films. Aluminum, silver and gold films from 2.5 to few tens of nm can be used, but gold film is the most frequently used one. With a prism near the metal interface, the incident light can excite optically the surface plasma wave at a well-defined angle at constant wavelength or conversely at a well-defined wavelength at constant angle. The excitation can be seen as a strong decrease in reflection for the transverse magnetic light and for a special angle of incidence, resulting in a minimum in the intensity of the reflected beam (Mol & Fischer, 2010). The direction of this strong decrease is related to the refractive index of the layer in contact with the film. This layer contains the immobilized molecule of interest, and the refractive index will change according to the concentration of the compound that will be retained by the immobilized molecule, resulting in a change in position of the minimum in reflectance accordingly. For studying the lectin-carbohydrate interaction, the lectin is immobilized onto the surface of the metal sensor and a solution of carbohydrate is flowed over the sensor. A part of the ligand will be retained by interacting with the lectin. This additional material increases the total amount of organic matter in the layer and as a consequence, the refractive index increases and a shift in the minimum position is observed (Mol & Fischer, 2010; SU & ZHANG, 2004)

1.4.5 Quartz crystal microbalance

Many important physical and chemical processes can be estimated from associated mass changes. The quartz crystal microbalance is a simple, efficient and high resolution mass sensing technique. When a mass binds to the surface, it tends to oscillate with the identical lateral displacement and frequency as the crystal surface.

A QCM consists of a thin quartz disc with metal electrodes plated onto the surface. Gold is usually used as electrode materials, deposited on the upper and lower quartz surfaces. When an alternating electric field is applied across the quartz crystal, through the upper and lower metal electrode, a mechanical oscillation of characteristic frequency (*f*) is generated in the crystal. In this method the lectin is immobilized on the surface of the crystal and a solution containing the carbohydrate is passed over the surface. The changes in the frequency are measured as the carbohydrates bind to the lectins on the surface. A reverse situation can be also implemented i.e. carbohydrates immobilized on the crystal.

The methods mentioned above are the most commonly used methods for assessing the lectin binding. There are other analytical techniques to choose from that even though are not used as frequently as the above mentioned techniques but are still effective in studying lectin-carbohydrate interaction. Turbidity assays using the UV-Vis spectroscopy to determine binding of lectins (Cairo, Gestwicki, Kanai, & Kiessling, 2002). Two dimension immune diffusion tests also known as double diffusion agar (DDA) technique can also be used (Kobayashi, Tsuchida, Usui, & Akaike, 1997).

2. Synthesis and Characterization of PVAm-GAL and its bonding with PVAm-PBA and phenylboronate silica surfaces

2.1 Introduction

Boronic acids have the ability to bind compounds containing diol functional groups through reversible ester formation. The condensation of borate ions and 1,2-diols or 1,3-diols occurs in alkaline condition. This process is reversible and pH can be used as a switch to run on or off the complex formation (Springsteen & Wang, 2002). Therefore the properties of borate ester have been exploited in numerous applications such as affinity chromatography (GILDENGORN, 1996), drug delivery (F. Liu, Song, Mix, Baudys, & Kim, 1997; S. Zhang, Trokowski, & Sherry, 2003) receptor and sensor for carbohydrates (Gray & Houston, 2002; Zhao, Fyles, & James, 2004) , labeling of proteins and cells (Chang, Pralle, Isacoff, & Chang, 2004) and bioconjugation (Wiley et al., 2001). The mechanism of interaction of pheynlboronic acid and diols is demonstrated in the Figure 2.1 (Springsteen & Wang, 2002).

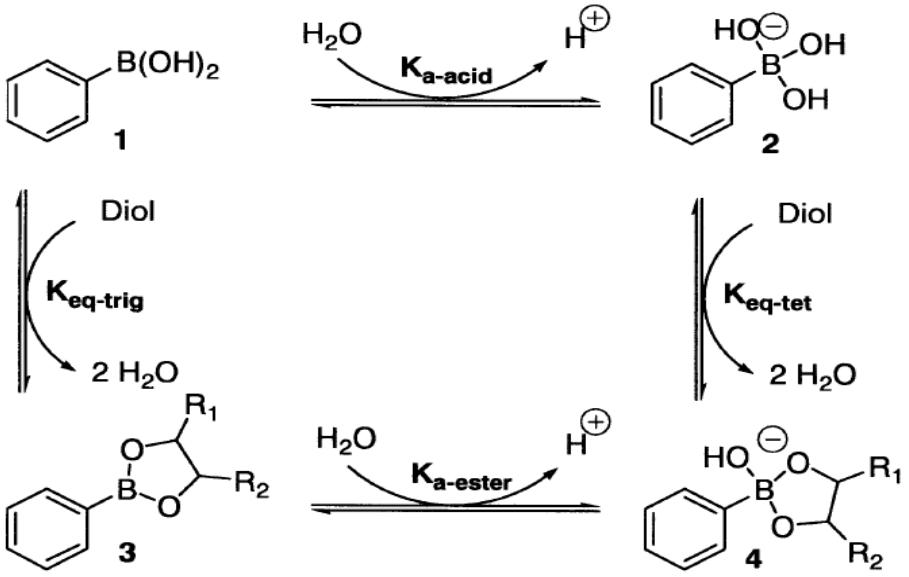

Figure 2.1 The interaction of phenylboronic acid and diols (Springsteen & Wang, 2002)

A phenylboronic acid in a trigonal form (1 in Figure 2.1) can transform to an anionic tetrahedral from (2 in Figure 2.1) in water. Boronate ester also shows two different forms in water (3 and 4 in Figure 2.1). Each of these diol binding steps is represented with a specific binding constant as shown in the scheme ($K_{eq\text{-}trig}$ and $K_{eq\text{-}tet}$). The overall binding constant of boronic acid and diols is usually represented with K_{eq} in literature. Boronate ester formation is generally occurs at pH values above 8, however there are some reports on boronate-diol bond formation at lower pH values (Gregory, 1995).

The well-documented ability of phenylboronic acid groups to condense with carbohydrates to give covalent rings was the inspirations for derivatization of polyvinylamine with pheynlboronic acid. As mentioned before boronate-carbohydrate reactions are promoted by alkaline pH, when the boronic acid is ionized (Springsteen & Wang, 2002). However, due to presence of nearby amine groups on the polymer backbone lowers the pK_a of phenylboronic acid and the bond formations can occur at

lower pH values (Zhu et al., 2006). The borate-carbohydrate bonds are weak in nature and the binding constants usually range from 10 to 5000 M^{-1} which are typically for hydrogen bands rather than covalent bonds (Springsteen & Wang, 2002). For example, the equilibrium constant for phenyl boronic acid bonding to glucose at pH 9 is reported to be 26 M^{-1} as calculated by competitive binding with a fluorescent probe. This association constant corresponds to a free energy of 1.9 kcal/mol, which is less that of a hydrogen bond (Springsteen & Wang, 2002). However polymeric phenylboronates form much more stable complexes with water soluble polyols as the can have multiple points of attachment (Keita, Ricard, & Audebert, 1995).

In this chapter the interaction of PVAm-GAL with phenylboronic acid modified PVAm (PVAm-PBA) as well as with phenylboronate modified silica surface will be discussed. PVAm-PBA has been previously reported to form complexes with polyol such as cellulose by our group(Chen, Leung, Kroener, & Pelton, 2009). Galactose groups on PVAm-GAL bond with PBA groups, resulting in multi-layer formation on the silica sensor as observed by QCM-D.

2.2 Experimental: Materials and Methods

2.2.1 Materials Used

Polyvinylamine (PVAm) with molecular weight of 340 kDa was provided by BASF with commercial trademark name of Lupamin® 9095. Lactabionic acid (4-O-ß-D-galactopyranosyl-D-gluconic acid), *N*-(3-(dimethylamino)propyl) -*N*'-ethylcarbodiimide hydrochloride (EDC), 4-carboxyphenylboronic acid, 4-vinyl phenylboronic acid, dimethyl L-tartrate, platinum(0)-1.3-divinyl-1,1,3,3-tetramethyldisiloxane (Karstedt's catalyst), D-(-)-Fructose, D-Sorbitol, and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Sigma Aldrich and used as received. Toluene and dimethyl sulfoxide (DMSO) were purchased from Caledon Labs. Spectra/Por Dialysis membranes

with molecular weight cut off of 12000-14000 and 3500 were purchased from Spectrum Laboratories Inc. All experiments were performed with water from a Millipore Milli-Q system.

2.2.2 Synthesis of PVAm-GAL

A series of polyvinylamine-g-galactose with different molecular weight and varying degree of galactose substitution were prepared by grafting lactobionic acid to the amine groups of PVAm by carbodiimide chemistry. In a typical experiment 0.15 g PVAm was dissolved in 80 ml of 0.1 M MES buffer at pH=5.5 and 20 ml of lactobionic acid in the same buffer with concentrations ranging from 1.2 to 9.6 g/L (depending on the desired degree of galactosylation) was added. 3 g of EDC was added to the solution and the mixture was stirred using a magnet stirrer for 240 min at room temperature. The product was then dialyzed against Milli-Q water for two weeks to ensure the removal of the undesired formic salts and was subsequently lyophilized.

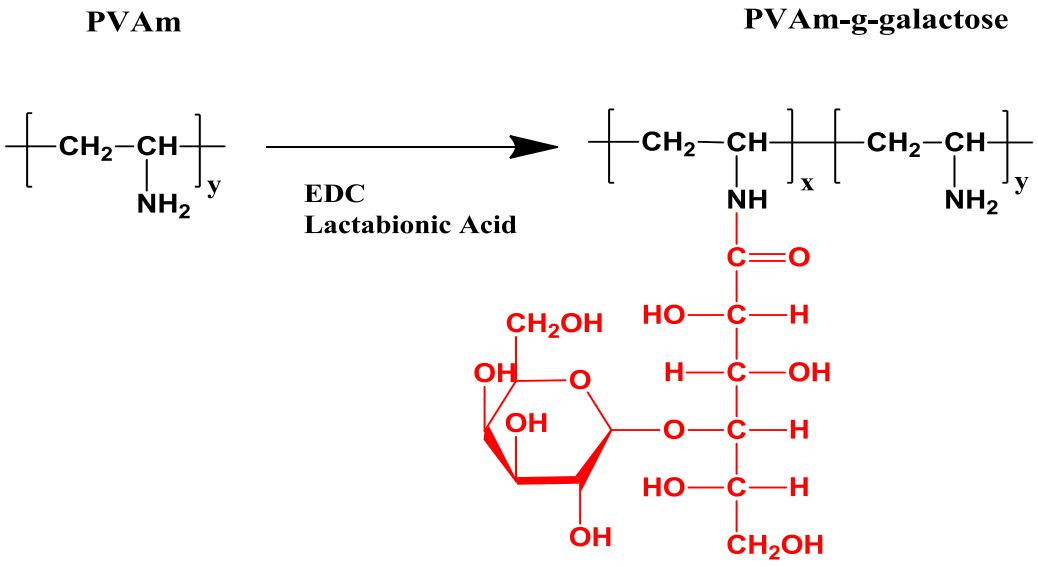

Figure 2.2 PVAm-GAL synthesis scheme

A table consisting of the name of all the galactosylated polymers synthesized in this work and their corresponding molecular weight and degree of substitution will be presented in the results and discussion section.

2.2.3 Synthesis of PVAm-PBA

PVAm-PBA was synthesized according to the procedure described previously by our group (Chen, Pelton, & Leung, 2009). In a typical coupling reaction of PBA to PVAm backbone, 0.4 g of PVAm was dissolved in 10 ml of MES buffer at pH=6 and 90 ml of 4-carboxyphenylboronic acid with concentration of 4.4 g/L at pH=6 in the same buffer was added. 7.5 g of EDC was then added and the mixture was stirred for 2 hours at room temperature. The product was dialyzed against Milli-Q water for two weeks and lyophylized subsequently.

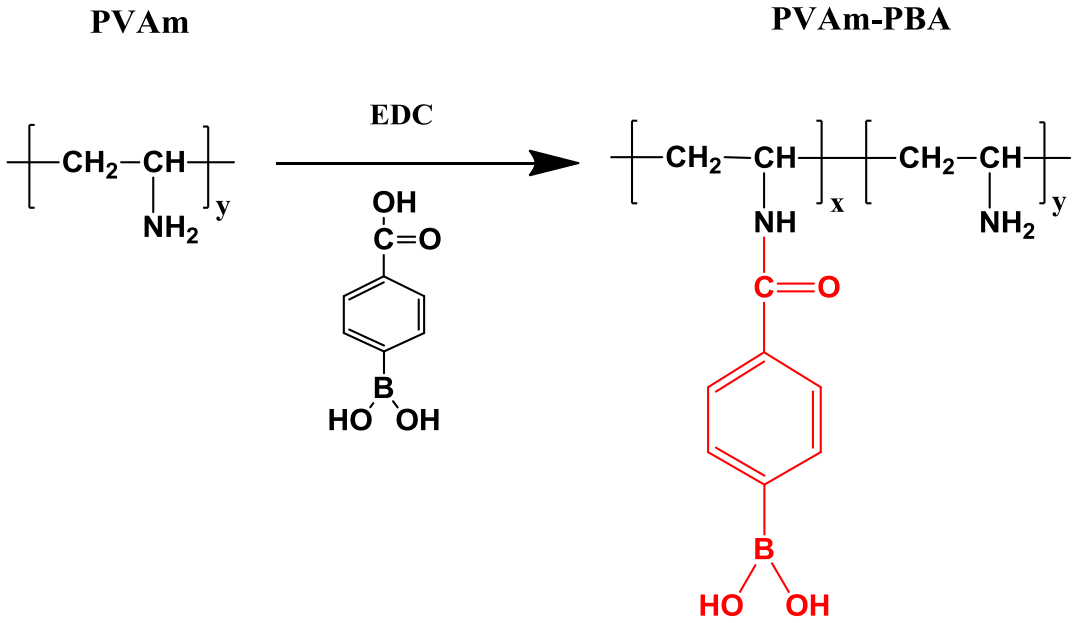

Figure 2.3 Synthesis reaction scheme of PVAm-PBA

Properties of PVAm-PBA used in this study will be presented in the results and discussion section.

2.2.4 Characterization by ^{1}H NMR

The chemical composition of the polymers was confirmed by ^{1}H NMR recorded on a 200 MHz Bruker spectrometer for low molecular weight polymers and on a 500 MHz device for higher molecular weight polymers at room temperature. Lyophilized polymers were dissolved in 1 mL of deuterium oxide (D_2O) to prepare 5 mg/ml samples.

2.2.5 Potentiometric and conductometric titration

Potentiometric-conductometric titrations of samples were carried out by dissolving 20 mg of polymer into 50 ml of 0.001 M KCl solution. HCl was added drop-wise to adjust the initial pH of the samples to 3. Samples were then titrated with 0.1 M NaOH to a maximum pH of 11 on a PC-Titration Plus equipment (ManTec Associates) at room temperature and the pH and conductivity as the function of the volume base added were recorded.

2.2.6 Preparation of phenylboronate modified silica

Tartrate protected boronate silane solution and modification of silica surfaces were performed according to the method described by Pelton et al. (Pelton et al., 2012). The reaction scheme for synthesis of tartrate protected phenylboronate is presented in Figure 2.4.

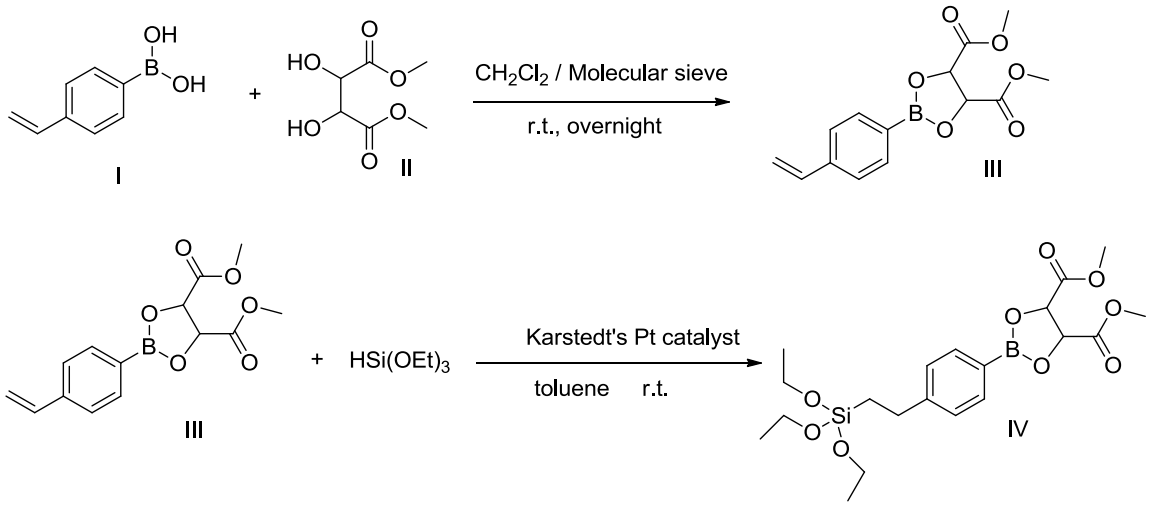

Figure 2.4 Synthesis reaction scheme for tartrate protected boronate silane (Pelton et al., 2012)

The reaction steps are as follows:

a) A mixture of 4-vinyl phenylboronic acid (I) and 1 equivalent of dimethyl L-tartrate (II) in dichloromethane (DCM) and molecular sieve were stirred at room temperature overnight to yield tartrate protected boronate ester (III).
b) 2 drops of Karstedt's catalyst, $Pt_2\{[(CH2=CH)Me_2Si]_2O\}_3$, (platinum(0)-1.3-divinyl-1,1,3,3-tetramethyldisiloxane) was added to a mixture of III and 1.05 equivalence of tiethoxy silane ($HSi(OEt)_3$) in toluene at controlled temperature of 50 °C.
c) After reaction was over, toluene and excess $HSi(OEt)_3$ were removed under vacuum giving final product tartrate protected 1-(4-ethylphenylboronic acid) triethoxy silane (IV) as colorless oil.

To prepare phenylboronate surfaces, silicon dioxide QCM-D sensors were immersed in 2.5 wt% solution of 1-(4-ethylphenylboronic acid) triethoxy silane using toluene as solvent for two hours at 50 °C. Then the sensors were rinsed with dry toluene

once, followed by rinse with dry DCM twice. Finally the sensors were placed in oven at 80 °C to remove the excess solvent.

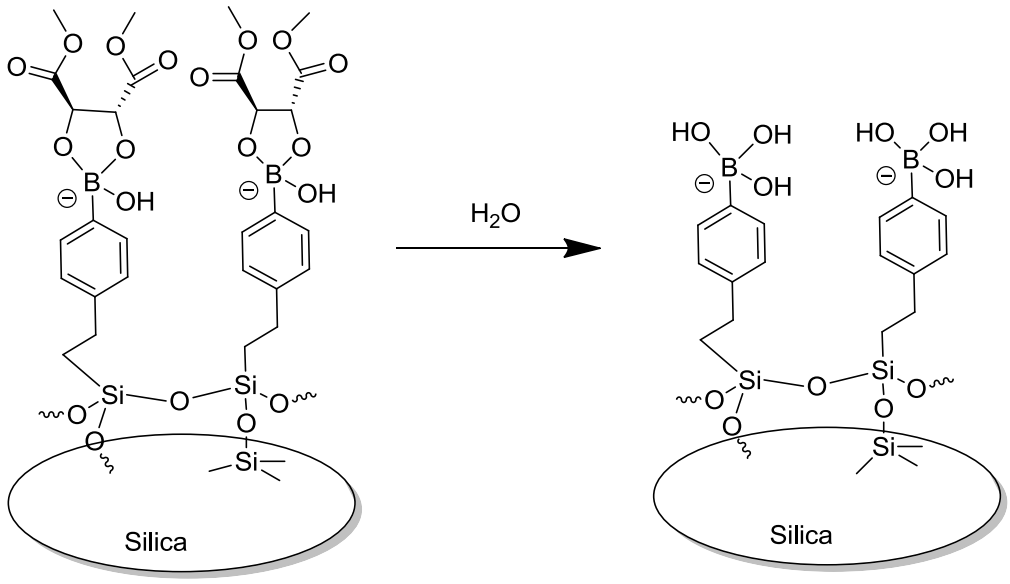

Figure 2.5 Phenylboronate functionalization of silica sensor by tartrate protected silaneboronate groups

2.3 Results and Discussion

2.3.1 Characterization of PVAm-GAL by ^{1}H NMR

The ^{1}H NMR spectra of PVAm and PVAm-g-galactose are presented in figure 2.6. The results indicate that the galactose was successfully introduced on to the PVAm backbone. For PVAm the peak for (−CH_2−) group is around δ=1.6-2 ppm and for (−CH−) proton depending on the cis or trans conformation of the amine groups there are two peaks at δ= 3.2 ppm and δ=3.8 ppm. After grafting lactobionic acid pendant groups

on the PVAm, the appearance of new signals in the range of 3.2-4.5 ppm was assigned to proton adsorption of the sugar group.

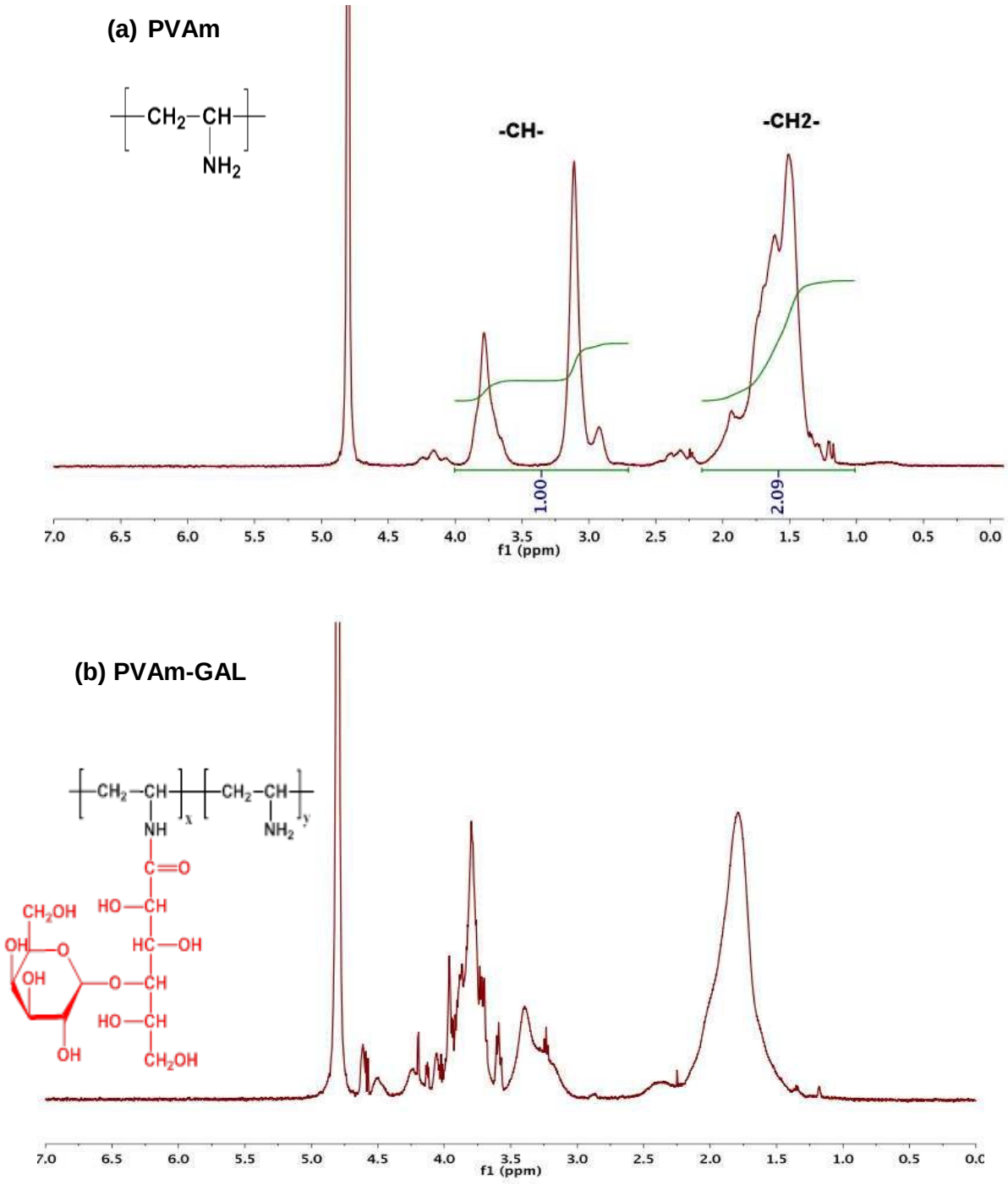

Figure 2.6 ^{1}H NMR spectra of (a) PVAm340 kDa and (b) PVAm340-GAL2 recorded on a 500 MHz Bruker spectrometer at room temperature

By comparing the ^{1}H NMR spectra of the polymer before and after galactosylation with ^{1}H NMR spectra of lactobionic acid, it is obvious that the lactobionic acid has been incorporated onto PVAm backbone.

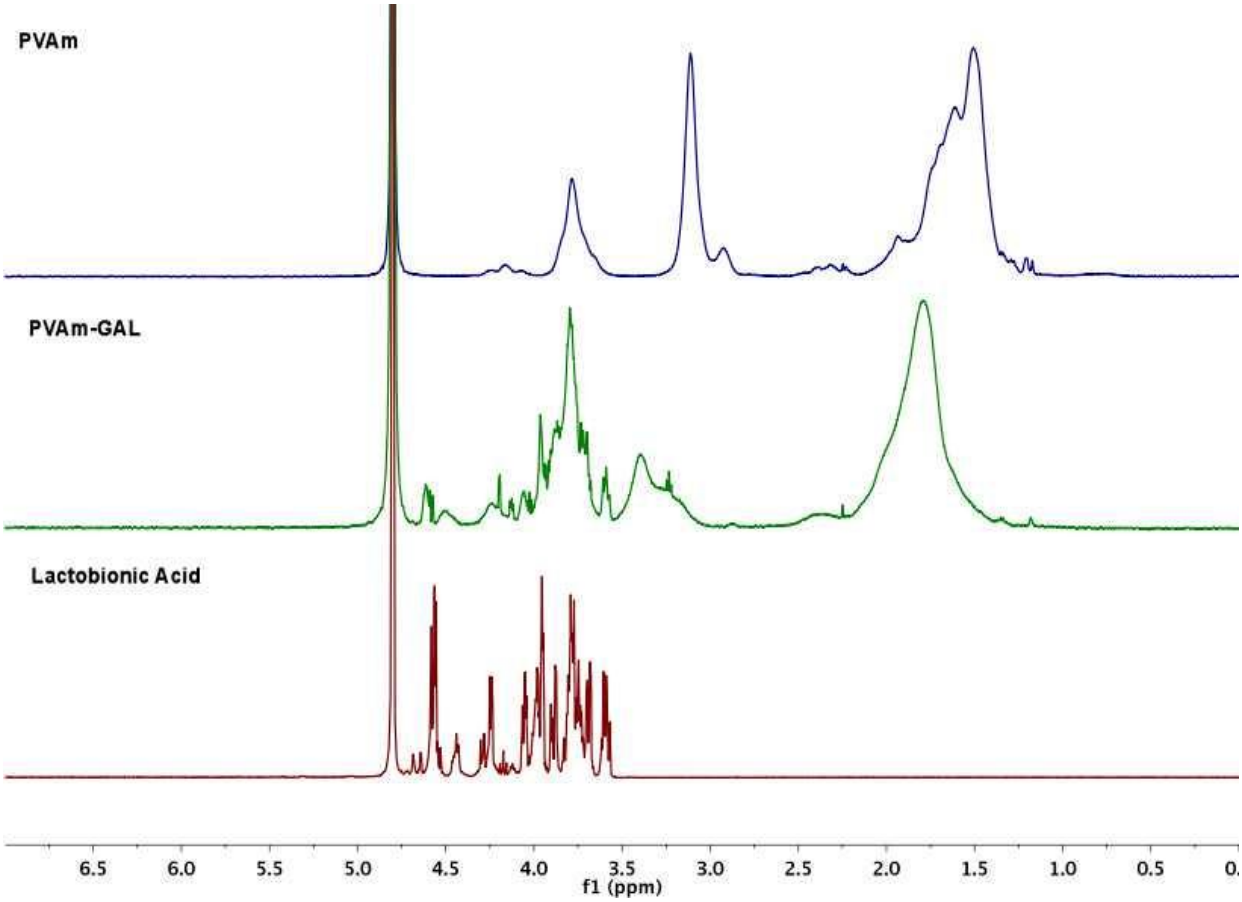

Figure 2.7 Comparison of ^{1}H NMR spectra of PVAm 340 kDa (top), PVAm340-GAL2 (middle), and Lactobionic Acid (bottom)

PVAm samples with 10 kDa molecular weight and increasing sugar contents were examined with ^{1}H NMR as presented in 2.8. It is obvious from the results that there are more intense lactobionic acid signals as the degree of galactose substitution increases. Due to overlapping of the lactobionic acid peaks with those of PVAm as well as shifting

and broadening of the polymer signals after sugar modification, choosing a diagnostic peak to track for calculating the degree of substitution is difficult. However, degree of galactose substitution was calculated based on integral of peaks in the galactosylated region of the spectra and compared to results obtained from titration. Conductometric titration provides a more reliable way for calculating the degree of galactosylation in this case.

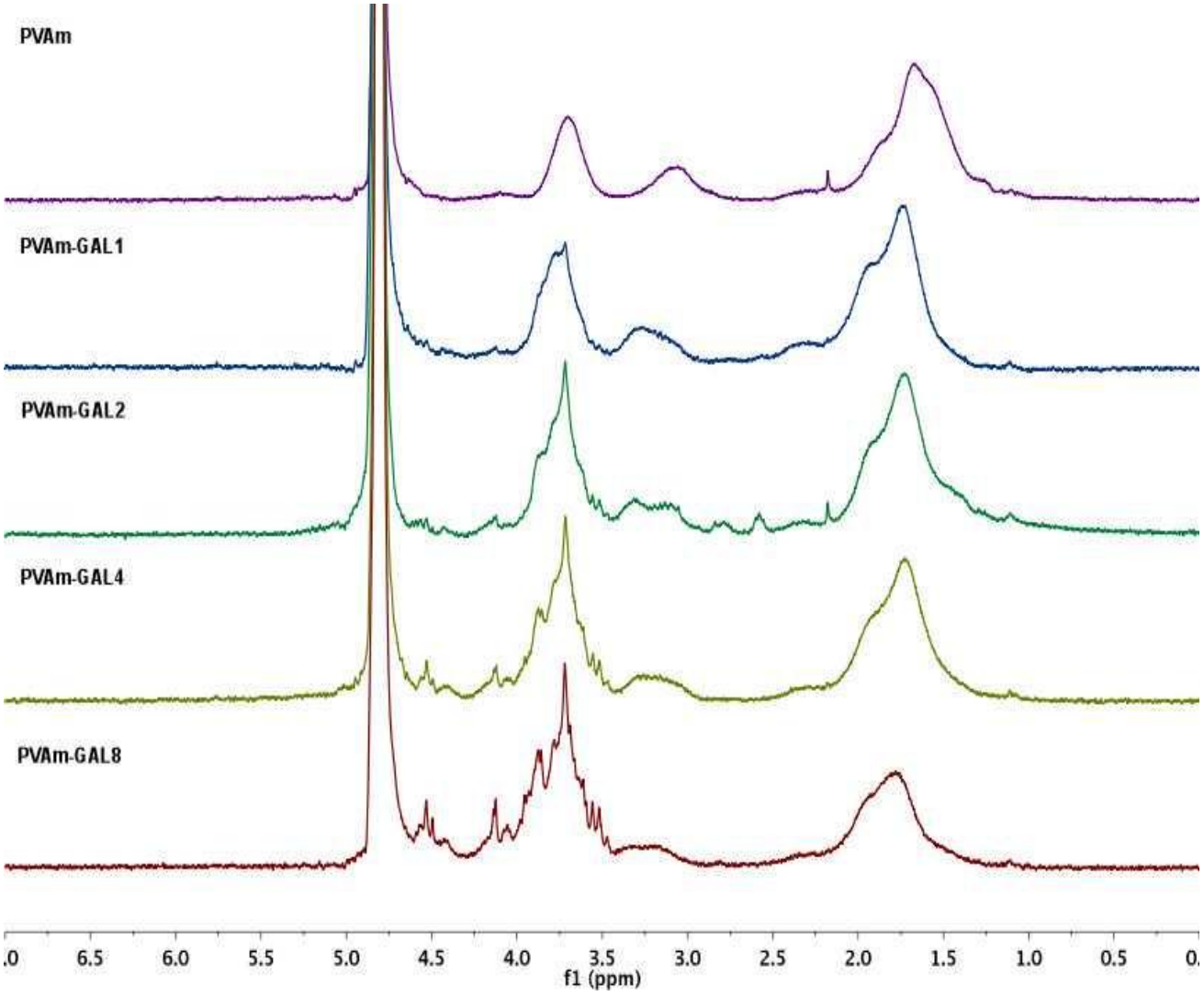

Figure 2.8 ^{1}H NMR spectra of 10 kDa PVAm and PVAm-GAL with increasing degree of galactosylation from top to bottom

PVAm-PBA with molecular weight of 340 kDa was also characterized with ^{1}H NMR as presented in Figure. A small amount of DCL in D_2O was used to dissolve the polymer as PBA substitution lowers the solubility of PVAm

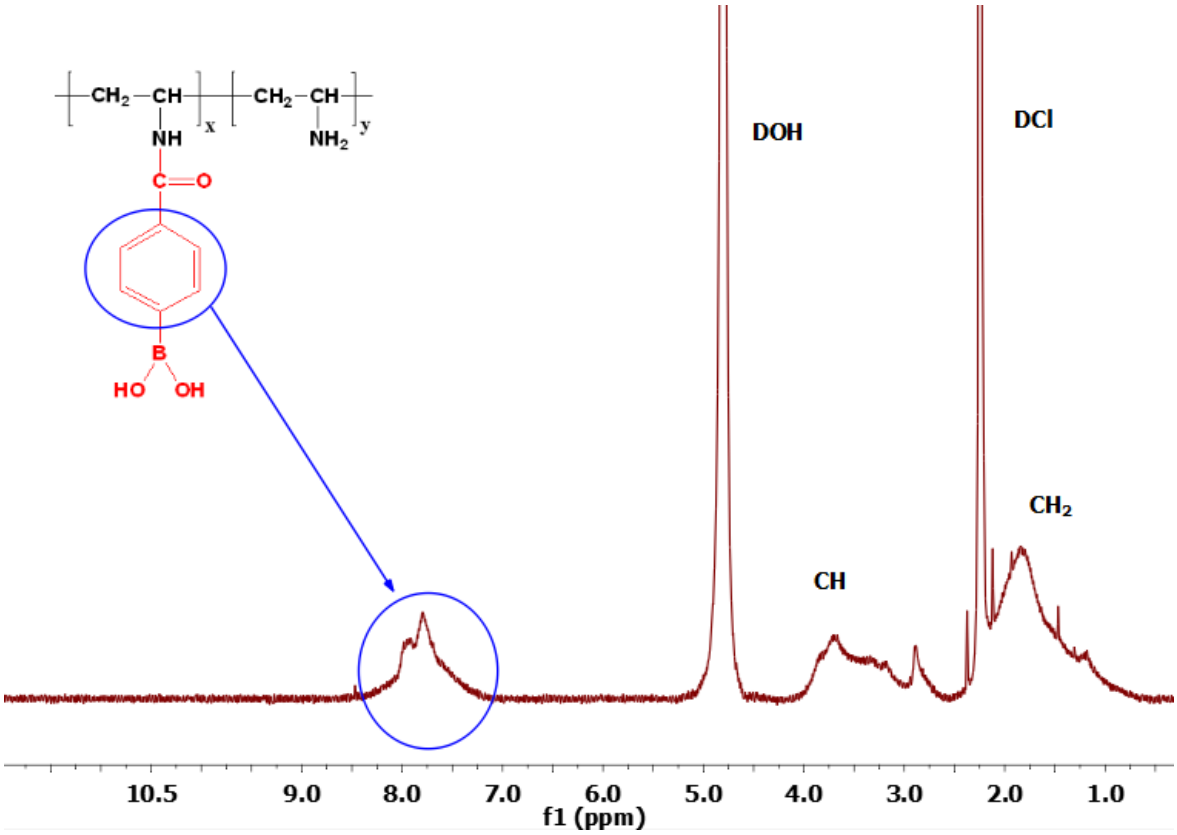

Figure 2.9 1H NMR spectra of PVAm-PBA with molecular weight of 340 kDa, recorded on a 500 MHz spectrometer

Degree of PBA substitution for PVAm-PBA polymer used in this study was calculate from the $^{1}HNMR$ spectra as reported in table

Table 1 Properteis of PVAm-PBA polymer synthesized

Name	**Molecular weight (kDa)**	**ǂ Degree of substitution from ^{1}H NMR % (mole percent)**
PVAm-PBA	340	12.6

2.3.2 Characterization of PVAm-GAL by FTIR

FTIR spectra of PVAm 340 kDa and PVAm-GAL polymers are presented in Figure 2.10. The adsorption at 1575 cm^{-1} was assigned to N-H bending vibration. Compared to that of PVAm, galactose modified PVAm shows a new signal at 1020 cm^{-1} which was attributed to C-O stretching vibration of galactose groups. This indicated that the amine groups were partially substituted by lactobionic acid.

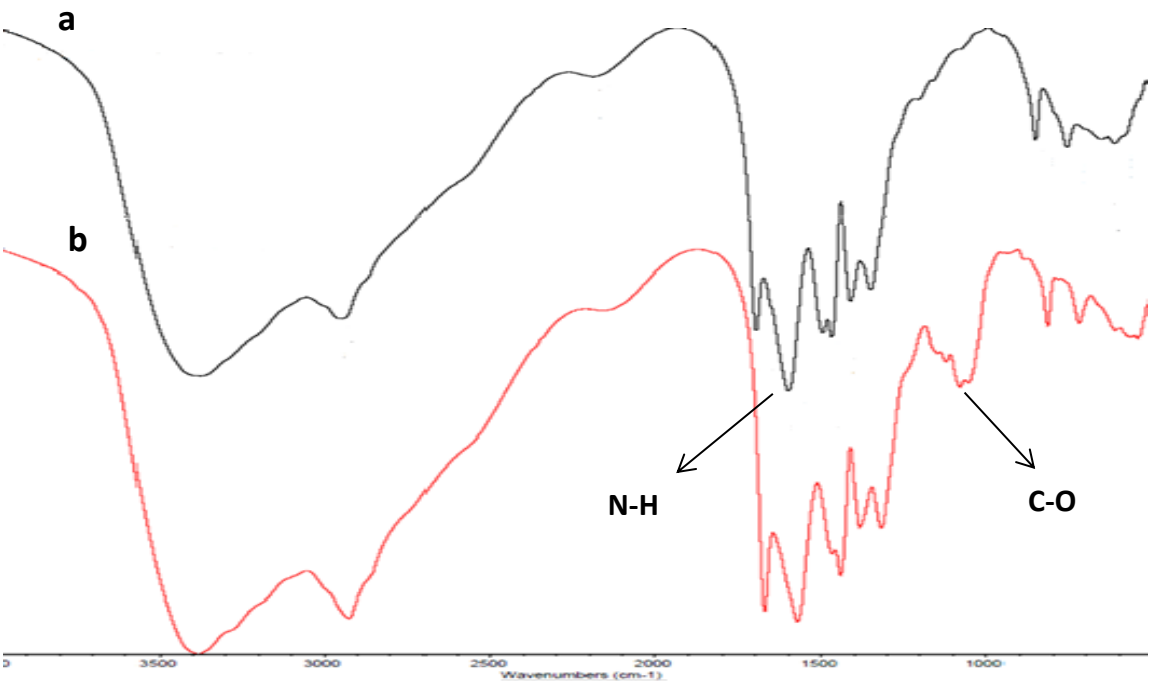

Figure 2.10 FTIR spectra of 340 kDa PVAm (a) before and (b) after galactosylation

2.3.3 Characterization of PVAm-GAL by potentiometric and conductometric titration

The Charge-pH behaviors of PVAm and PVAm-GAL with various degrees of substitution and different molecular weights were examined by potentiometric and conductometric titration. An example is presented in Figure 2.10 for 340 kDa PVAm. At low pH values the amine groups of PVAm-GAL are protonated due to the polyelectrolyte

effect of polymer backbone. Titration with 0.1 M NaOH was carried out up to pH=12 where all the amine groups are neutralized.

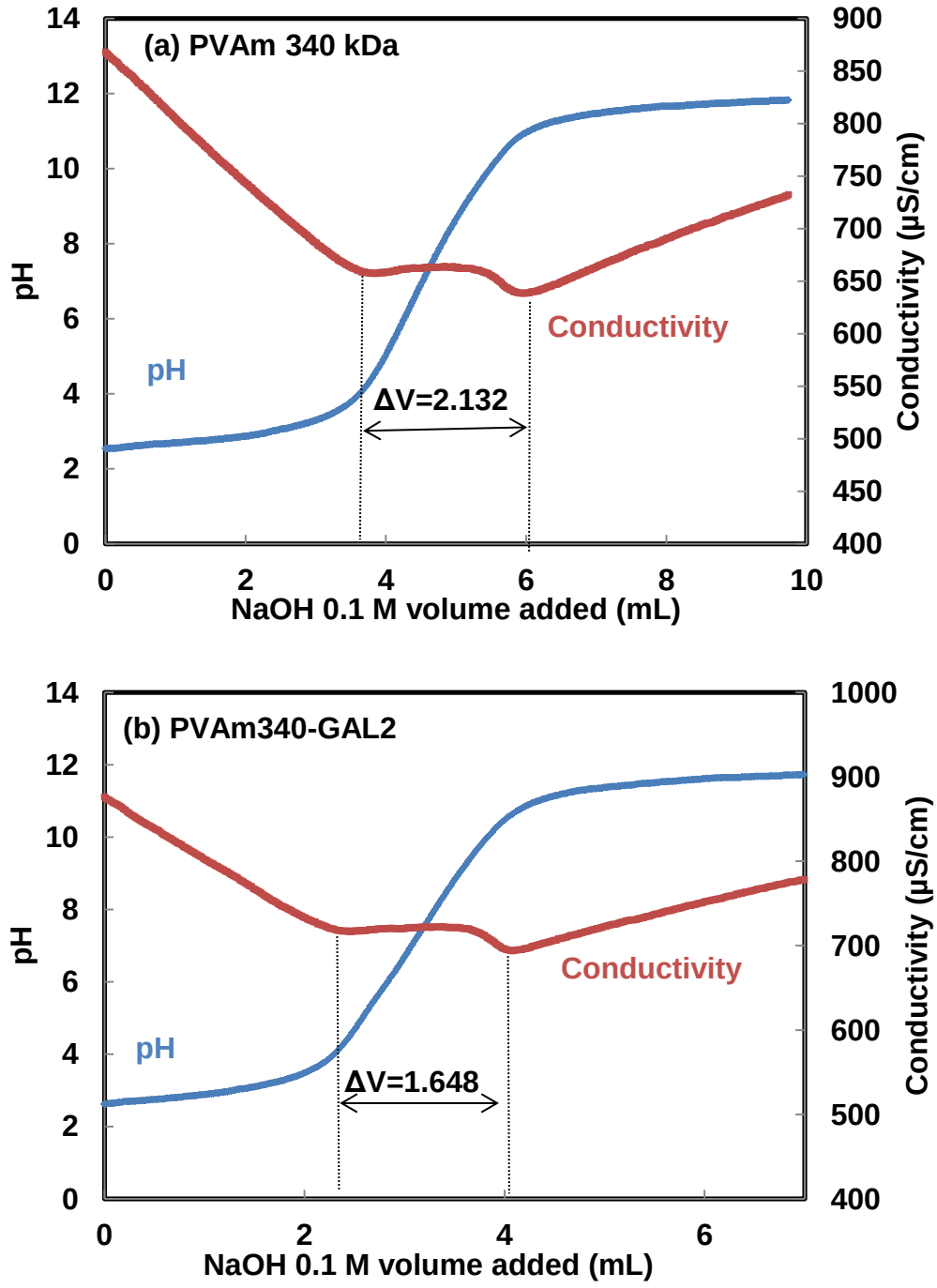

Figure 2.11 Potentiometric and conductometric titration for 20 mg of (a) PVAm 340 kDA and (b) PVAm340-GAL2 in 50 ml 0.001 M KCl

By comparing the volume of titrant required to neutralize PVAm-GAL to that of unmodified PVAm, the amine mole content and degree of galactose substitution can be calculated. Table 2 presents the list of polymers synthesized along with their corresponding degree of galactosylation calculated from titration and NMR results. A sample calculation is available in Appendix A.

Table 2 List of polymers synthesized, their molecular weight and degree of substitution

Polymer Name	**Molecular weight (kDa)**	**Degree of substitution % (mole percent)**		**Lactobionic acid concentration (g/L) in the recipe**
		Titration	NMR	
PVAm10-GAL1	10	27.7	22.6	1.2
PVAm10-GAL2	10	38.8	-	2.4
PVAm10-GAL4	10	43.1	26.6	4.8
PVAm10-GAL8	10	42.5	34.4	9.6
PVAm45-GAL1	45	28.4	-	1.2
PVAm45-GAL2	45	42.0	-	2.4
PVAm45-GAL4	45	63.6	-	4.8
PVAm45-GAL8	45	67.2	-	9.6
PVAm340-GAL2	340	22.6	23.3	2.4
PVAm340-GAL4	340	22.5	22.1	4.8
PVAm340-GAL8	340	22.6	24.6	9.6

Comparison of potentiometric titration curves of PVAm with increasing degree of galactose substitution is presented in Figure 2.12. A clear decrease in titratable amine groups is observed as the degree of galactose substitution is increased.

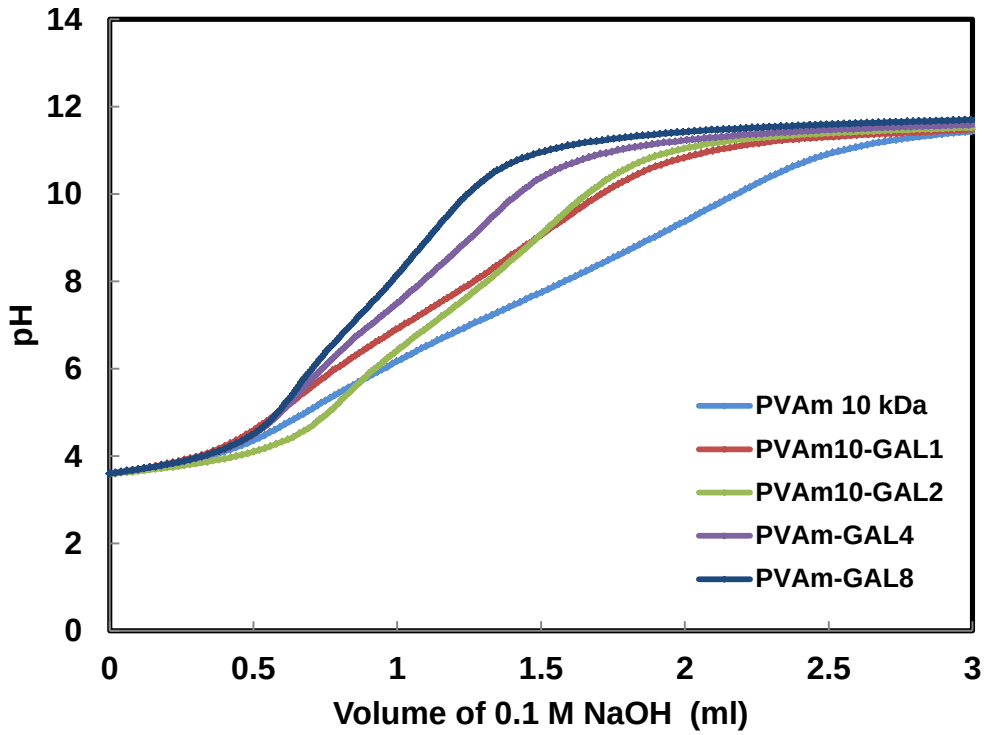

Figure 2.12 Comparison of potentiometric titration curves for PVAm 10 kDa with various degree of galactosylation

Amine content of the polymers was also calculated based on conductometric curves. The results are summarized in table 2.

Table 3 Amine contents of PVAm-GAL samples in this study

Polymer name	Amine content (mmol NH_2/g sample)
PVAm10-GAL1	7.7
PVAm10-GAL2	6.5
PVAm10-GAL4	5.9
PVAm10-GAL8	6.1
PVAm45-GAL1	10.7
PVAm45-GAL4	5.4
PVAm45-GAL8	4.9
PVAm340-GAL2	8.2
PVAm340-GAL4	8.3
PVAm340-GAL8	8.2

2.3.4 Adsorption of PVAm-GAL on phenylboronate surfaces:

Adsorption of galactose modified PVAm on phenylboronate modified silica sensor was observed with QCM-D. The activation of the phenylboronate surfaces was achieved by washing off the tartrate groups with 5 mM NaCl at pH=7. PVAm-GAL was then immobilized on boronate silane surface at pH=9.2 to ensure the ionization of the boronic acid groups. Figure 2.12 presents the schematic complexation of PVAm-GAL to the surface

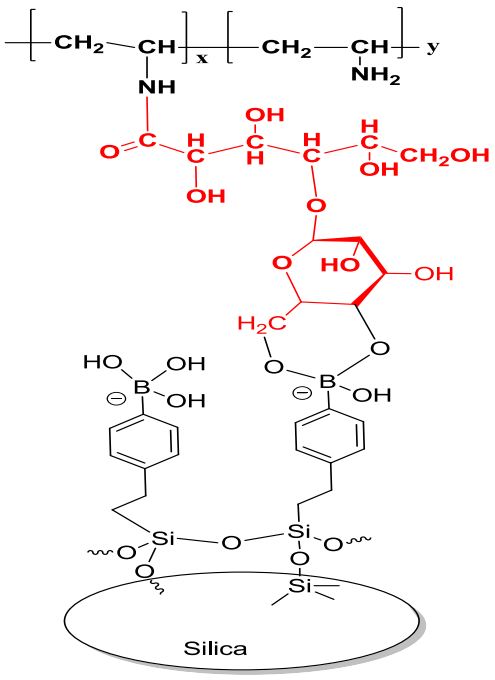

Figure 2.13 Schematic complexation of PVAm-GAL with phenylboronates groups on a silica sensor

The adsorption is represented by a near 40 Hz drop in frequency as a result of mass adsorbed on the surface. Passing of the buffer did not wash off the polymer, indicating firm attachment to the surface.

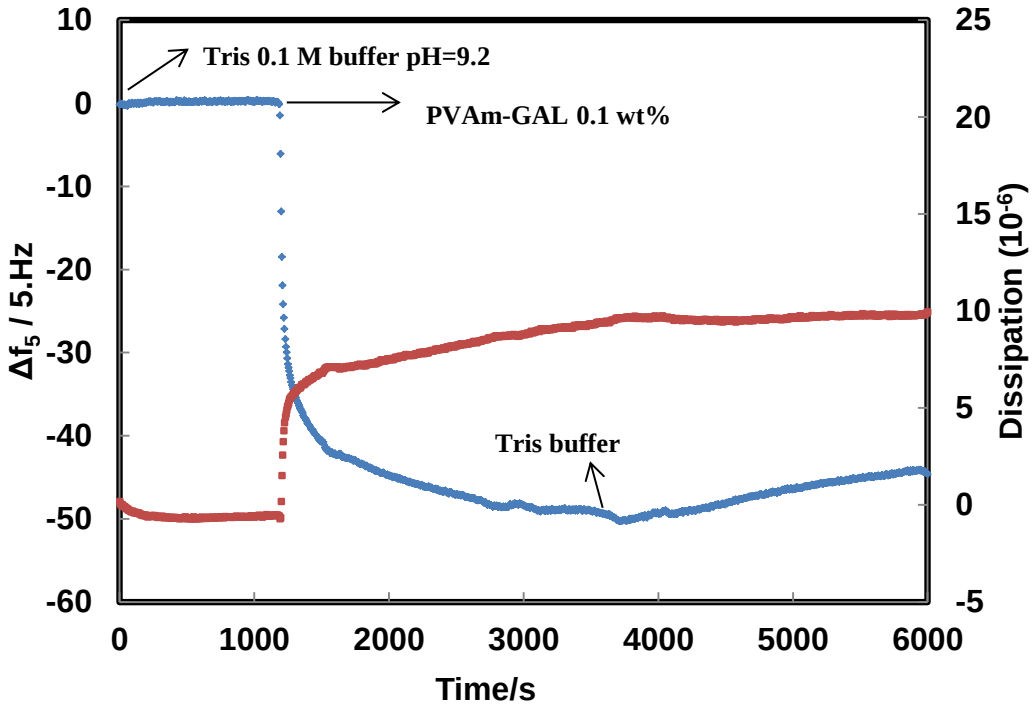

Figure 2.14 Adsorption of PVAm-g-galactose on boronate silane silica

Control studies were performed by studying the adsorption of unmodified PVAm on boronate silane silica chips as presented in Figure 2.12. The graph suggests a drop in the oscillating frequency due to the high molecular weight of the polymer solution. However it appears to follow an ascending trend corresponding to mass desorption from the surface, observed when unbound polymer is detaching from the surface. When compared to the plateau following the frequency drop observed when PVAm-GAL was introduced to the systems, it is safe to conclude that the unmodified polymer does not adsorb on the boronate silane.

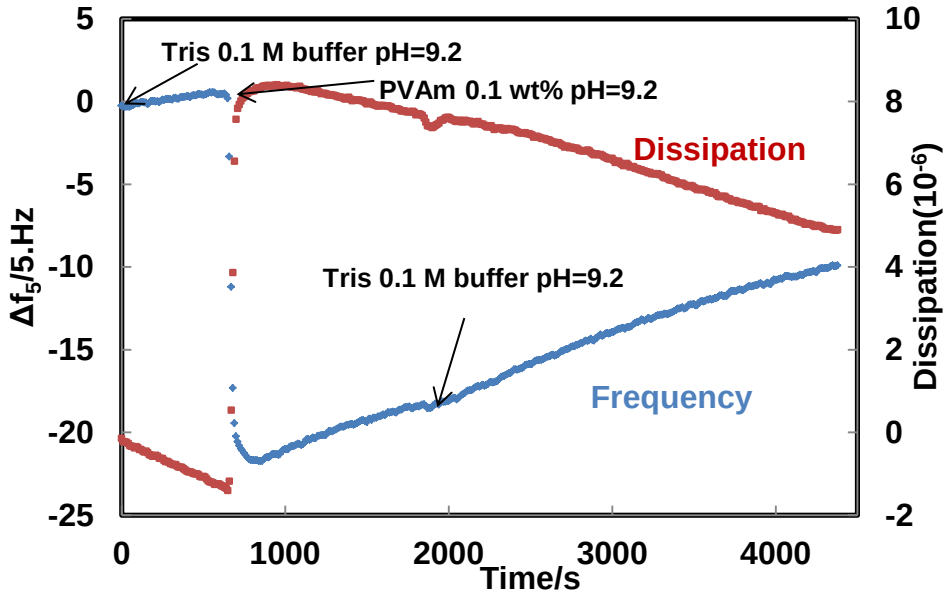

Figure 2.15 Adsorption behavior of PVAm 340 kDa on phenylboronate silica at pH=9.2 and T= 25°C

Another control study was performed by adjusting the pH of the PVAm-GAL solution below the pKa of PBA groups as presented in Figure 2.16. For this purpose, pH was adjusted to 6.5 which is well below the pH value necessary for the polyol binding to occur. As most of the boronic groups are not ionized at pH=6.5 we expected to see no binding happening between galactose groups and boronate groups of the surface.

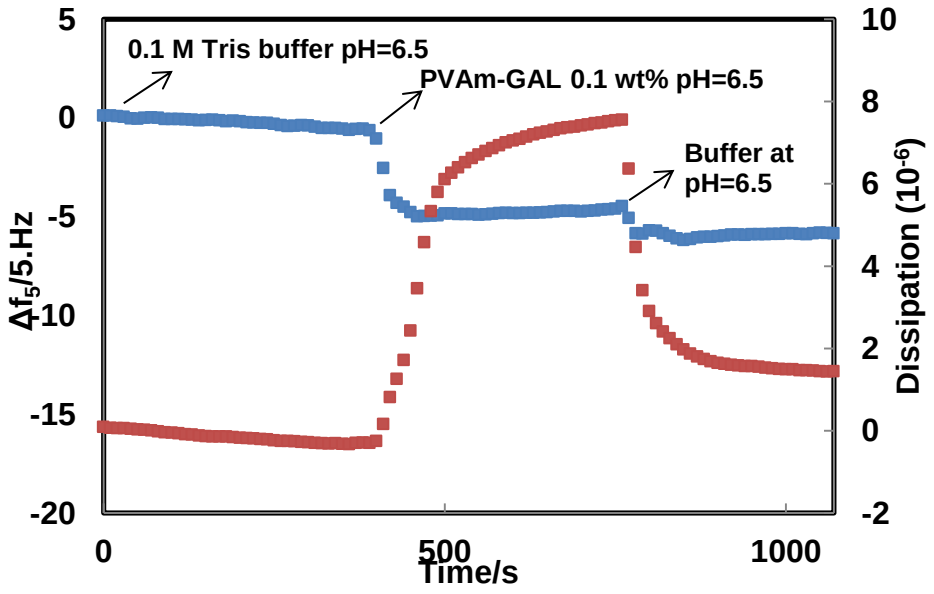

Figure 2.16 Adsorption behavior of PVAm340-GAL2 on phenylboronate silica sensor at pH=6.5 and T=25

The frequency change is much less than that of pH=9.2 indicating less adsorption. The dissipation increases after introducing the PVAm-GAL solution, however, after passing of the buffer it return to almost the starting point. This behavior is observed when the polymer sits at the surface due to its high molecular weight but there is no bound formation. This creates a more viscoelastic system but the unbound polymer is washed away after passing the buffer causing the dissipation to drop back to the rigid system before introducing the polymer.

2.3.5 Multilayer formation of PVAm-Galactose and PVAm-PBA

It is expected that the phenylboronic acid groups on PVAm-PBA form complexes with polyol groups of lactobionic acid on PVAm-GAL as presented in Figure 2.15.

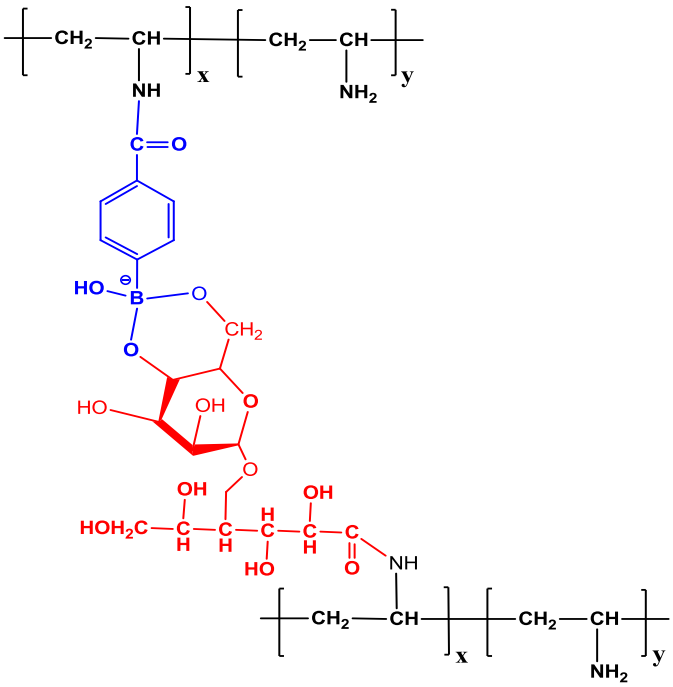

Figure 2.17 Schematic bond formation between PVAm-GAL and PVAm-PBA

The ability of PVAm-GAL to form multi layers with PVAm-PBA was studied on the QCM-D sensors as depicted in Figure 2.8. PVAm340-GAL2 was first immobilized on phenylboronate silica surface. PVAm-PBA was then introduced to the system which resulted in bond formation with the galactose pendant groups of the previous layer, observed by further drop in frequency. This second layer held together after passing the buffer. Finally another layer of PVAm340-GAL2 was introduced which resulted in further bonding with the PBA groups. There was an obvious increase in dissipation as each layer was built up on the previous one. This indicates a more viscoelastic system expected of a multilayer formation on the surface as compared to a more rigid system observed for single layer adsorption.

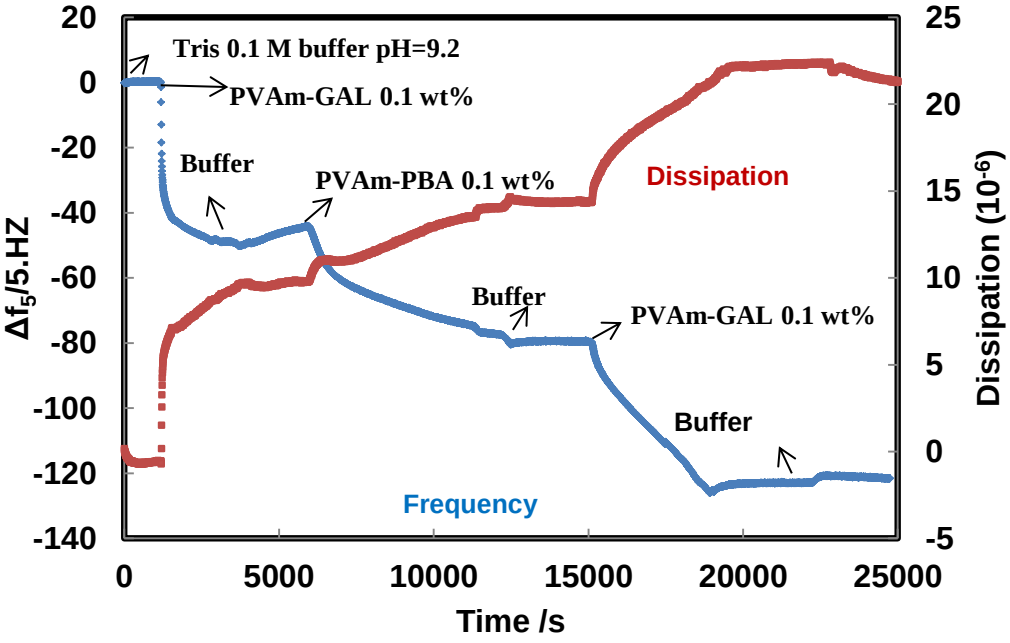

Figure 2.18 Multilayer formation of PVAm-GAL and PVAm-PBA on phenylboronate silica in 0.1 M Tris buffer at pH=9.2

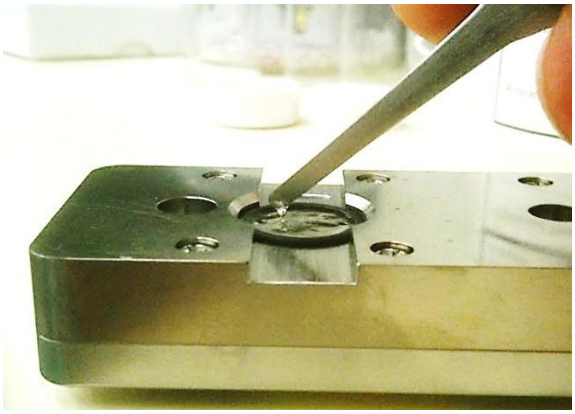

Figure 2.19 Photograph of PVAm-GAL and PVAm-PBA multilayer formation on silica sensor resulting in a hydrogel surface coating

Due to the nearby amine groups on the polymer backbone it is expected that the phenylboronic acid binding to occur at lower pH values than the pKa of PBA (around 9). The pH was adjusted to 7.4 and adsorption of PVAm-PBA on top of the PVAm-GAL

coated surfaces was observed. Decreasing the pH value causes the PBA groups to deionize and switch from anionic tetrahedral structure to trigonal structure. This should result in the PVAm-GAL and PVAm-PBA complexation to fall apart. Therefore pH of the washing buffer was adjusted to 3 which resulted in a rise in the frequency of the system, corresponding to destruction of GAL/PBA bond and desorption from the surface.

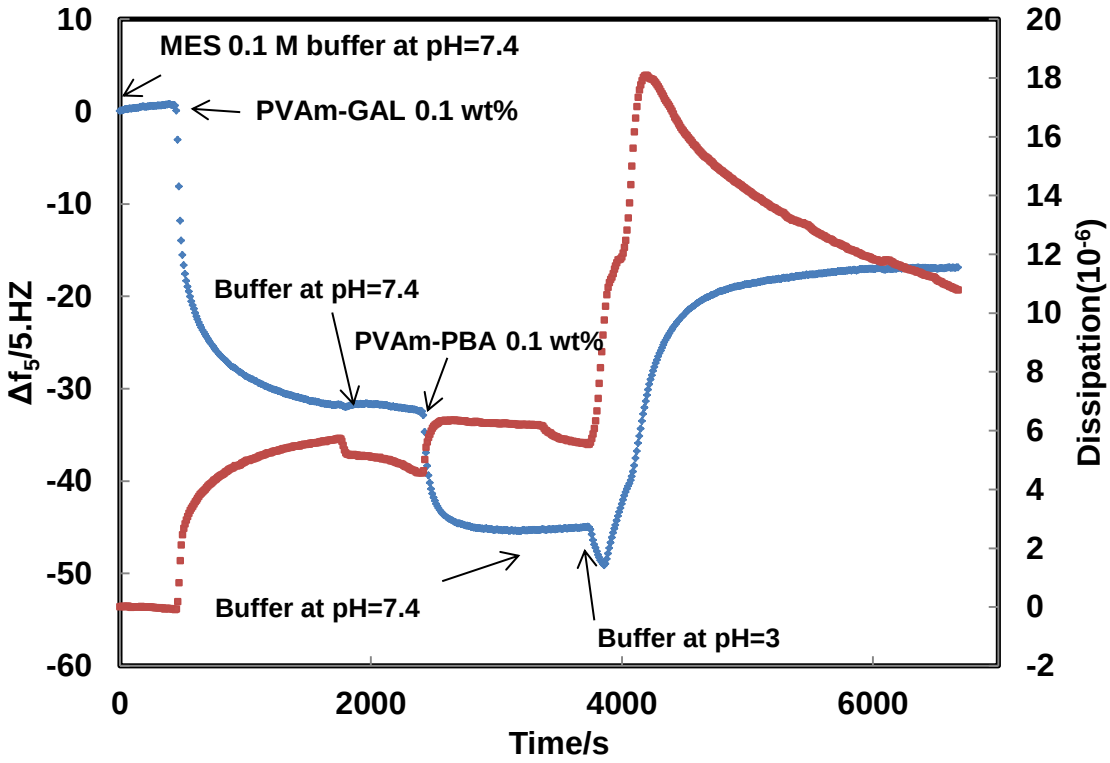

Figure 2.20 Effect of low pH on PVAm-GAL and PVAm-PBA complex

Since the frequency reached a plateau higher than of that the starting point, it is suggested that some of the PVAm-Gal may be washed away from the surface due to the very low pH of the buffer. Therefore, another experiment was designed to reduce the pH value of the buffer in a step wise manner. After passing a buffer at pH=7.4 and observing no desorption, a buffer of pH=6 was used and there was a decrease in frequency, indicating an increase in mass adsorbed. This behavior can be justified by assuming that

the bonds still hold together at pH=6 due to the effect of nearby amine groups. The hydrogel structure formed at the surface expands, allowing more water molecules to be held inside the gel, resulting is an increase in the mass adsorbed. By decreasing the pH to 5 and then to 4, we see desorption of the polymer layer.

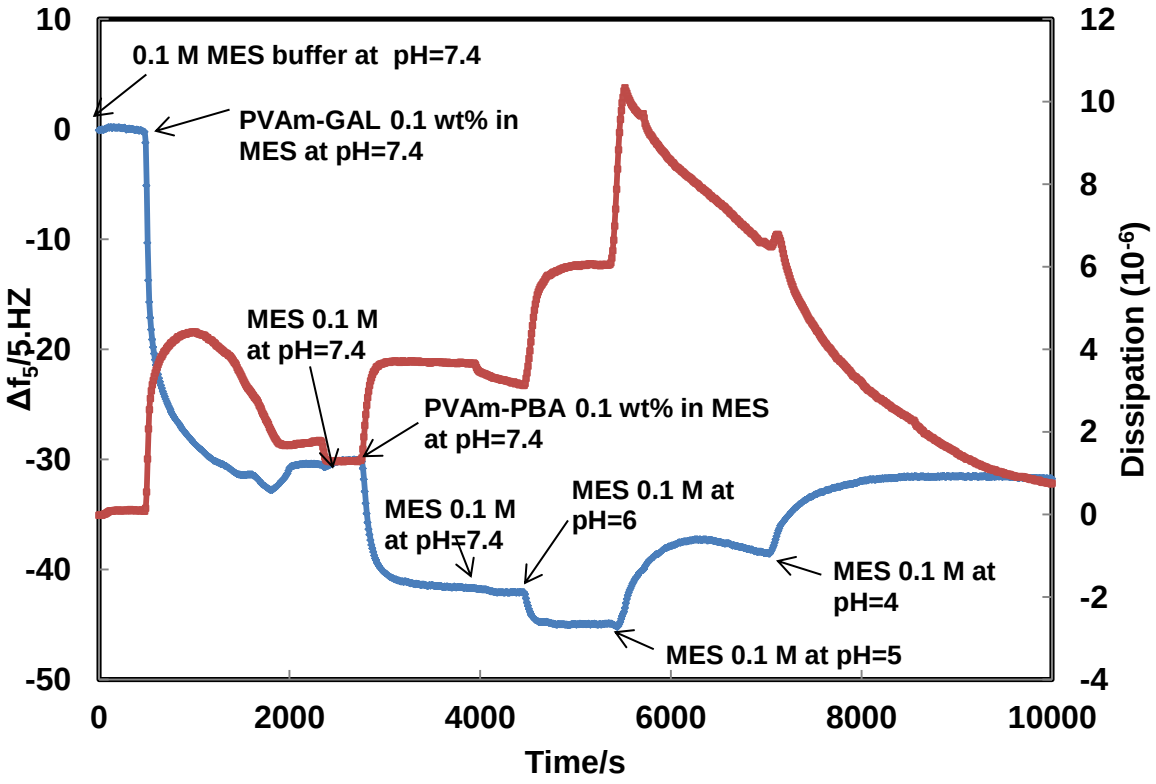

Figure 2.21 Effect of a stepwise decrease of pH on PVAm-GAL and PVAm-PBA complex

To study the gel formation ability of the two polymers in solution, two solutions of 0.3 wt% of PVAm340-GAL2 and PVAm340-PBA were mixed and the pH was increased from 5.5 to 8.5. At the beginning due to the high molecular weight of the polymer the mixture was a viscous solution, by increasing the pH, there was bulk hydrogels formed and the polymers started to coacervate as presented in Figure 2.21. By

lowering the pH from from 8.5 to 4, the gel structure first expanded increasing in size, and then the next day was completely dissolved.

Figure 2.22 Photograph of PVAm-GAL and PVAm-PBA coacervate

2.3.6 Competitive binding of sorbitol and fructose:

Different diols show different affinities toward PBA. Springsteen and Wang have reported the K_{eq} for a number of diols as shown in Table 2 below. Dihydroxyphenyl containing compounds, such as ARS and catechol, show very high affinities for PBA with binding constants (K_{eq}) as high as 1300 and 830 M^{-1} respectively. Compounds such as sorbitol, fructose, mannitol, and sorbose are next in line with K_{eq} values in the range of 110-370 M^{-1}. Compounds such as arabinose, ribose, sialic acid, glucoronic acid, galactose and mannose with K_{eq} in range of 13-30 M^{-1} have moderate affinities for PBA. Finally compounds such as D-glucose, maltose, lactose, and sucrose with K_{eq} values of 0.67-4.6 M^{-1} show weak affinities for PBA (Springsteen & Wang, 2002).

Table 4 Association constants with phenylboronic acid in 0.1 M PBS buffer at pH=7.4 adopted from (Springsteen & Wang, 2002)

Diol	K_{eq} (M^{-1})	Diol	K_{eq} (M^{-1})
Alizarin Red S.	1300	Sialic acid	21
Catechol	830	*cis*-1,2-Cyclopentane diol	20
D-sorbitol	370	Glucoronic acid	16
D-fructose	160	D-galactose	15
D-tagatose	130	D-xylose	14
D-mannitol	120	D-mannose	13
L-sorbose	120	D-glucose	4.6
1,4-Anhydroerythritol	110	Diethyl tartrate	3.7
D-erythronic-γ-lactone	30	Maltose	2.5
L-arabinose	25	Lactose	1.6
D-ribose	24	Sucrose	0.67

Competitive binding experiments were designed to study whether addition of small molecule diols such as D-fructose and D-sorbitol would result in displacement of the adsorbed PVAm-GAL monolayer from the boronate functionalized QCM sensor. As it can be seen from the graph below after introducing a solution of 10 g/L of sorbitol to the system followed by passing of buffer, there is a notable jump in frequency indicating mass desorption from the surface. This is explained by the higher affinity of the boronate silane groups of the surface for the sorbitol molecule as compared to the D-galactose modified PVAm. Association constant of free sorbitol to PBA is 370 M^{-1} which is strong enough to cause displacement of PVAm-GAL from the surface. The dissipation data also confirms a sudden drop associated with a less viscoelastic film on the QCM sensor as the PVAm-GAL layer is removed.

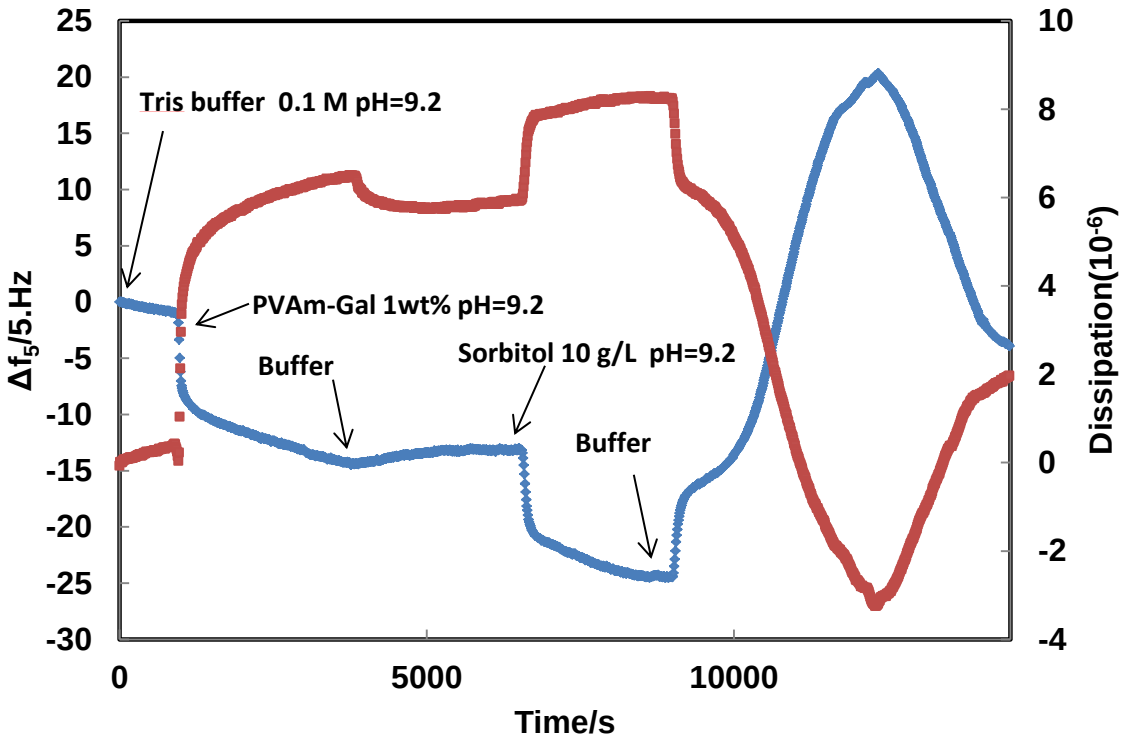

Figure 2.23 Displacement of the bound PVAm-GAL from phenylboronate silica by sorbitol in Tris 0.1 M at Ph=9.2

Same competitive experiments were repeated using D-fructose instead of sorbitol. As it can be seen from Figure 2.23 the high concentration fructose solution did not result in displacement of the adsorbed PVAm-GAL. This indicates that PBA groups on the QCM sensor show higher affinity toward the PVAm-GAL as compared to D-fructose. Free D-fructose diol has an association constant of 160 M^{-1} which is not strong enough to form new complex with PBA.

As D-sorbitol displayed the ability to displace the adsorbed PVAm-GAL, whereas D-fructose failed to do so, it can be concluded that the affinity of PBA towards PVAm-GAL lies between the two small diol molecules. Therefore the strength of polyol complexes of phenylboronate surface and its corresponding ester is as follows: D-sorbitol>PVAm-GAL>D-fructose.

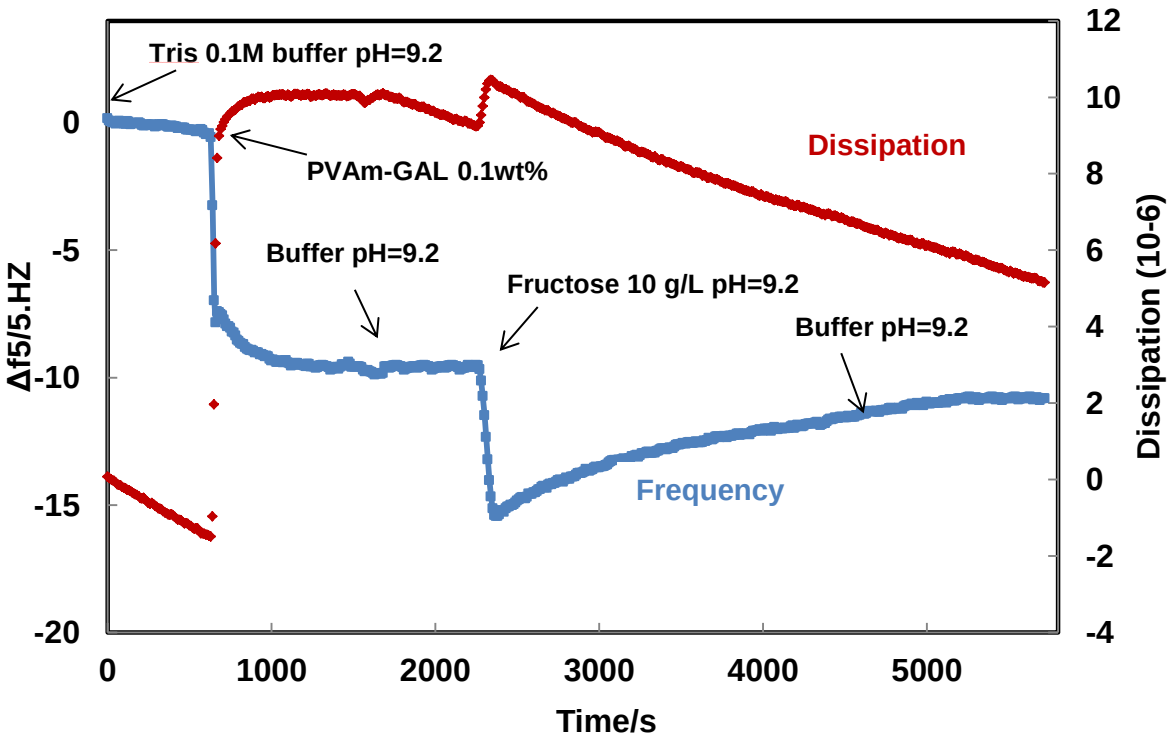

Figure 2.24 Competitive binding of fructose and PVAm340-GAL2 in Tris 0.1 M at pH=9.2

2.4 Conclusions

1) The adsorption of PVAm-GAL onto a phenylboronate derivatized silica surface is enhanced by the condensation of boronate groups with the pendant galactose groups on PVAm-GAL.

2) Much of the adsorbed PVAm-GAL can be removed by destroying GAL/boronic acid bonds either by rinsing at low pH or by sorbitol addition. Whereas the bonding energy of fructose to PBA was too low to displace bound galactose groups.

3) PVAm multilayer structure was assembled by alternating layers of PVAm-GAL and PVAm-PBA. To our knowledge, this is the first reported example of a reversible multilayer hydrogel surface coating based entirely on polyvinylamine.

4) Aqueous mixtures of PVAm-GAL and PVAm-PBA gave bulk hydrogels in the form of coacervate. Lowering the pH destroyed the gel structures.

3. PVAm-GAL a Novel Protein Binding Glycopolymer with Potential Application in Target Delivery to Hepatocyte

3.1 Introduction

3.1.1 The need for polymeric target drug delivery systems

Most drugs are rapidly degraded by enzymatic and metabolic processes and need to be encapsulated for protection against external medium. Polymeric carriers in the form of biodegradable and water-soluble matrices have gained a lot of attention for this purpose recently. The active agent or drug is entrapped within the matrix and is released in a sustained fashion as the polymer is dissolved or degraded or by diffusion through the matrix (Brannon-Peppas, 1995; Finch, 1993). Therefore the polymer acts as a transient mask, protecting the therapeutic agent from physiological degradation until the desirable tissue is reached where the drug is released usually by environmental stimuli.

The motivation behind the target delivery techniques stems from the need to reduce the doses of administrated drugs to eliminate the problems raised by free drug in the body. This is especially critical in the field of cancer therapy where the ultimate goal is to deliver a highly cytotoxic drug to the cancer cells and for only those cells to be destroyed. The use of an excess drug in the body can raise problems related to systemic side effects and bioavailability. In case of conventional direct injection, the drug is rapidly metabolized which results in an exceedingly high drug concentration at the site of action followed by a rapid decreased to sub-therapeutic drug concentration (Uhrich, Cannizzaro, Langer, & Shakesheff, 1999). Therefore, there is a need for controlled drug delivery to achieve effective release of the drugs at the right tissue in order to decrease the damage on healthy tissues.

3.1.2 Ligand-receptor mediated drug delivery

To address the issues discussed above in term of targeting a specific tissue usually the drug carrier surface is decorated with a pilot molecule selected for affinity for the tissue of interest. This results in a selective recognition interaction between the carrier and the receptors available on the surface of the target tissue (Sutton, Nasongkla, Blanco, & Gao, 2007). Upon the complexation of the pilot moiety with the receptor, the carrier is trapped within the tissue where it is supposed to release the drug. Once the ligand has docked with a surface receptor, the internalization process begins. The cell membrane closes around the ligand-receptor complex to form an enclosed vesicle which forms the endosome later on. The endocytosis process continues and the complex then moves to the lysosome, which has a lower pH than endosome and is rich in enzymes, to be digested (Bareford & Swaan, 2007).

Polymeric carriers have emerged as materials of choice due to their ease of functionalization (Freichels et al., 2011) making it possible to modify them with a variety of ligands. Some of the most commonly used pilot moieties are sugars, peptides, antibodies, and folic acid (Fahmy, Fong, Goyal, & Saltzman, 2005; Jagur-Grodzinski, 2009; Shi, Lu, & Shoichet, 2009). Among them sugars are receiving an increasing attention and will be addressed in the next section.

3.1.3 Sugars as targeting moieties

Various sugar binding proteins, i.e. lectins, have been found on the mammalian cell surfaces. Different cell tissues express different types of lectins and when the cells are diseased or cancerous, the type of the glycan arrays expressed on their surface would differ from their normal state (KOYAMA et al., 1993). Sugar specificity of the lectins and their tissue specific distribution inside the body make it possible to design targetable drug carriers by incorporating sugar moieties into the macromolecular drug carrier.

Carbohydrate-protein interactions are expected to be one of the most promising routes in cellular-specific drug targeting. The type of sugar used for labeling the carrier dictates the receptors involved in the recognition process. For example, mannose is one of the sugars used for carrier labeling. Mannose receptors are found plentifully on macrophages, which are involved in the phagocytosis process which causes the uptake, degradation, and eventual clearance of particles from bloodstream (Chellat, Merhi, Moreau, & Yahia, 2005). Since macrophages are host cells of bacteria and parasites, targeting them is a way of treating bacterial infections and inflammatory diseases. Another sugar ligand used commonly for labeling the polymeric carriers is galactose, which will be discussed in detail in the next section.

3.1.4 ASGP-R mediated drug delivery to hepatocytes

D-Galactose is a commonly used targeting ligand, directing to the hepatic cells through binding with the large number of Asialoglycoprotein receptors which are exclusively expressed by liver cells (F. Zhang et al., 2006). The Asialoglycoprotein receptor (ASGP-R), also known as hepatic lectin is predominantly expressed on the surface of mammalian hepatocyte and is in charge of clearance of glycoproteins with galactose and lactose (a disaccharide of galactose and glucose) from the circulation system by receptor-mediated endocytosis (Wu, Nantz, & Zern, 2002) . These receptors can be used in the treatment of diseases associated to liver, i.e., Wilson's disease, hereditary hemochromatosis, and α1-antitrypsin deficiency (Pathak, Vyas, & Gupta, 2008). Moreover, liver is a primary location of tumor metastases, making targeting of ASGP-R for the delivery of antitumor drugs even more intriguing. Galactose binding lectins are overexpressed in various tumor cells (Lahm et al., 2001), which is a motivation for developing targeted systems in cancer therapy.

3.1.5 Targeting hepatocytes using galactosylated materials

Since galactose binding proteins were reported in hepatocyte by Ashwell and Morell (Ashwell & Morell, 1974) a wide range of drug carriers such as proteins and synthetic polymers have been designed and labeled with ASGP-R specific ligands such as galactose, lactose, and acetylgalactosamine. These galactosylated materials were attempted for targeting the liver and successfully accumulated in the liver after injections. Terada et al .developed galactosylated liposomes containing PEGylated groups for selective targeting of hepatocellular carcinoma(Terada, Iwai, Kawakami, Yamashita, & Hashida, 2006). Jeong et al. synthesized diblock copolymer of poly(γ-benzyl l-glutamate)-PEG and conjugated galactose for application of liver specific targeted delivery (Jeong et al., 2005). Sugar conjugated PEG-poly (D,L-lactic acid) (PDLLA) micelles were developed by Kataoka research group and were investigated for their specific interaction with ricinus communis agglutinin lectin (Nagasaki, Yasugi, Yamamoto, Harada, & Kataoka, 2001). Core-shell type polymeric nanoparticles based on diamine-terminated poly(ethylene glycol) (ATPEG) with a galactose moiety from lactobionic acid were synthesized for liver-specific drug delivery systems (I.-S. Kim & Kim, 2002).

3.2 Experimental: Materials and Methods

3.2.1 Materials used

Polyvinylamine (PVAm) with molecular weights of 10, 45, and 340 kDa were provided by BASF with commercial trademark names of Lupamin® 1595, Lupamin® 5095, and Lupamin® 9095 respectively. Lactabionic acid (4-O-ß-D-galactopyranosyl-D-gluconic acid), *N*-(3-(dimethylamino)propyl) -*N*'-ethylcarbodiimide hydrochloride (EDC), and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Sigma Aldrich and used as received. PVAm-GAL polymers were synthesized according to the procedure described in the previous chapter. Lectin from ricinus communis agglutinin with molecular weight of 120 kDa (RCA_{120}), and Concanavalin A lectin (Con A) were also purchased from Sigma Aldrich and stored in the fridge upon arrival. All experiments were performed with water from a Millipore Milli-Q system.

3.2.2 Preparation of polymer and lectin samples

Preparing samples for a typical QCM experiment involved dissolving 10 mg of PVAm-GAL in 0.1 M MES buffer (or other appropriate buffer) and adjusting the pH to the desired value using 0.1 M NaOH and 0.1 M HCl. Lectin solutions were kept refrigerated upon arrival as proteins undergo conformational changes at elevated temperatures. 0.1 mg/ml RCA_{120} solution was prepared in the same buffer after sterile filtration. Table 3 presents the name and basic properties of the protein samples used for the experiments.

Table 5 Properties of proteins used for this study

Name of protein	Abbreviation	Molecular weight (kDa)	Carbohydrate specificity	Isoelectric Point in water at 25 °C
Ricinus communis agglutinin	RCA_{120}	120	β-Galactose	7.5-7.9
Concanavalin A	Con A	102	α-Mannose	4.5-5.5
Bovine serum albumin	BSA	66.5	-	4.7

3.2.3 Sensor surface preparation

SiO_2 sensors were used for the QCM-D studies and they were cleaned using the following protocol prior to use: silica sensors were first treated with UV/ozone for 10 minutes followed by immersing them in a solutions of 2% sodium dodecyl sulfate (SDS) in milli-Q water for 30 minutes at room temperature. Then they were rinsed with milli-Q water and dried with nitrogen gas. Finally they were treated for 10 minutes under UV/ozone light prior to placing them inside the QCM-D modules. Phenylboronate modified sensors were prepared according to the procedure described in previous chapter.

3.2.4 QCM-D measurements

The binding of RCA_{120} lectin to galactose modified PVAm was studied using a quartz crystal microbalance with dissipation (QCM-D) technology from Q-Sense, BiolinScientific. In a typical QCM experiment, the baseline was acquired by flowing a 0.1 M MES buffer solution at pH=6.5 through the chamber. The same MES buffer was used for preparing all the solutions for the experiment. When the frequency shift detected over the time scale of 10 minutes is less than ±1 Hz, it is regarded as a stable baseline. Next step is immobilizing the polymer on the QCM chips. 0.1 wt% galactose-g-PVAm solution with flow rate of 0.150 ml/min was passed over the sensor which is accompanied by a drop in frequency. After achieving a new baseline, buffer solution was passed

through the chamber to remove the loosely bound polymers until a flat line in frequency was observed. Lectin solution with concentration of 1 mg/ml was introduced to the system, followed by another wash step with the MES buffer until another baseline was achieved. The flow rate and the temperature in the flow modules were kept constant at 0.150 ml/min and 25 °C respectively throughout the whole process.

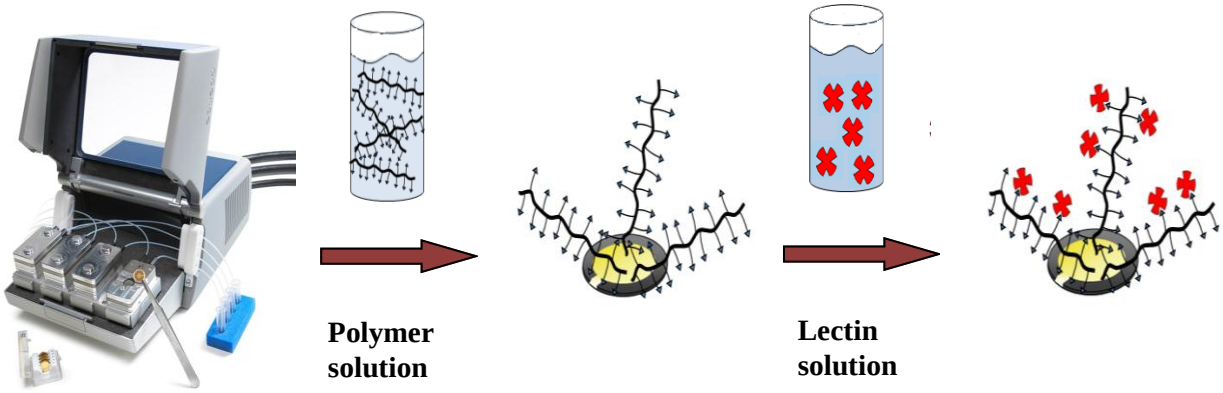

Figure 3.1 Schematic of QCM-D experimental setup

3.3 Results and Discussion

3.3.1 Adsorption of RCA_{120} lection on PVAm-GAL

Lectin from Ricinus Communis Agglutinin (RCA_{120}) is a galactose specific lectin derived from the seed of Ricinus communis, the castor bean plant. RCA_{120} lectin is a tetramer with a molecular weight of 120 kDa. It binds preferentially to oligosaccharides ending in β-galactose but may also interact with N-acetylgalactosamin. Studies on binding of simple sugars to the agglutinin using equilibrium dialysis (Olsnes, 1974), fluorescence polarization (Khan, Mathew, Balaram, & Surolia, 1980), and site directed mutagenesis (Lord, 1995) have shown that each molecule of RCA possesses two identical and independent carbohydrate binding sites.

In this study affinity of RCA_{120} lectin for a galactose modified PVAm was examined using QCM-D. First PVAm-GAL was immobilized onto the silica chip surface using phenylboronate modification. Boronate surfaces were prepared as described in the previous section. After washing off the tartrate groups with a buffer solution at pH of 6.5, PVAm-GAL was adsorbed onto the surface followed by injection of a 1mg/ml solution of RCA_{120} lectin. Lectin injection into the system was associated with a frequency drop up to the point of saturation of the surface. Injection was conducted until there was a baseline achieved in the frequency which in this case was after a near 120 Hz frequency drop. After rinsing with buffer, the frequency remained unchained, indicating the immobilized lectin molecules were firmly attached to the polymer.

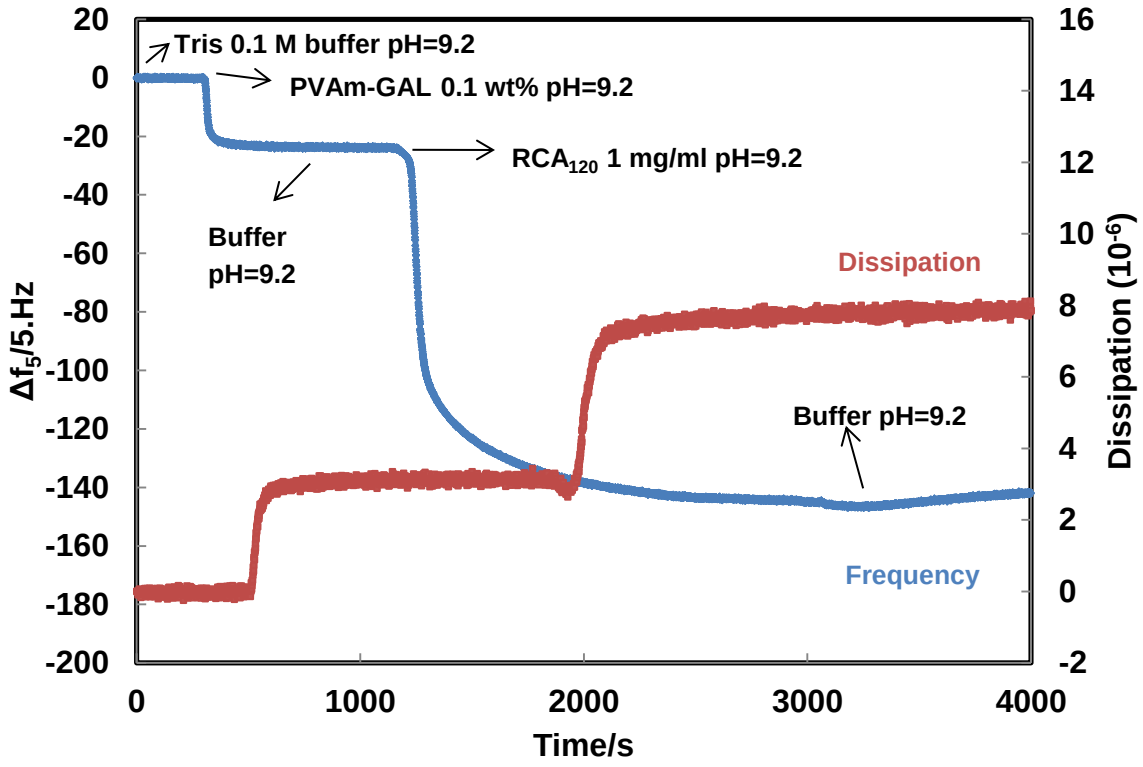

Figure 3.2 Adsorption of RCA_{120} letin on PVAm340-GAL2 studied on phenylboronate modified silica sensors at pH=9.2 and T=25 °C

Biological amphoteric molecules such as proteins contain both acidic and basic functional groups. The net charge of the molecule is affected by the pH of the environment as the amino acids that make up the protein become positive or negative or neutral (Righetti, 2004). The isoelectric point, abbreviated as pI, is the pH value at which the protein has no net electrical charge. At a pH value below the pI, proteins are positively charges and at values above the PI they carry a net negative charge. For RCA_{120} the isoelectric point is in the range of 7.5-7.9 (Karamanska, Mukhopadhyay, Russell, & Field, 2005). PVAm-GAL is a cationic polymer due to its amine groups and it is not completely neutralized at pH=9.2. Therefore, to avoid an electrostatic interaction between the positively charged polymer layer and the negatively charged protein the pH

was adjusted to 6.5, well below the RCA_{120} pI value. There was still adsorption of protein as presented in Figure 3.3. Even though the net charge of the protein is positive below it pI, however there are still negatively charged patches left on the protein which could still result in interaction with the cationic polymer.

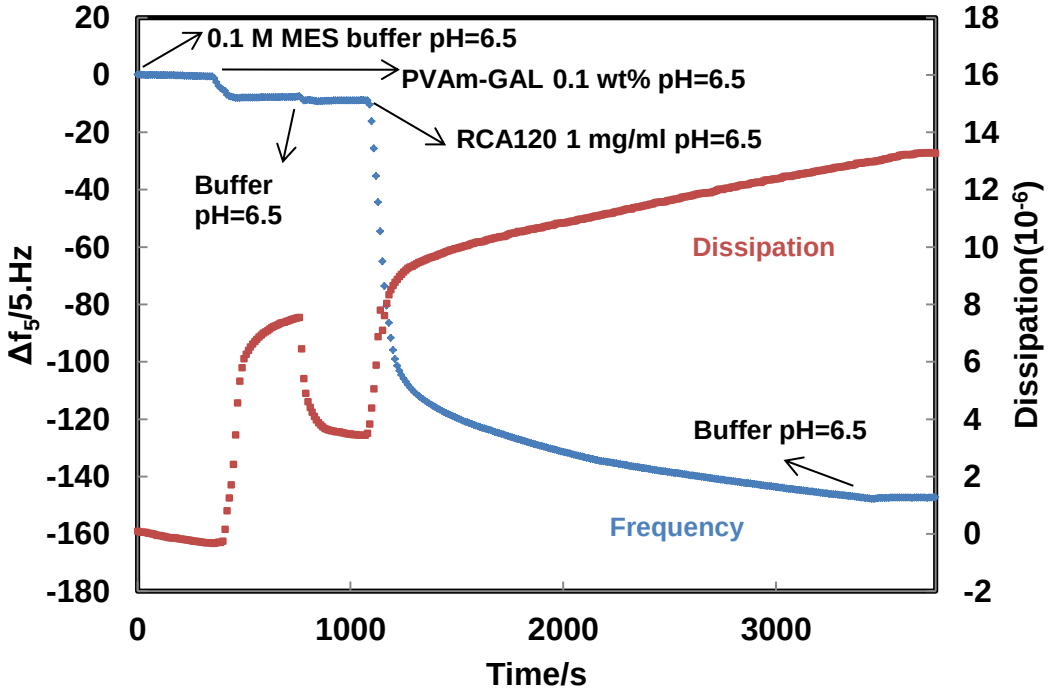

Figure 3.3 Adsorption of RCA_{120} on PVAm340-GAL2 at pH =6.5, below the isoelectric point of the lectin

3.3.2 PVAm-GAL adsorption on unmodified silica

Due to the high content of primary amine functions along the polymer chain, it is expected for PVAm-GAL to adsorb easily on most surfaces. Therefore, even on a bare silica surface with no phenylboronate modification, the polymer showed satisfactory adsorption which did not wash off with passing of the buffer. Therefore a physical adsorption of polymer on the silica sensor was used to avoid a chemical modification step. This provides a facile way of providing galactose modified silica surfaces by coating with PVAm-GAL polymer. QCM-D graphs for adsorption of PVAm-GAL and

PVAm on the unmodified silica chips are presented in Figure 3.4 and Figure 3.5 respectively.

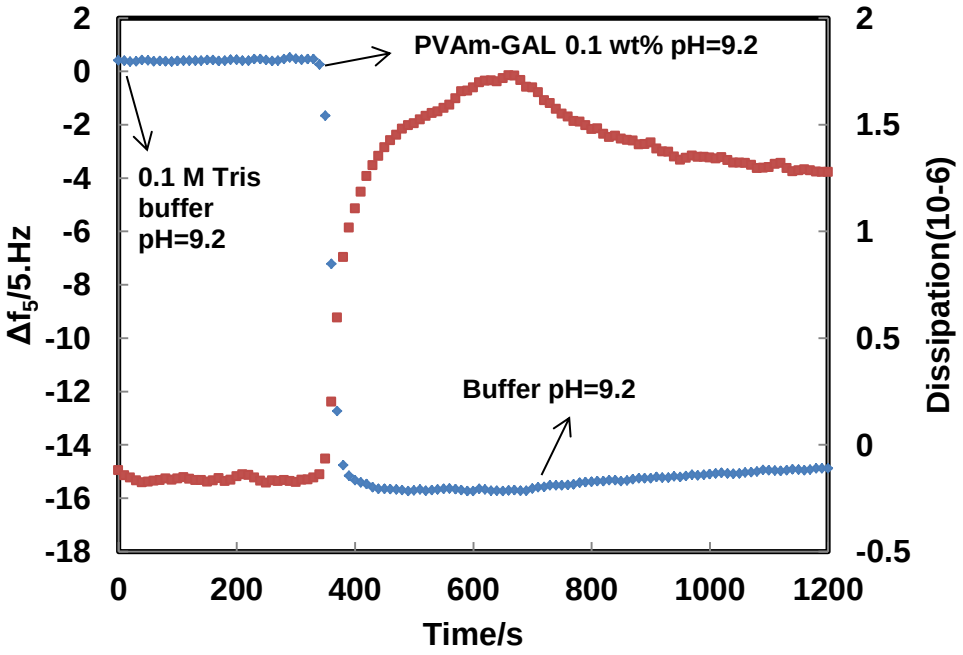

Figure 3.4 Adsorption of PVAm 340-GAL2 on unmodified silica sensor at pH=9.2 in 0.1 M Tris buffer

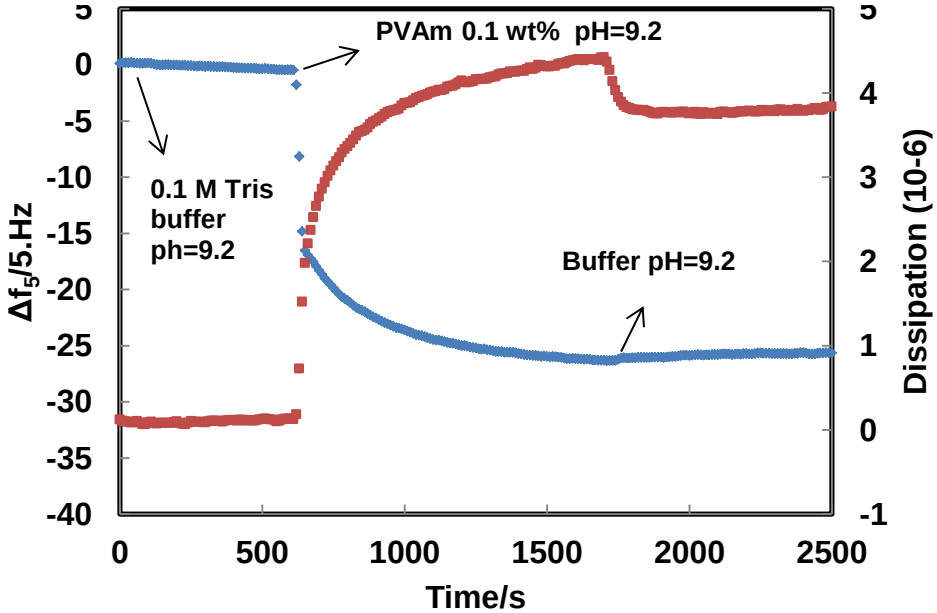

Figure 3.5 Adsorption of PVAm 340 kDa on unmodified silica sensor at pH=9.2 in 0.1 M Tris buffer

3.3.3 Association constant for binding of RCA_{120} to PVAm-GAL

In biomolecular interactions in solution, ligands and receptor form ligand-receptor complexes which can be characterized with an association constant. The binding reaction between PVAm-g-galactose and RCA_{120} lectin can be described in a manner similar to ligand-receptor binding, by assuming that RCA_{120} is a ligand molecule and PVAm-GAL is a receptor immobilized on the silica surface.

3.3.3.1 The Langmuir isotherm

The Langmuir adsorption equation relates the adsorption of molecules on a solid surface to the concentration of the solution above the solid surface. It is the most common isotherm used to model the adsorption processes due to its simplicity and good agreement with a lot the experimental data. The Langmuir isotherm, models the monolayer adsorption process and assumes that adsorption takes place at specific homogeneous sites on the surface, adsorption is reversible, and there is no lateral interaction between adsorbed molecules on the surface (Keles, Volden, & Sjo, 2012).

$$\Delta f = \Delta f_{max} \frac{K_a[Ligand]}{1+K_a[Ligand]} \qquad \text{(Equation 3.1)}$$

Where Δf is the frequency change, Δf_{max} is the frequency change upon saturation, [ligand] is the concentration of a ligand solution, and K_a is an association constant.

This model can be applied to our system by considering the galactose groups on the surface as equivalent binding sites of the same size and shape for adsorption of proteins. The resonant frequency shift of RCA_{120} lectin on PVAm-GAL coated sensors was recorded while increasing the concentrations of the lectin solution. The amount of the adsorbed RCA_{120} lectin increased when increasing the concentration and reaches a plateau at higher concentrations. The obtained dependency of frequency shift on the lectin concentration is plotted in Figure 3.4. It follows the Langmuir-type adsorption

isotherm, making it possible to calculate the association constant for binding of the lectin to the surface.

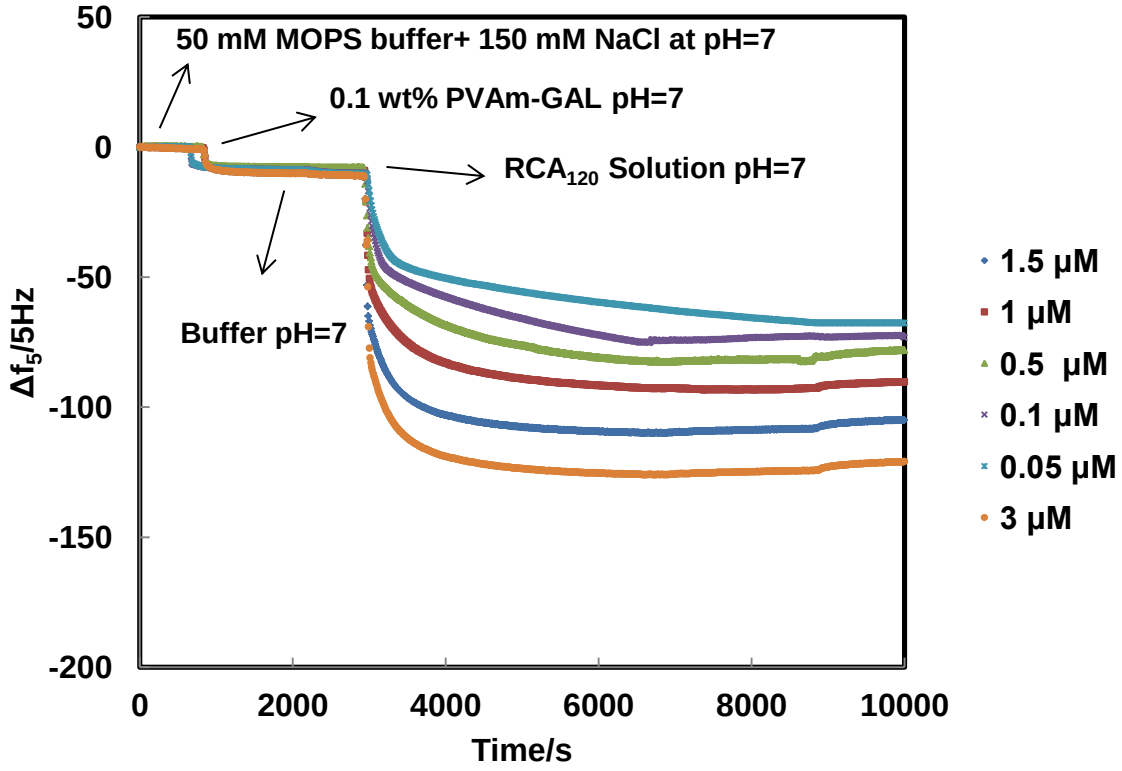

Figure 3.6 Saturation binding experiments for RCA_{120} on PVAm340-GAL2 on silica sensor in 50 mM MOPS buffer at 150 mM NaCl , pH=7 and T= 25 °C

The resulted frequency change (Δf) as function of the RCA_{120} lectin concentration was plotted. To obtain the Langmuir isotherm for adsorption of the RCA_{120} lectin on the PVAm-GAL, the data was fitted using root mean square deviation (rmsd) non-linear fitting.

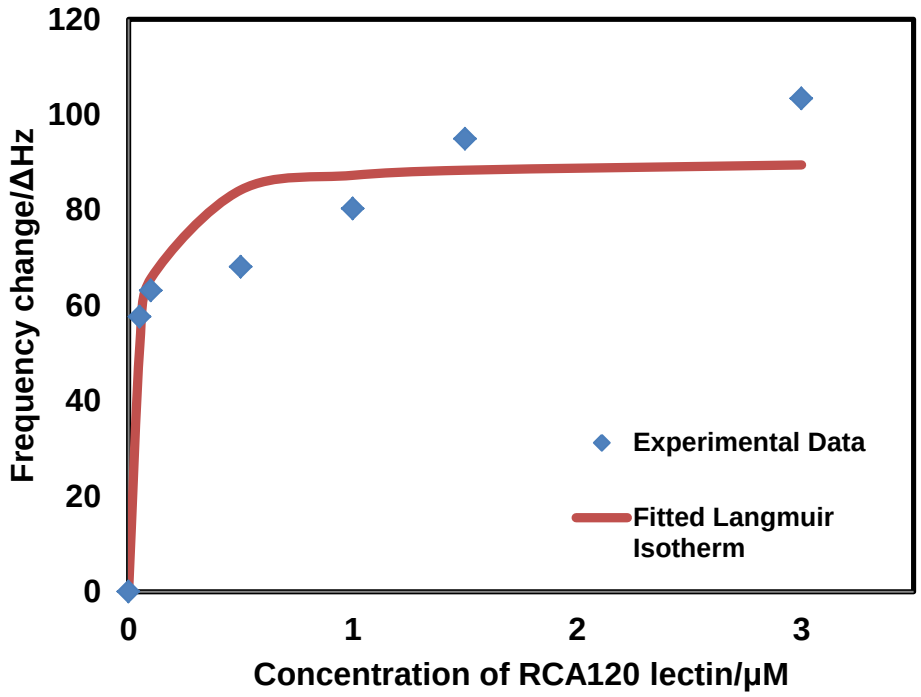

Figure 3.7 Langmuir Isotherm for RCA_{120} adsorption on PVAm340-GAL2 on silica sensor

The graph above provides an estimate of how the adsorption isotherm for this system looks like. However, to be able to use a non-linear fitting to arrive at the actual Langmuir equation, many more data points are needed which is not practical to obtain experimentally. Therefore usually a linear fitting of the experimental data is used. There are two different linear fitting used in literatures for calculating the association constant of ligand-receptor systems which would result in two different plots; the Line-weaver-Burk plot which is a double reciprocal plot, and the Scatchard plot, which is a single reciprocal plot.

The Scatchard plot is the most commonly used method for calculating the association constant for the lectin-carbohydrate system. It is obtained by rearranging the Langmuir equation as below and plotting the [ligand]/Δf as function of ligand concentration.

$$\frac{[ligand]}{\Delta f} = \frac{K_a[ligand]}{\Delta f_{max}} + \frac{1}{\Delta f_{max}} \qquad \text{(Equation 3.2)}$$

Therefore the slope of the graph would be $K_a/ \Delta f_{max}$ and the intercept would be $1/ \Delta f_{max}$.

Figure 3.8 shows the Scatchard plot for binding of RCA_{120} lection to PVAm-GAL coated silica surface. The data fits the Scatchard regression perfectly and the association constant was calculated to be $K_a = 6.266 \times 10^6\ M^{-1}$.

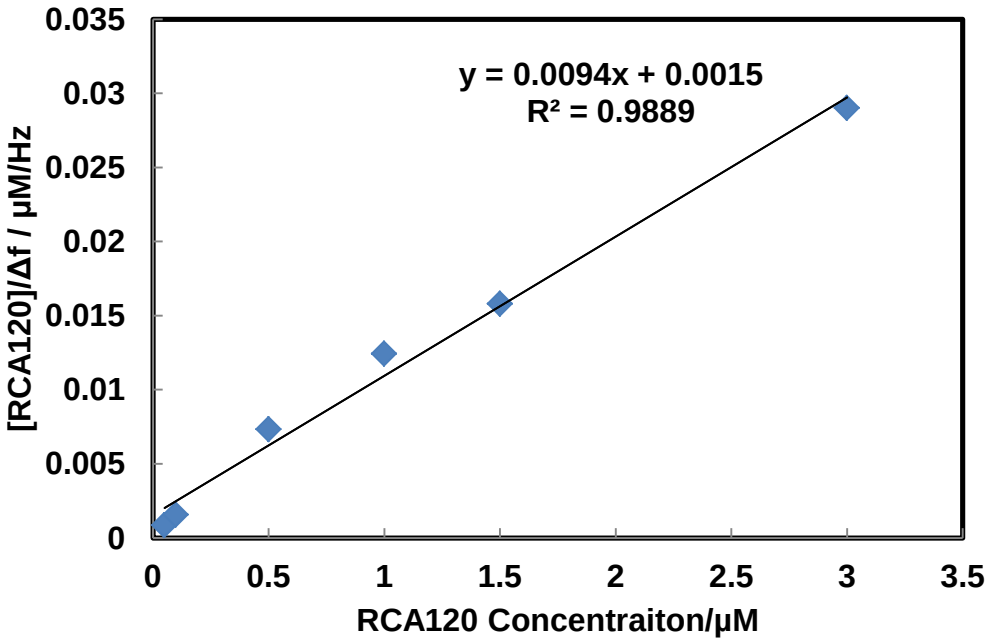

Figure 3.8 Scatchard plot of reciprocal linear regression for calculating the net association constant for adsorption of RCA_{120} on PVAm340-GAL2

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This value for the association constant is reasonable when compared to the literature. Even though the association constant for complexation of RCA_{120} with small sugar derivatives of D-galactose is reported to be $1.\ 2 \times 10^3 M^{-1}$ (Mizukami, Takakura,

Matsunaga, & Kitano, 2008), the association constant for RCA_{120} with glycopolymers is expected to be substantially larger due to the "cluster glycoside effect" which was discussed in previous chapters. The multivalent binding of RCA_{120} to the galactose binding sites of the PVAm-GAL increases the association constant when compared to the monovalent binding of small sugars. In the work of Mizukami et al. the binding on RCA_{120} to a galactose-carrying polymer brush on gold sensor was studied. The association constants for the glycopolymers were reported $4.2 \times 10^6 M^{-1}$ and $8.5 \times 10^6 M^{-1}$ based on their degree of polymerization which is in similar order of magnitude as the association constant for our system.

3.3.4 Non-specific binding

In addition to binding to the receptors of interest, the lectin may also bind to other binding sites available in the system. Binding to receptors of interest, in this case the galactose groups, is called specific binding, while binding to other sites is called nonspecific binding. In this system, the occurrence of two types of non-specific binding is likely; adsorption of lectin on polymer due to the non-specific protein-amine interaction and adsorption of lectin on the sensor due to the nonspecific protein-surface interaction.

3.3.4.1 Non-specific protein-amine binding

Control studies were performed by studying the adsorption of RCA120 lectin on unmodified PVAm. The results indicated that there was still adsorption of lectin on the polymer due to the non-specific binding with the highly active amine groups of the backbone.

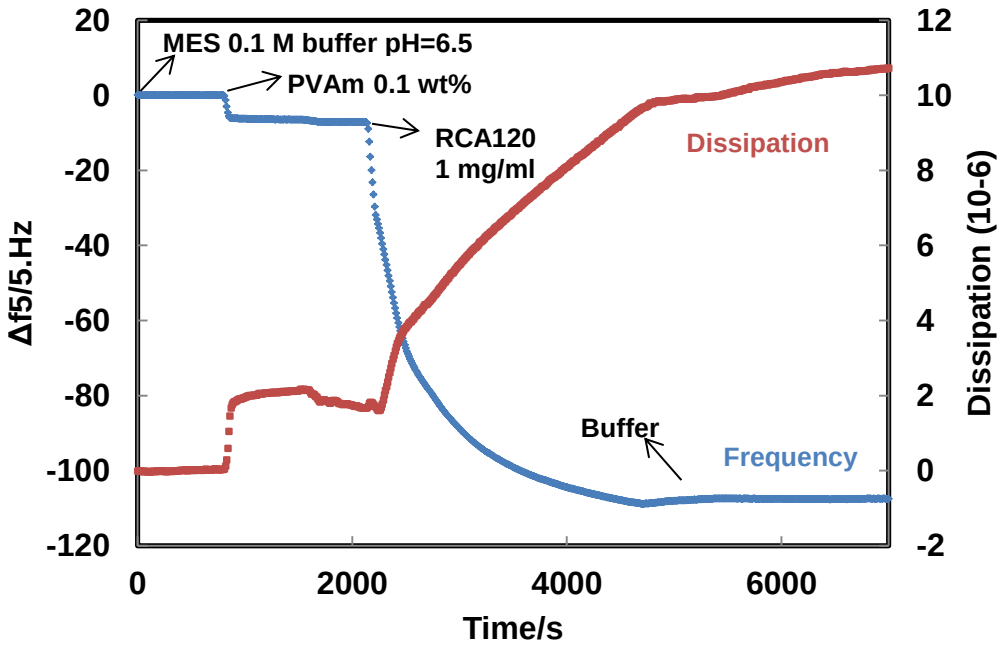

Figure 3.9 RCA_{120} adsorption on PVAm 340 kDa on unmodified silicain MES 0.1 M at pH=6.5 and T=25 °C

However it appears that galactosylation of PVAm enhances the binding with the protein. When two parallel experiments were performed comparing the adsorption behavior of the lectin on unmodified PVAm and PVAm-g-galactose with 48% degree of substitution, there frequency drop for the PVAm-GAL polymer was higher as well as the change in the dissipation of the system. It is expected as the grafted galactose groups create a more viscoelastic system when bounded with the protein as compared to the amine groups on the backbone of the polymer.

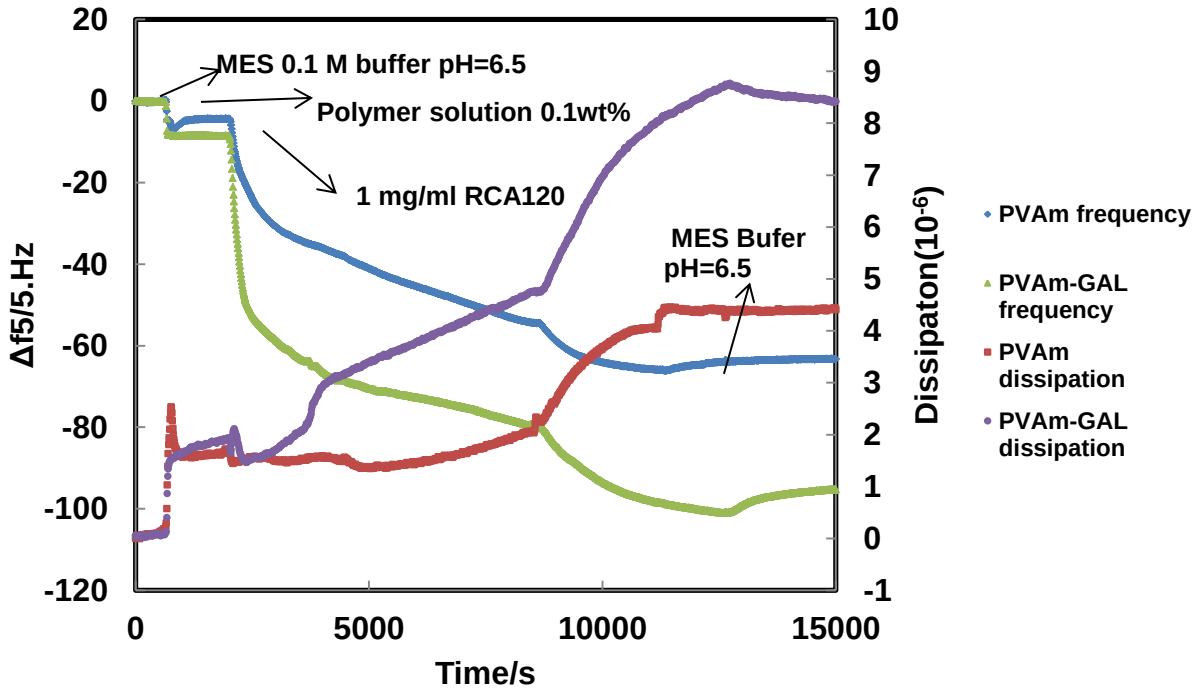

Figure 3.10 Effect of galactosylation of PVAm on adsorption behavior of RCA_{120} lectin observed on silica sensor in 0.1 M MES buffer at pH=6.5 and T=25 °C

Another control study was performed by studying the interaction of a protein that has no affinity for galactose with PVAm-GAL. Concanavalin A (ConA) lectin interacts with receptors containing mannose carbohydrates as it has four binding sites specifically for α-D-mannosyl and α-D-glucosyl residues. Ideally, there should be no interaction between this protein and galactose containing polymer, however, we expected that the amine groups would bind to the ConA lectin due to the nonspecific interaction. QCM-D result is presented in the graph below. The adsorption of ConA however results in less frequency drop as compared to RCA_{120} indicating less adsorption.

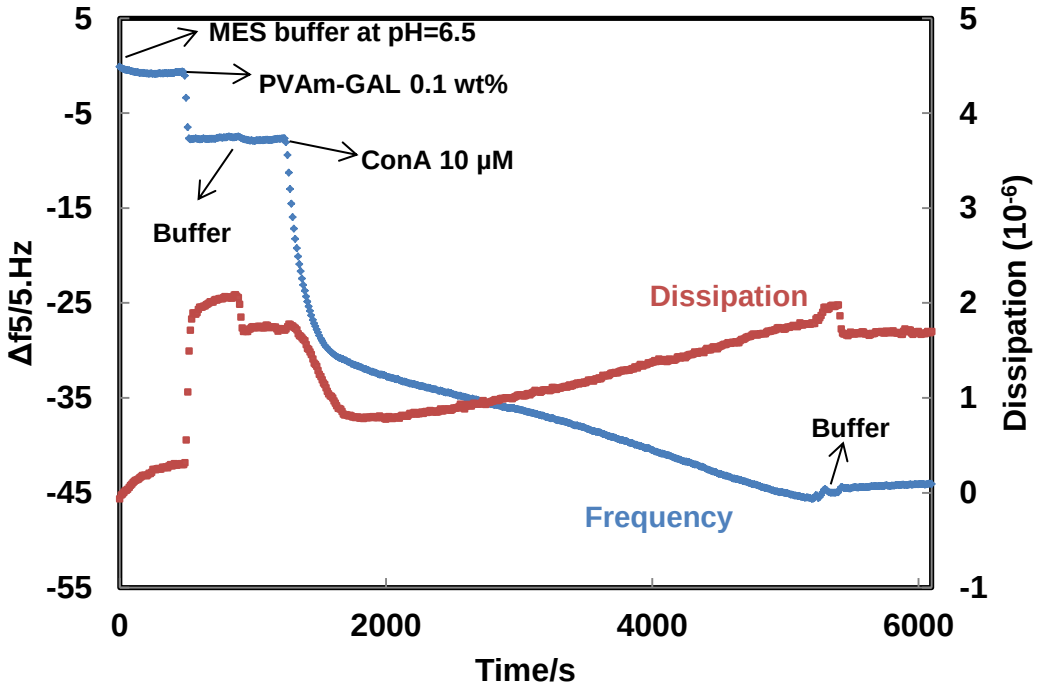

Figure 3.11 Adsorption behavior of ConA lectin on PVAm340-GAL2 observed on silica surface in MES buffer at pH=6.5 and T=25 °C

3.3.4.2 Non-specific protein-surface binding

Even though PVAm-GAL adsorbs onto the silica chip immediately, however there might not be complete coverage of the surfaces, which would result in the nonspecific interaction of protein with the bare patches left on the silica surface. Therefore the adsorption of RCA_{120} lectin on silica surface was studied and the graph below presents the results

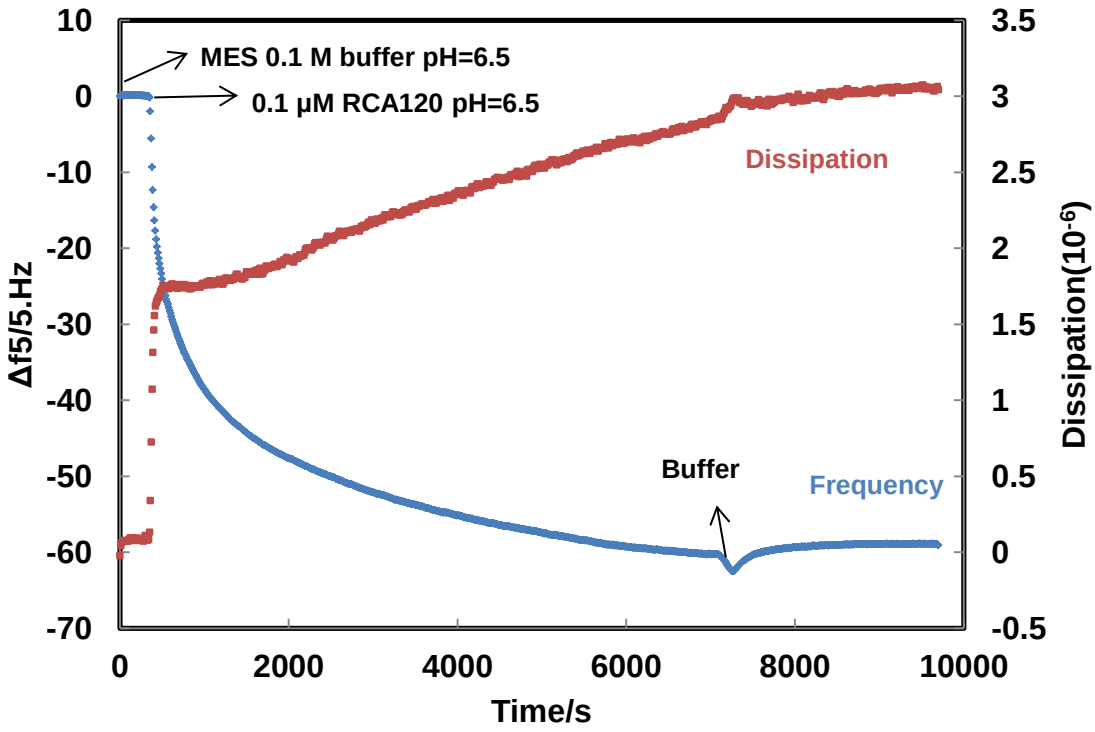

Figure 3.12 Adsorption of RCA_{120} on silica sensor in 0.1 M MES buffer at pH=6.5

RCA_{120} protein shows adsorption on silica sensor indorsing the assumption of nonspecific protein-surface interaction. To address this issue, further study needs to be performed using X-ray photoelectron spectroscopy (XPS) to analyze the elemental composition of the surface of the QCM chips and confirm the bare silica patches remained on the surface after immobilizing the polymer layer.

3.3.5 Blocking nonspecific binding sites

Nonspecific binding can be minimized by saturating the unoccupied binding sites with a blocking agent, without taking active part in specific binding reaction (Sawyer, 1997). A number of chemical and biological reagents are commonly used to block the

nonspecific sites including detergent blockers such as Tween20 and Triton X-100, polymer based blockers such as Polyethylene glycol (PEG), and protein blockers such as milk protein, fish serum, and serum albumin (Reimhult, Petersson, & Krozer, 2008; Steinitz, 2000). Bovine serum albumin (BSA) is the most widely used blocking agent due to its low cost and reduced steric hindrance of specifically binding proteins (Reimhult et al., 2008). It can be used for prevent nonspecific protein-surface binding on both hydrophobic and hydrophilic surfaces, in addition to many preactivated covalent surfaces (Brorson, 1997; Gibbs & Kennebunk, 2001).

BSA with an average molecular weight of 66 kDa belongs to the soft proteins class, which can easily change their structure and conformation. The adsorption of BSA on different surfaces has been widely investigated. On a charged surface, the adsorption is attributed to the electrostatic interaction between the surface and the oppositely charged functional groups of the polypeptide chains of BSA molecule. On a hydrophobic surface, the adsorption is driven by affinity of the nonpolar part of BSA molecule toward the surface. BSA adsorption is restricted to a monolayer, which usually takes less than 2 hours of incubation time to form with concentration of 10mg/ml or less (Giacomelli, Avena, & De Pauli, 1997; Ishiguro et al., 2005). At Higher concentrations of protein or longer adsorption time, as crowding of BSA molecules happens, conformational changes in the monolayer occurs due to the flexible nature of BSA (Militello et al., 2004).

3.3.5.1 Adsorption of RCA_{120} protein on pre-adsorbed BSA layer

To examine the effectiveness of the blocking agent, first silica sensor was coated with a BSA solution with concentration of 10 mg/ml followed by RCA_{120} protein introduced on top of the previous layer. As expected BSA saturated all the binding sites on surface and RCA_{120} did not adsorb on silica. Due to the non-reactive nature of BSA towards other proteins, there was no adsorption of RCA_{120} on BSA either.

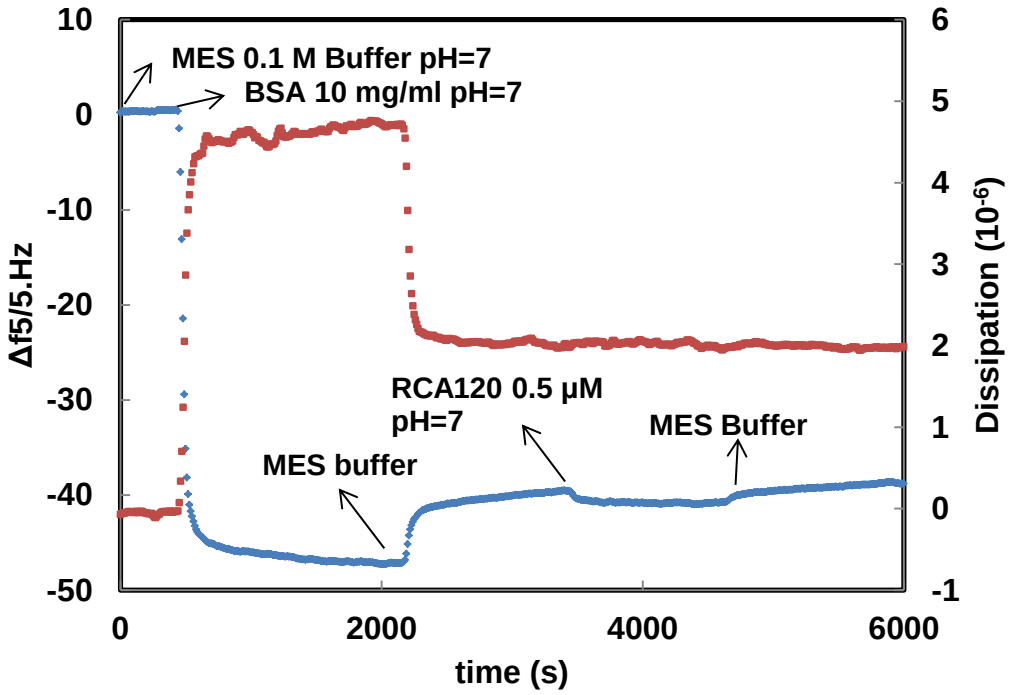

Figure 3.13 Adsorption of RCA_{120} lectin on silica sensor treated with BSA in MES 0.1 M at pH=7 and T= 25 °C

3.3.5.2 RCA_{120} adsorption on PVAm-GAL with BSA pretreatment

PVAm-GAL was coated on silica sensor as described in previous sections, followed by interacting with a 10 mg/ml BSA protein. RCA_{120} protein was then introduced to the system and adsorption behavior was monitored with QCM-D as presented in Figure 3.14.

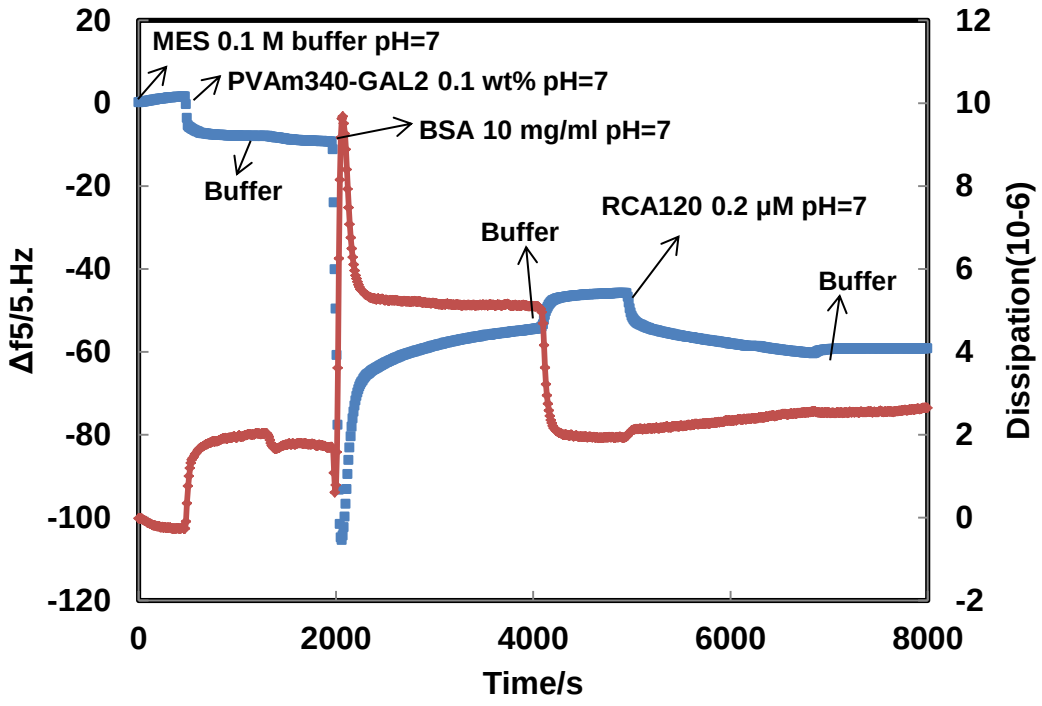

Figure 3.14 RCA_{120} Adsorption on PVAm340-GAL2 with BSA pretreatment in MES 0.1 M buffer at pH=7 and T=25°C

BSA protein adsorbs on the polymer coated sensor, blocking not only the bare patches on the sensor but also the polymer backbone covering the highly active amine groups of PVAm. However, it is observed that the RCA_{120} lectin solution still adsorbs on the sensor resulting in frequency drop in the system. This phenomenon can only be explained in this way that even though BSA protein layer covers the pre-adsorbed polymer layer, it does not cover the galactose pendant groups, making them available for recognition by the RCA_{120} protein. Therefore, the galactose groups protrude above the BSA layer providing binding site for the lectin.

To confirm this theory, a series of control studies were performed. First the same experiment was repeated with unmodified PVAm. We expect that after introducing the

BSA on top of pre-adsorbed PVAm layer there would be no binding sites left as there are no galactose side groups. Therefore introducing the RCA_{120} lectin should result in no change in the frequency or dissipation of the system. Figure 3.15 shows the result for this experiment which is in perfect agreement with our expectation. The RCA_{120} lectin passed over the BSA blocked layer with no adsorption.

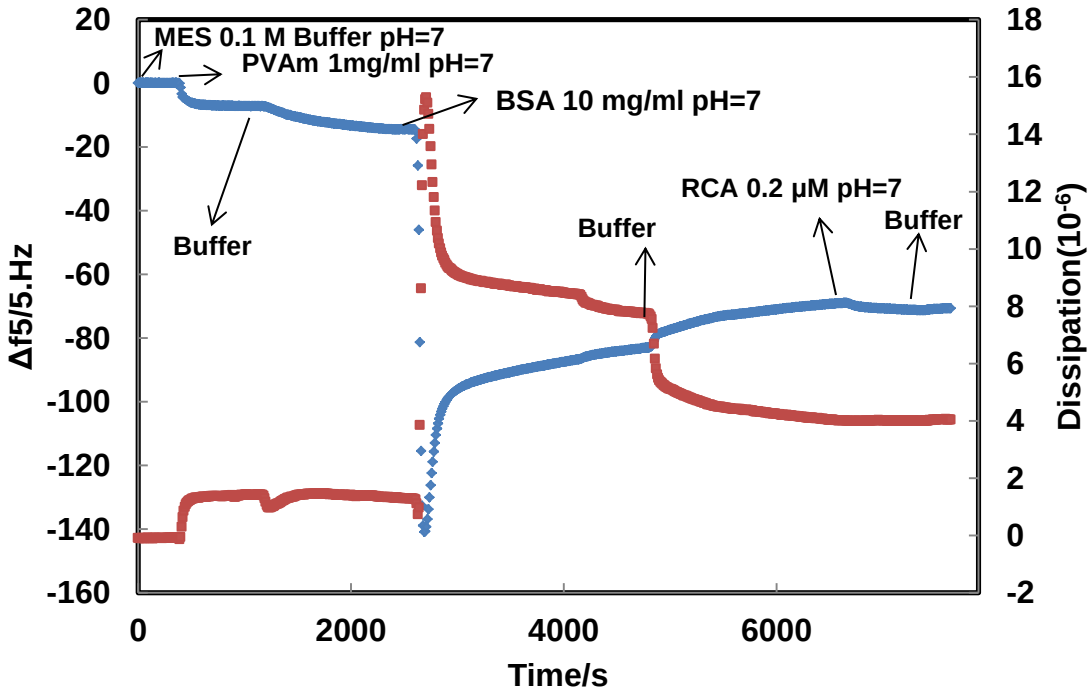

Figure 3.15 RCA_{120} adsorption on PVAm 340 kDa with BSA pretreatment in MES 0.1 M at pH=7 and T=25°C

Another control study was performed using ConA lectin instead of RCA_{120}. After coating the sensor with PVAm-g-galactose and blocking the surface with BSA as described earlier, ConA lectin was passed over the system. Even though the galactose groups are protruding above the BSA layer, providing a binding platform, however, since the ConA lectin is not responsive towards galactose there should be no binding happening. The graph below shows the result for this experiment.

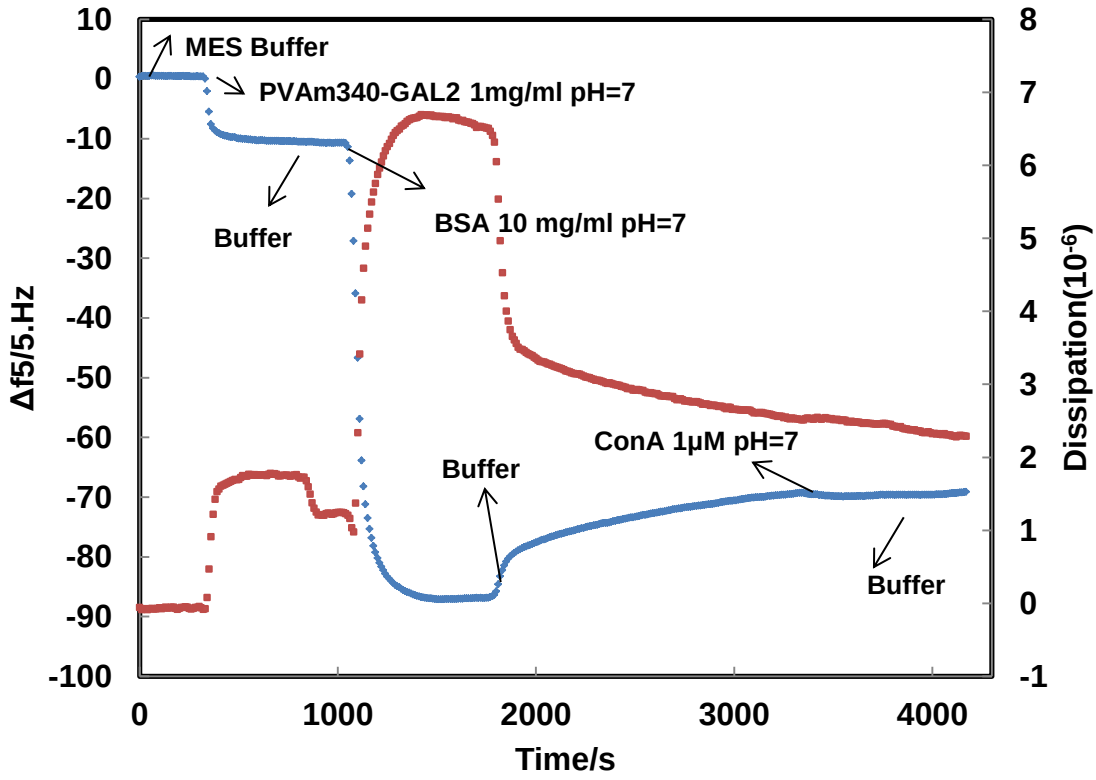

Figure 3.16 Adsorption behavior of ConA lectin on PVAm340-GAL2 with BSA pretreatment in MES 0.1 M at pH=7

Con A, a lectin with mannose binding sites, does not recognize the galactose groups protruding above the BSA and no additional adsorption occurs. This proves that the binding of RCA_{120} to the PVAm-GAL is due to the specific carbohydrate-protein interaction of the lectin with the galactose pendant groups on the polymer.

3.4 Conclusions

1. Stable, galactose-rich surfaces are obtained by the adsorption of PVAm-GAL onto silica.
2. RCA_{120} lectin, a protein with galactose specific binding sites, spontaneously binds to PVAm-GAL adsorbed on silica. The binding constant was calculated $K_a = 6.266 \times 10^6\ M^{-1}$, a value consistent with the literature.
3. Galactosylation improves affinity of RCA_{120} lectin toward PVAm.
4. Pretreatment with BSA prevented non-specific adsorption of RCA_{120} lectin onto silica-supported PVAm-GAL.

4. Preliminary Studies on Complexation of PVAm-GAL with DNA for Gene Therapy applications

4.1 Introduction

Gene therapy is based on the idea of delivering therapeutic genes inside the body in order to correct a disease through the expression or knockdown of a protein. It is usually an important step for studies that involve expression of an exogenous gene in a target cell (Fernandez & Rice, 2009). Currently three different methods of gene delivery are available: viral vector, non-viral vector, and physical vector. Viral gene therapy consists of using replication deficient and genetically modified viruses to deliver oligonucleotides to cells and tissues. This method has the advantage of high transfection efficiency but suffers from severe limitations due to the immunological response to the viral proteins (Glover, Lipps, & Jans, 2005; Jackson, Juranek, & Lipps, 2006). Non-viral delivery strategies on the other hand, use synthetic carriers that are able to package oligonucleotides into nano-sized complexes to facilitate transport inside the body. Non-viral vectors, are less toxic compared to the viral vectors, have more flexibility in the size of the gene to be delivered and have low immunogenicity. The physical methods employ physical force for gene transfer into cells through techniques such as microinjection, gene gun and hydrostatic pressure (Gardlík et al., 2005).

4.1.1 Barriers to gene delivery

After administration of DNA which contains the therapeutic gene, there are a series of complex barriers to overcome for the DNA to be delivered to the target cell and taken up by the nucleus. First of all DNA is rapidly degraded in the extracellular

environment. Therefore, there needs to be a carrier to mask the DNA from degradation upon administration both intravenously (Kawabata, Takakura, & Hashida, 1995) and intramuscularly (Mumper et al., 1996). Second, DNA is a macromolecule which restricts its entry to the cell to an endocytotic pathway. However, since both the DNA and the surface of the cells are negatively charged (each nucleotide in DNA contains a phosphoric acid group and the cell surface is covered with anionic proteoglycans), the association of DNA with the cell is very low which results in a non-efficient endocytotic uptake. The next step after internalization of the DNA by the endosome is to escape the endosomal compartment to prevent trafficking to lysosomal compartments, where DNA is degraded due to the low pH and higher concentrations of enzymes. However, since the DNA is large in size and carries negative charges, the penetration through the negative surface of the endosomal membrane is low. After the endosomal escape, the DNA must diffuse within the intracellular fluid (cytosol) to access nucleus. This diffusion is size-dependent and DNA with larger numbers of base pairs happens to be immobile in the cytosol network. The last step is entry of DNA into nucleus where transcription of gene into mRNA happens (Nishikawa & Huang, 2001; Wiethoff & Middaugh, 2003).

4.1.2 Polymer-mediated gene delivery

4.1.3 Cationic glycopolymers

Due to the less immune and inflammatory response induced by polymers as compared to the viral-based systems, the lower cost of synthesis of polymeric carriers, as well as their larger nucleic acid storage capacity, polymer-mediated gene delivery system has gained a lot of attention recently as an alternative to the viral-based transfection methods (Y. Liu, Wenning, Lynch, & Reineke, 2004). The ever growing need for designing an ideal non-viral gene carrier to overcome the intercellular and extracellular barriers to gene delivery process, has put great emphasis on synthesizing various cationic polymers that are able to efficiently condense DNA. Generally, cationic polymers interact with DNA through electrostatic interactions with its negatively charged phosphoric acid

groups. The resulting complex which is formed through an entropically driven process is a nanometer scale aggregate usually referred to as polyplex. The complexation of anionic DNA with cationic polymers results in neutralization of the complex which improves the efficacy of gene delivery through increasing cellular uptake by the negatively charged plasma membrane. The cationic polymer masks the DNA, protecting it from degradation in the extracellular environment as well as in intracellular fluid. The cationic polyplexes undergo enforced endocytosis due to the electrostatic interaction between the positively charged carrier and the negatively charged host cell surface, thus contributing to higher gene transfer efficiency. Moreover, upon complexation with DNA, the cationic polymer compact DNA into smaller sized particles which aids the movement of DNA in cytosol and into the nucleus. Transfection studies with cationic polymer-DNA complex show much higher gene expression as compared to naked plasmid, suggesting that a positively charged vector promotes the movement of polyplexes inside the nucleus (Pouton & Seymour, 2001).

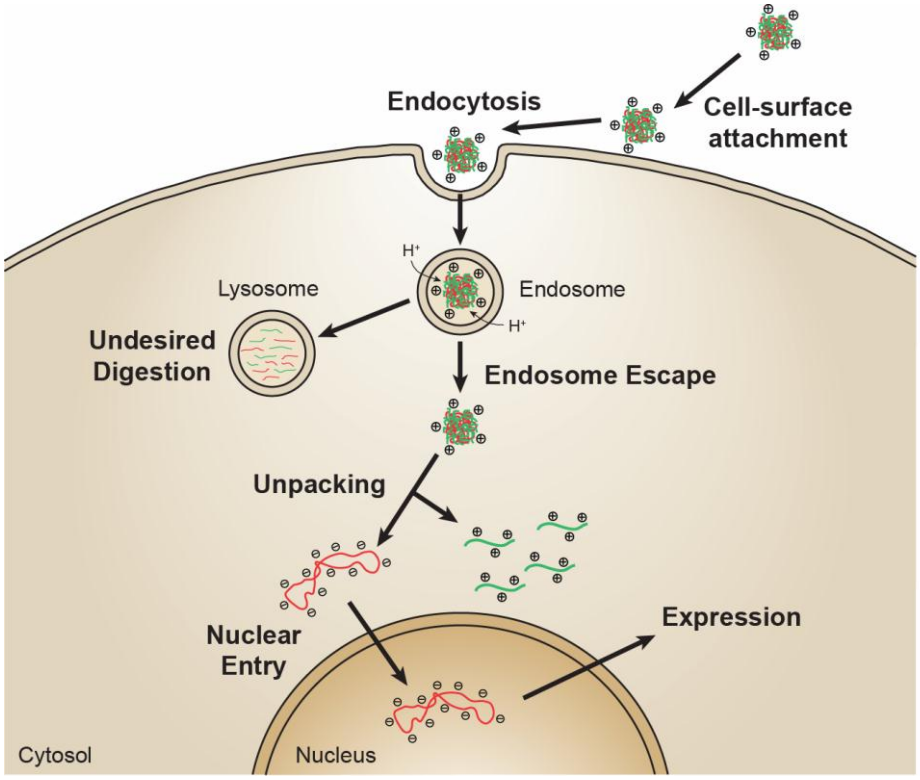

Figure 4.1 Schematic depiction of the cellular delivery of polyplexes (Kohman, 2009)

Even though polyplexes offer a lot of advantages for gene delivery in vitro but their use can be limited by instability of the complexes in physiological conditions. At higher salt concentrations (150 mM or higher), the polyplexes tend to aggregate which results in vascular blockage in vivo. Also they can bind to serum proteins, which results in phagocytosis and rapid clearance from the circulation system. Another problem with polyplex-mediated gene delivery is the "DNA unpackaging". Once the polyplex carrier has transported the DNA to the nucleus of the cell, the cationic polymer has to ultimately release DNA, as the cationic component is no longer required. If the DNA unpackaging happens to be an inefficient process, the polymer component can actually hinder the transfection process by limiting the access of DNA to cellular uptake (X. Liu, Yang, & Lynn, 2008). Some of the most commonly used cationic polymers for the purpose of gene delivery that can enhance the cellular uptake of DNA are polyethylenimine (PEI), poly(L-lysine) (PLL) and chitosan.

4.1.4 Modification of cationic polymers

To improve the efficacy of cationic polymer-based gene delivery carriers, a lot of effort has been put into incorporating new designs into the structure of cationic polymers. The modifications of cationic polymers are focused on reducing aggregations, increasing stability and circulation time, improving biocompatibility, and incorporating targeting moieties to achieve specificity (Harada, Togawa, & Kataoka, 2001; S. Park & Healy, n.d.). The most common modification of cationic polymers is incorporating Poly (ethyleneglycol) (PEG) onto the backbone of the polymer. Conjugation of PEG reduces the toxicity and aggregation of polyplexes, and increases circulation time in blood. PEG chains act as molecular brush and provide steric stabilization. However, it can decrease the surface interaction of the carriers with the cells which can lower the transfection efficiency and can also cause premature unpackaging of DNA from the complex (Mintzer & Simanek, 2009).

Cationic polymers can also be modified with targeting ligands such as asialoglycoproteins, transferrin, antibodies, and carbohydrates. Incorporation of cyclodextrin, a cyclic oligosaccharide, into polyethylenimine (PEI) results in much higher transfection levels compared to those polyplexes with just PEI. However based on the in vivo studies, these

polymers also tend to aggregate at elevated ionic strengths which were overcome by PEGylation of these polyplexes (Popielarski, Mishra, & Davis, 2003). Carbohydrate carrying polymers are widely used to improve gene delivery processes, due to the specific interaction of carbohydrate moieties with proteins on living cells. Galactose is one of the carbohydrates most commonly used as targeting ligand of polymeric gene carriers and will be discussed in the next section.

4.1.5 Liver Specific gene delivery

The specific interaction of galactose with ASGP-R receptor or hepatic lectins on the hepatocyte has been exploited for targeting the liver tissue. Galactose is the most extensively studied ligand recognized by ASGP-R and galactosylated polymers have been widely examines to deliver pharmaceutical agents such as genes to the liver. PEI has been reported to be modified with galactose for in vitro gene delivery to hepatocytes (Zanta, Boussif, Adib, & Behr, 1997) and for delivery of a functional DNA to the liver tissue (Chemin et al., 1998). Various PEI-GAL derivatives with MW ranging from 1800 to 70000 were synthesized and investigated as targetable vector to hepatic lectin and transfection efficiency was found higher in HepG2 cells (Morimoto et al., 2003). Poly(L-ornithine) was labeled with both galactose groups and a fusogenic peptide for complexation with plasmid DNA. These polyplexes were administrated intravenously for targeting the mice hepatocyte. These complexes were reported to be 100-150 nm in size and have higher gene expression compared to non galactosylated carriers (Nishikawa et al., 2000). poly(2-dimethylamino)ethyl methacrylate (DMAEMA)-co-N-vinyl-2-pyrrolidone (NVP) was conjugated with PEG and further galactosylation of PEG terminal ends was carried out. This polymer was complexes with DNA and further coated with KALA peptide and use for target gene delivery to hepatocyte. Transfection efficiency in HepG2 cells showed higher uptake of the DNA bye this polymer (D. W. Lim, Yeom, & Park, 2000). A particular cationic polymer of interest that has been widely investigated for gene delivery to the liver is chitosan, which will be studied in depth in the following chapter.

4.1.5.1 Chitosan for target gene delivery to the liver

Chitosan is a nontoxic, positively charged biodegradable and biocompatible polymer that has been widely investigated for application of gene delivery. It is derived from hydrolysis of chitin, which is a naturally occurring polymer found in crab and shrimp shells. There has been much research on improving the transfection efficiency of chitosan to target hepatocytes. Park and coworkers used coupling of lactobionic acid, which contains a galactose unit, to chitosan for creating liver specificity Galactosylated chitosan (GC) was further grafted with dextran for increasing water solubility which is a big issue with chitosan. Stability of complexes of this polymer with DNA was much higher and they were able to transfect HepG2 cells, indicating specific interaction between the ASGP-R on the cell surface and galactose ligand on chitosan (Y. K. Park et al., 2000) . The same researchers also grafted PEG to galactosylated chitosan and showed that upon complexation with DNA this polymer proved to be effective in protecting against enzymatic degradation as well as transfecting the liver cells(I.K Park et al., 2001). Galactosylated chitosan has been conjugated with Poly(vinyl pyrrolidone) (PVP) which shows better retention in blood. Two different molecular weight (10 and 50 kDa) PVP were tested and the results showed that lower molecular weight has better binding strength due to its higher flexibility attributed to its smaller size. However the low molecular weight showed inferior DNA degradation protection (I.K. Park et al., 2003). Gao et al also grafted lactobionic acid groups to low molecular weight chitosan and studied the effect of galactose content of the polymer on transfection efficiency of pSV-β-galactosidase reporter into HepG2 cells in vitro (Gao et al., 2003). The polyplexes proved to be very efficient in selectively transfecting hepatocyte and efficiency increased with the degree of galactosylation. Thiazolyl blue tetrazolium bromide (MTT) assay was performed on the modified polymers and showed relatively low cytotoxicity, providing evidence that the modified chitosan vector has the potential use as a safe gene delivery system. Kim and coworkers similarly coupled lactobionic bearing galactose groups on water soluble chitosan for hepatocyte specificity and tested transfection efficiency into HepG2 cells (T. H. Kim, Park, Nah, Choi, & Cho, 2004).

4.2 Experimental: Materials and Methods

4.2.1 Materials used

Polyvinylamine (PVAm) with molecular weight of 10 kDa was provided by BASF. Lactobionic acid (4-O-ß-D-galactopyranosyl-D-gluconic acid) was purchased from Sigma Aldrich. Low molecular weight PVAm-GAL (10 kDa) was prepared according to the procedure described in previous chapters. DNA oligonucleotides with 20 and 100 number of sequences were purchased from Integrated DNA Technologies (IDT) and stored in the freezer upon arrival. All samples were made with water from a Millipore Milli-Q system.

4.2.2 Formulation of polyplexes of PVAm-GAL and DNA

PVAm10-GAL2 stock solutions (1-6 mg/ml) were prepared by dissolving the polymer in sterile 100 mM NaCl buffer at pH=7. The DNA sample was dissolved in 1 ml of 100mM NaCl buffer at pH=7 to make the stock solution. PVAm-GAL polyplexes were formulated by drop-wise addition of the DNA solution to the polymer solution and adjusting the total volume to 2 mL with NaCl solution after mixing. The mixture was then vortexed for 30 seconds followed by spinning down using a bench centrifuge. Finally the mixture was incubated at room temperature on a shaker for 30 minutes.

4.2.3 Adjusting the N/P ratio

The physiochemical properties of polyplexes, including the surface charge and particle size is affected by the molar charge ratio of positive amine groups of the polymer to negative phosphate groups of the DNA (N/P ratio). In this study 6 different samples with N/P ratios varying from 1 to 6 were prepared. For all experiments the volume and

concentration of DNA solution was kept constant (100 μL of 0.36 mg/ml) and the desired N/P ratio was obtained by adjusting the concentration of the PVAm-GAL solution.

4.2.4 Dynamic Light scattering measurements

Particle size of the polyplexes were determined using dynamic light scattering (DLS) with a BI-9000 AT digital correlator apparatus from Brookhaven Instruments Corporation. Detection was conducted at 25 °C with a 633 nm light source fixed at a 90° angle and photomultiplier tube detector. The data was analyzed with a BIC TurboCorr digital correlator and software 9kdlsw32 version 3.34. All measurements had an intensity count rate between 60-250 kilocounts per second which is sufficiently large for statistical accuracy but low enough that multiple scattering is avoided.

4.2.5 Electrophoretic mobility measurements

The electrophoretic mobility measurements were made at 25 °C using a ZetaPlus zeta potential analyzer from Brookhaven Instrument Corp in the phase analysis light scattering mode. BIC Pals Zeta Potential Analyzer software version 2.5 was used for analyzing the data. The reported values were based on 3 measurements with 10 cycles for each. Smoluchowski model was used for the conversion of electrophoretic mobility into zeta potential.

4.3 Results and Discussion

4.3.1 Calculation of phosphate mole content and N/P adjustment

The phosphate molar content [$PO4^{3-}$] of DNA was calculated based on the quantity of DNA bases in the sequence as each base carries one mole of phosphate groups with a negative charge. The phosphate content of the T100 DNA sample, containing 100 bases, was calculated to be 0.33×10^{-5} mol/mg. The amine mole content [$NH3^{+}$] of the PVAm10-GAL2 was calculated from titration results to be 0.65×10^{-5} mol/mg. By varying the concentration of PVAm-GAL solution, a series of polyplexes with different N/P ratios were synthesized. Table 3 shows the properties of DNA samples used in this study. A sample calculation is available in Appendix B.

Table 6 Properties of DNA samples used for preparation of PECs

Name of DNA sample	Number of bases	Molecular weight (kDa)	Phosphate content (mol PO_4^{3-}/mg)
T100 DNA	100	30.36	0.33×10^{-5}
T20 DNA	20	6.02	0.34×10^{-7}

Table 7 Properties of PVAm-GAL polymer used for preparation of PECs

Name of polymer	Molecular weight (kDa)	Degree of galactosylation (%)	Amine content (mol NH_3^{+}/mg)
PVAm10-GAL2	10	38.8	$\mathbf{0.65 \times 10^{-5}}$

4.3.2 Physicochemical properties of PVAm-GAL polyplexes

A series of polyplexes with different N/P ratios were synthesized and their size variation was determined using DLS. Figure 4.2 shows the results for effect of N/P molar

ratio on diameter of polyplexes formed. The data suggests an increase in the effective diameter of the particles as the N/P ratio increases. Despite the fact that polyelectrolyte complexes have been around for more than 50 years since first introduced by Fuoss and Sadek (Fuoss & Sadek, 1949), there is no quantitative theory that can be used for predicting the size of these complexes as the dilute solution of oppositely charged polyelectrolytes are mixed together. Increasing the charge ratio in the polyplex implies an increase in the PVAm-GAL concentration in the complex. At higher N/P ratios the excess cationic polymer results in higher nucleation of the soluble components and creates larger aggregates. It is reported that higher amount of cationic polymer in the polyplexes may lead to a higher osmotic pressure in the endosomes and consequently an increase in efficiency of DNA release from the system (Köping-Höggård et al., 2001) .

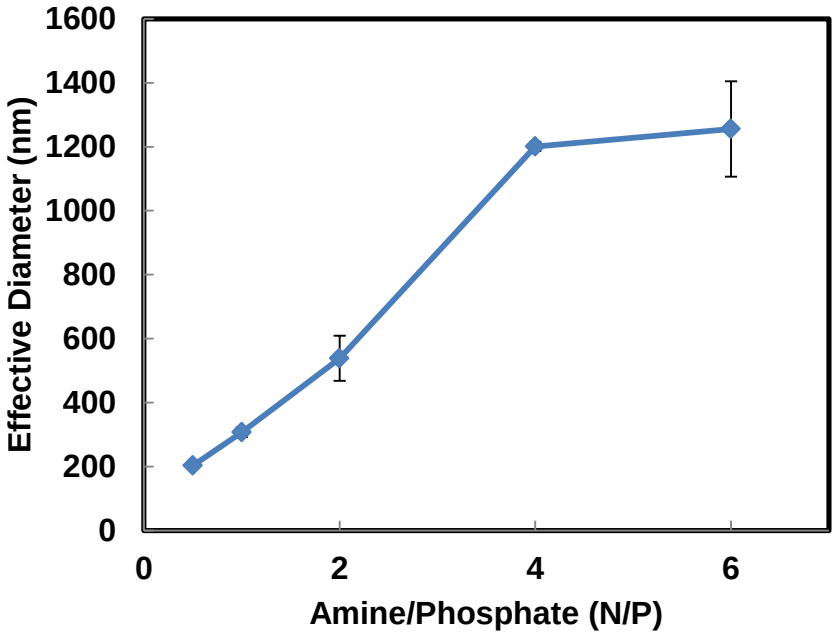

Figure 4.2 Effect of N/P ratio on size of polyplexes of PVAm10-GAL2 and T100 DNA at pH=7 and T=25 °C in 0.1M NaCl.

The surface charge of the polyplexes depend on the mixing stoichiometry of PVAm-GAL to DNA. The surface charge of the particles is important since it influence the ability of polyplexes to interact with cells which have a negatively charged

membrane(Huang, Fong, Khor, & Lim, 2005). Polyplexes with a neutral surface charge tend to aggregate due to the lack on inter-particular repulsive force (De Smedt, Demeester, & Hennink, 2000). For chitosan, there is an optimal range for the N/P ratio since too low ratio will result in physically unstable complexes and poor transfection, whereas a N/P ratio that is too high may yield overly stable complexes which show reduced transfection (Köping-Höggård, Mel'nikova, Vårum, Lindman, & Artursson, 2003; Sato, Ishii, & Okahata, 2001)

Table 8 Effect of N/P ratio on mean mobility and zeta potential of polyplexes of PVAm10-GAL2 and T100 DNA in 100 mM NaCl at pH=7 and T= 25 °C.

N/P	Mean Mobility	ǂ Mean Zeta Potential
0.5	-2.59 ± 0.14	-33.24 ± 1.85
1	-3.16 ± 0.13	-40.46 ± 1.75
2	-2.84 ± 0.15	-36.41 ± 1.87
3	1.96 ± 0.19	25.19 ± 2.43
4	1.92 ± 0.14	24.51 ± 1.90
6	2.13 ± 0.15	28.24 ± 1.97

ǂ Smoluchowski model was used for calculation of zeta potential values.

Table 6 shows the results of electrophoretic mobility of PVAm-GAL polyplexes as the N/P molar ratio increases. At low N/P ratio, in the excess DNA region, the electrophoretic mobility values are negative. As the molar ratio of cationic polymer to DNA increases, the mean mobility of the particles as well as their zeta potential values switch from negative to positive. At high N/P ratio, in the excess PVAm-GAL region, the polyplexes are positively charged.

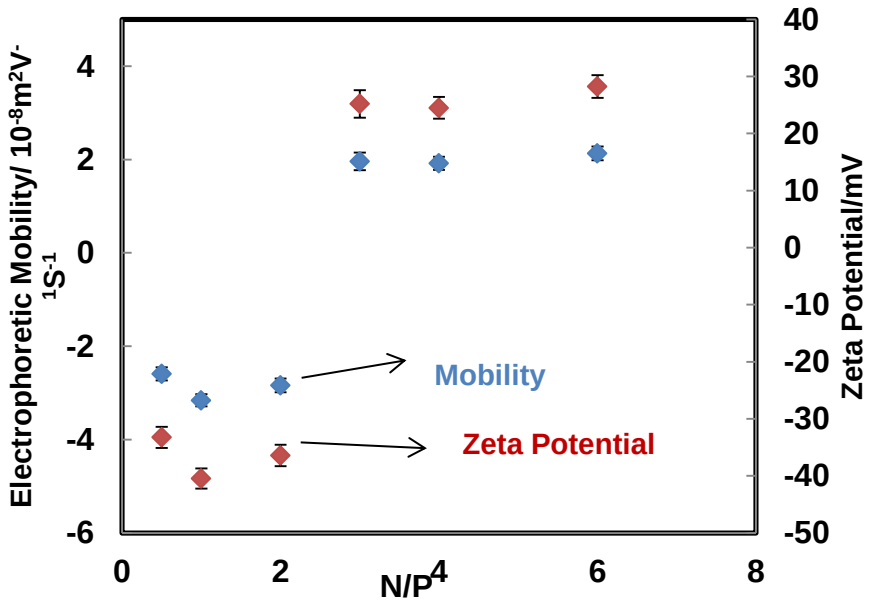

Figure 4.3 Effect of N/P ratio on electrophoretic mobility and zeta potential of polyplexes of PVAm10-GAL2 and T100 DNA at pH=7 and T=25 °C in MOPS 50 mM and NaCl 100 mM

Similar experiments were repeated with a DNA sample that consisted of 20 bases of repeating T sequences (T20) to examine the effect of the DNA chain length on the physiochemical properties of the polyplexes. The phosphate mole content for this DNA was calculated to be 3.3833×10^{-6}.

Table 9 Effect of N/P ratio on size of polyplexes of PVAm10-GAL2 and T20 DNA in 100 mM NaCl at pH=7 and T=25 °C

N/P	Effective Diameter (nm)	Poly Dispersity
0.5	432.6	0.236
1	456.1	0.284
2	467.2	0.272
4	535.6	0.231
6	498.2	0.221

The effective diameter of the polyplexes shows slight increase as the molar content of PVAm-GAL is increased. However the changes in diameter are not as significant as compared to T100 DNA. Higher molecular weight of either polymer in complexation may result in an increase in size of particles. There have been reports for chitosan polyplexes where a decrease in the size of the complexes was observed as the molecular weight of the chitosan decreased (MacLaughlin et al., 1998). However size of these complexes is highly dependent on reaction procedure such as order of addition and method of mixing.

Electrophoretic mobility results for complexes based on the T20 DNA are presented in table 8. Results are consistent with those of T100 DNA sample tested previously. The low N/P creates a DNA rich system causing the particles to have negative surface charges. At N/P around 3 the surface charge switches and at higher molar ratios the complexes become positively charged.

Table 10 Effect of N/P ratio on size of polyplexes of PVAm10-GAL2 and T20 DNA in 100 mM NaCl , at pH=7 and T=25 °C

N/P	Mean Mobility	Mean Zeta Potential
0.5	-1.20 ± 0.09	-15.36 ± 1.11
1	-0.67 ± 0.08	-8.61 ± 1.15
2	-0.74 ± 0.07	-9.53 ± 0.94
4	1.78 ± 0.10	22.78 ± 1.30
6	1.13 ± 0.09	14.51 ± 1.10

In comparison with many polyelectrolytes, PVAm-GAL and oligonucleotides are very hydrophilic polymers. Therefore, it is expected that their complexes to have hydrogel characteristics. The water content of these particles affects their

physicochemical properties and it would be of importance to be measured for future studies.

4.3.3 Cytotoxicity studies

4.3.3.1 MTT assay

For practical use in vivo, PVAm-GAL polymer must not induce significant cytotoxicity to the body. To determine the effects of the polymer on cellular bodies, an in vitro thiazolyl blue tetrazolium bromide (MTT) assay was used. This assay is a metabolic process in which the concentration of the purple metabolite of the MTT dye can be related to the level of cell metabolism and thus the total number of viable cells. For these assays, Human Hepatocellular Carcinoma Cells (HEP-G2) were used.

"Complete" Eagle's Minimum Essential Medium (EMEM) (ATCC # 30-2003) was used as the proliferation media and was prepared by adding fetal bovine serum (FBS) (ATCC # 30-2020) to a final concentration of 10% to the base EMEM medium.

A detailed procedure for cell splitting and culturing is available in Appendix C. Tests were conducted in polystyrene 24 well plates ($2cm^2$) containing HEP-G2 cells at a concentration of **50,000** cells and 1 mL of the media per well. Cells were allowed to adhere for 24 hours.

Solutions of the unmodified and galactose modified PVAm polymer with two different molecular weights of 10 kDa and 340 kDa were made at the concentration of 2mg/mL and were sterilized using 0.2 μm for 10 kDa polymers and 1.2 μm for 340 kDa polymers. Cells were exposed to 1mL per well of the sterilized polymer solutions diluted with the media to the concentrations of 100, 250, 500, 750 and 1000μg/mL for 24 hours. A blank well containing no cells and wells containing cells but no polymer solutions were

used as controls. Experiments were replicated 3 times each to ensure consistent behavior among the cell response, with the error bars representing the standard deviation of the measurements.

The MTT stock solution (3mL) was reconstituted in PBS and sterile filtered. The concentration was 40mg/mL, prepared in l0 mM PBS. When applied to cells, the solution was diluted to 2.5 mg/mL with EMEM medium. After 24 hour exposure to polymers, cells were exposed to 100 µL of MTT solution and incubated for 24 hours. After the incubation, the formazan precipitate was solubilized by adding 200 µL DMSO to each well and shaking the plates for 10-20 minutes or until formazan was dissolved. 200 µL of the resulting solution was transferred to a 96 well plate and read in a micro-plate reader (Biorad, Model 550) at 570nm.

4.3.3.2 Cell Viability

Cell Viability was measured as a function of formazan absorption at 570nm compared to a blank well, in which no polymer was exposed to cells. Absorbance due to the plate and medium itself was used as a control. Cell viability was calculated based on the following equation:

$$Cell\ viability\ (\%) = \frac{Absorbance_{\ Polymer\ solution}}{Absorbance_{\ Blank}}$$

Figure 4.4 shows the percent cell viability of low molecular weight PVAm (10 kDa) with different extent of galactose modification. No significant cytotoxicity was observed specially at low concentrations of the polymer. Results indicate that the low molecular weight PVAm-GAL can be potentially used in vivo for treatment of liver diseases. Moreover galactosylation seems to slightly improve biocompatibility of PVAm as the percent viability is higher for PVAm-GAL polymers compared to unmodified PVAm.

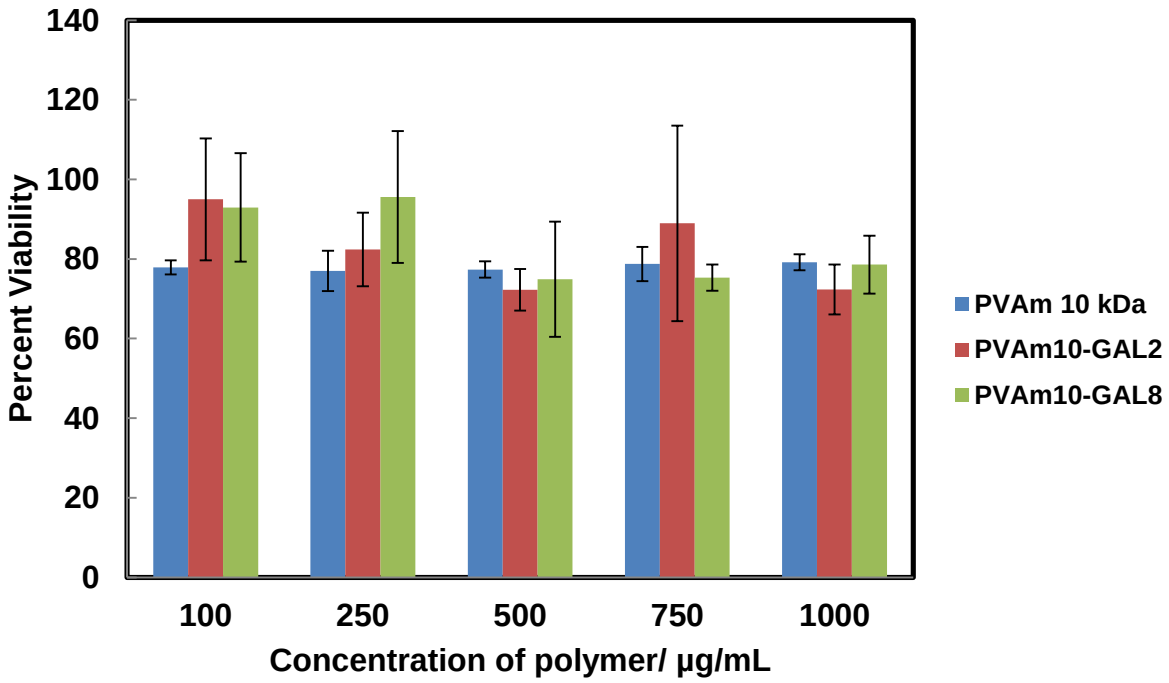

Figure 4.4 Percent cell viability of HepG2 cells exposed to 10 kDa PVAm with different sugar content

Figure 4.5 shows the MTT results for unmodified and galactosylated PVAm with high molecular weight (340 kDa). Cell viability is higher compared to low molecular weight PVAm. In a number of cases there is further growth of the cells after exposing to the polymer resulting in values higher than 100%. This could be due to the penetration of low M.W. polymers inside the cells whereas the high M.W. polymers are restricted to the surface of the cells. PVAm340-GAL2 shows no significant cytotoxicity.

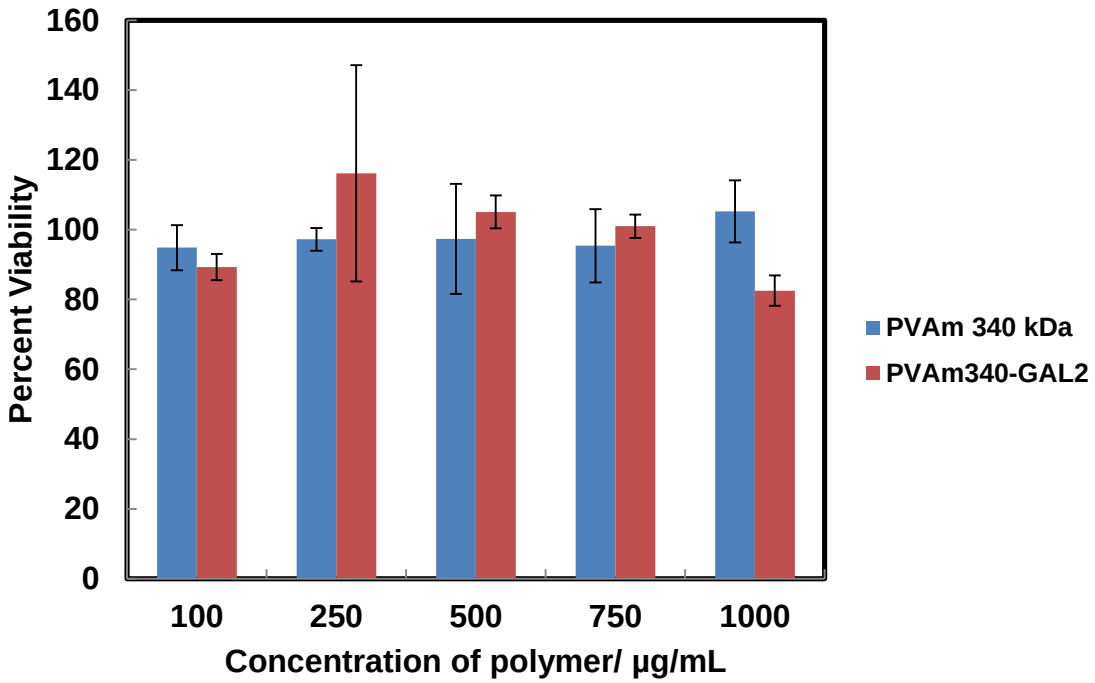

Figure 4.5 Percent cell viability of HepG2 cells exposed to PVAm 340 kDa and PVAm340-GAL2

4.4 Conclusions

1. Low molecular weight PVAm-GAL shows ability to form polyelectrolyte complexes with DNA.
2. Physicochemical properties of PVAm-GAL polyelectrolyte complexes with DNA such as surface charge and particle size is dependent on the N/P molar ratio.
3. Further studies on effect of pH, ionic strength, and molecular weight of PVAm-GAL and DNA on physicochemical properties of polyplexes needs to be carried out for optimization of size and charge of particles for gene delivery purposes.
4. Low molecular weight PVAm-GAL shows low cytotoxicity. Cell viability was determined to be high after exposing HepG2 cells to the polymer. Galactosylation improves biocompatibility of PVAm.

5. PVAm-GAL as Coating for Cell Encapsulation Carriers

5.1 Introduction

5.1.1 Cell encapsulation

Cell encapsulation is a process by which living cells or other biologically active compounds are entrapped within semipermeable membranes for effective protection of the immobilized material for the purpose of biomedical applications (Hubner, 2007; Torre, Faustini, Attilio, & Vigo, 2007). Cell therapy treatments involve the replacement of repair of damaged cells by means of transplantation of therapeutically designed human or animal cells. Cell transplantation is used for various diseases where the host body shows inability to produce the required amount of an essential molecule such as a hormone or an enzyme. The capsule used for entrapment of cells plays a crucial role as it protects the transplanted cells from the host's immune system by providing physical isolation while still permitting metabolic exchange and release of therapeutic proteins (F. Lim & Sun, 1980). In addition of being stable over extend time periods, the material for construction of the immunoisolating capsule should be sufficiently permeable to allow nutrients to enter and therapeutic proteins to escape from the system. The capsule needs to be compatible with both host and implanted cells and not degrade in vivo.

5.1.2 Alginate microcapsules

Various synthetic and natural materials have been used for cell encapsulation including poly(glycolic acid) (PGA) (B. S. Kim & Mooney, 1998), agarose (Shoichet, Li, White, & Winn, 1996), polyacrylates (Li, 1998) and alginate (Martinsen, Storrø, & Skjårk-Braek, 1992), among which alginate is the most commonly investigated. Alginate is a natural polysaccharide in the family of linear binary copolymers of glycosidically linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues. A schematic depiction of structure of alginate is available in

Appendix D. Alginate possesses the striking ability to form hydrogels rapidly and under physiological conditions by ionic crosslinking of G-rich regions in the presence of multivalent cations such as Ca^{2+}, Ba^{2+} and Sr^{2+} (Smidsrod, 1974). The mechanism of binding is usually explained as an "egg box" model (Grant, Morris, Rees, Smith, & Thom, 1973; Sikorski, Mo, Skjåk-Braek, & Stokke, 2007) as the diaxially linked G regions form cavities which is the binding site for ions, similar to eggs in an egg box. Figure 5.1shows a schematic depiction of this model.

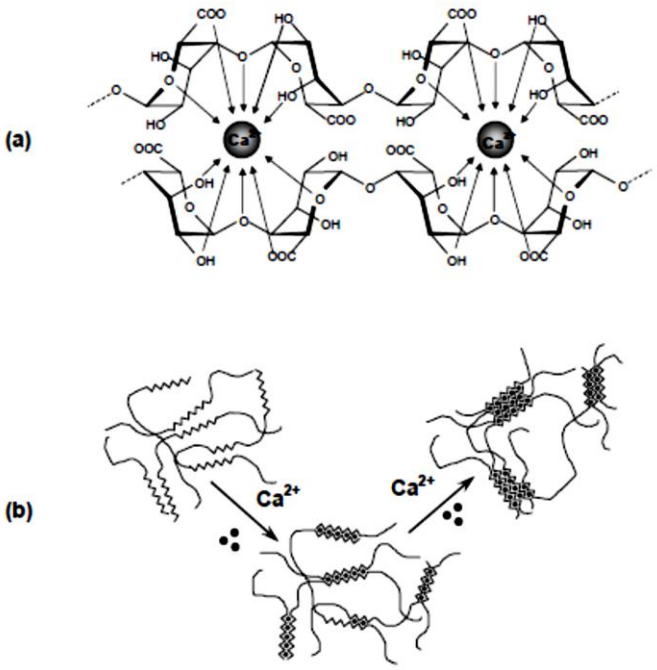

Figure 5.1 The eggbox model for binding of divalent ions to alginate. (a) Chelation of divalent cations, (b) Lateral association of chains (Mørch, 2008)

5.1.3 Interactions of alginate with polycations

Since alginates are negatively charged polymers, they can form strong complexes with cationic polymers such as chitosan (Lawrie et al., 2007; Taqieddin & Amiji, 2004), poly-L-lysine (F. Lim & Sun, 1980; B. Thu et al., 1996), and poly(ethyleneimine)

(Tanaka, Kurosawa, Kokufuta, & Veliky, 1984) through electrostatic interactions. These complexes do not dissolve in the presence of non-gelling cations such as monovalent ions (e.g. Sodium), and therefore have been extensively used to stabilize the alginate beads. These polymer coatings re crucial as they strengthen the outer surface of the capsules, increasing the long-term stability of the beads, and reduce the porosity of Ca-Alginate beads, providing control over permeability.

Of all the polycations used for the purpose of coating the alginate beads, poly-L-lysine (PLL) is the most extensively studied. The coating is usually done by transferring the beads into a solution of polycation, forming a capsule with a polycation/alginate complex membrane around the bead. As most of polycations are cytotoxic and the presence of the positive charge can also lead to attachment of cells to the surface in vivo, alginate-PLL beads are usually coated with another layer of alginate resulting in alginate-PLL-alginate capsules (Bünger et al., 2003). While such capsules provide the immune-isolation needed for implanted cells, buy they have shown insufficient mechanical stability when used in vivo. The PLL coating failed to provide the necessary strength for the alginate beads when implanted in larger animals such as dogs (Peirone, Delaney, Kwiecin, Fletch, & Chang, 1998). This may be contributed to the gradual loss of the crosslinking ions (calcium) or the polyelectrolyte overcoat.

The capsules used for immobilization of islets are usually based on a liquid core. In this system the crosslinking Ca^{2+} ions are replaced with non-gelling Na^{+} ions by the aid of agents such as citrate, which causes the gel core to dissolve. This will result in a polyanion/polycation complex that acts as a semi-permeable membrane (Orive, Hernández, Gascón, Igartua, & Pedraz, 2003). Many modifications of alginate microcapsules have been proposed over the years including use of other polycations (Orive, 2003), formation of multilayer capsules (Schneider et al., 2001), addition of an outer gel layer (Wong & Chang, 1991), and surface modification of the alginate/PLL membrane (Kung, Wang, Chang, & Wang, 1995).

5.2 Experimental: Materials and Methods

5.2.1 Materials used

Ca-Alginate beads were provided by Dr. Hoare's lab and stored in the gelling bath solution consisting of 1.1% calcium chloride and 0.45% NaCl. PVAm10-GAL2 polymer was synthesized according to the procedure described in previous chapters. Fluorescein-5-Isothiocyanate (FITC) was purchase from Invitrogen Life Technologies. Dimethyl sulfoxide (DMSO) was obtained from Caledon Labs. Sodium citrate from Sigma Aldrich and sodium bicarbonate from EMD Millipore were used for the experiments.

5.2.2 FITC labeling of PVAm-G

To label the PVAm-GAL polymer, FITC was first dissolved in DMSO to achieve a 1 mg/ml solution. PVAm10-GAL2 was dissolved in sodium bicarbonate buffer at pH=9 to form a 2 mg/ml solution. 0.1 ml FITC solution was added to 10 ml of polymer solution and the mixture was stirred for 10 hours at room temperature followed by quenching with 0.26 mL of 2 M NH_4Cl for 2 hours. The unreacted FITC was removed using centrifuge filtration method with Allegra X-12R centrifuge from Beckman Coulter at 3750 rpm for 15 minutes. Millipore centrifugal units with 3000 MWCO were used for this purpose. The polymer was then dialyzed against Milli-Q water for 3 days and then lyophilized.

5.2.3 Preparing and Coating of alginate beads

The beads prior to coating were washed in sequence with (a) 1.1% CaCl2, 0.45% NaCl for 2 min; (b) 0.55% CaCl2, 0.68% NaCl for 2 min; (c) 0.28% CaCl2, 0.78% NaCl for 2 min; (d) 0.45% NaCl for 3 min; (e) 0.9% NaCl for 2 min, and then stored in saline. The PVAm-GAL coating was performed by exposing the Ca-alginate beads to 0.05 wt% PVAm-GAL in saline for 6 minutes and washing them in sequence with 8 ml of wash solutions (d), (a) and (e) above.

5.2.4 Observation under microscope

A solution of the beads was placed on a glass slide and observed under an Olympus BX51optical microscope with fluorescence and bright field filters. The images were analyzed using QCapture and Image-pro plus 7.0software.

5.3 Results and discussion

5.3.1 PVAm-Gal/Alginate polyelectrolyte complexation

Upon exposing of the alginate beads to a solution of fluorescein labeled PVAm-GAL at pH=7, the cationic polymer adsorbs on the surface of the beads due to electrostatic interaction with the negatively charged alginate. After 2 washing steps with saline solution to ensure the removal of the unreacted polymer, the beads were observed under an optical microscope with both fluorescent and bright field monitoring channels as presented in figure 5.3. Fluorescence intensity line profile of the microcapsules was obtained by plotting grey values versus distance across the bead (pixels). The intensity profile, presented in part (c) of Figure 5.2, shows the distribution of FITC labeled PVAm-GAL polycation in the microcapsule. The highest concentration of PVAm-GAL is on the surface of the beads. However the polycation shows penetration inside the bead with rapid decrease in concentration as we approach the center of the beads. Penetrating of the polymer coating inside the alginate gel is reported in literature for low molecular weight PLL (1-10 kDa) (Goosen, O'Shea, Gharapetian, Chou, & Sun, 1985; King, Daugulis, Faulkner, & Goosen, 1987).

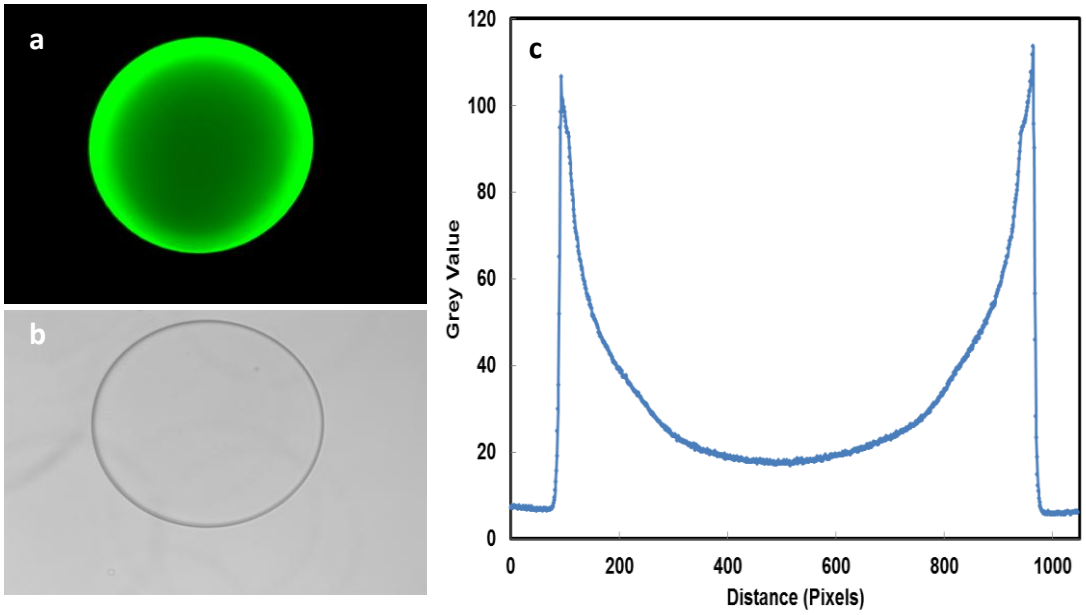

Figure 5.2 PVAm-GAL coated alginate bead as observed under (a) fluorescent microscope and (b) bright field microscope (c) fluorescence intensities as a function of distance through the beads

Due to the electrostatic nature of the interaction between the polycation and the alginate beads, it is expected that at elevated pH, where most of the amine groups on the PVAm-GAL are neutralized and the polymer has little or no positive charge, there would be less adsorption of the polymer on the beads. The pH of the coated beads was adjusted to 11.5 and they were observed under microscope as presented in figure 5.3. Immediately after exposure of the beads to high pH saline solution there was wrinkling of the surface, explained by desorption of the polymer from the surface of the beads. However, over time the beads gained their original spherical shape.

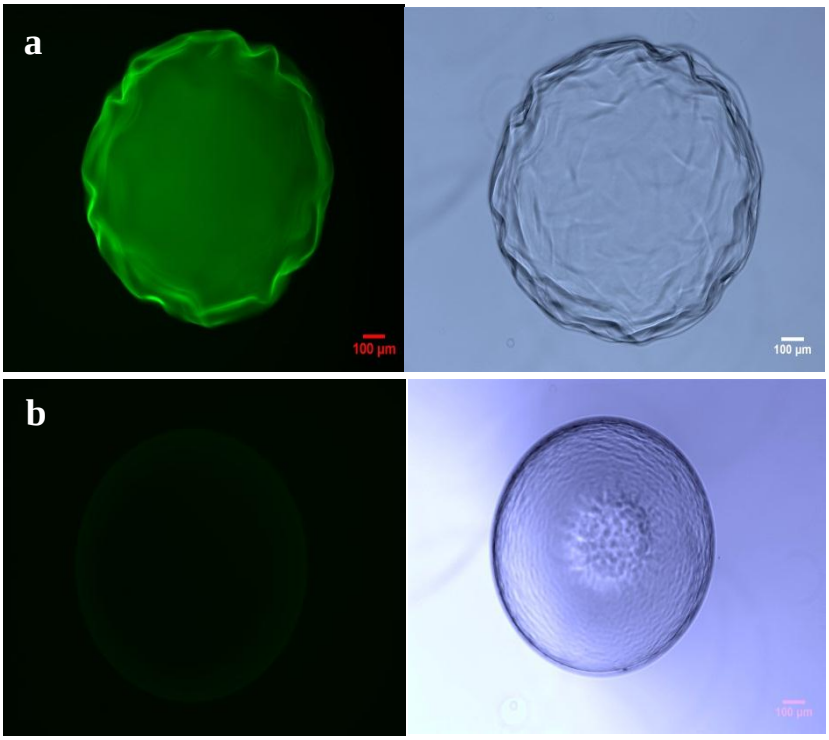

Figure 5.3 Fluorescence (left) and bright field microscopy (right) images of PVAm10-GAL2 coated alginate beads (a) immediately upon exposure to normal saline solution at pH=11.5 and (b) after 15 minutes

By comparing the fluorescence intensities of PVAm-GAL coated beads at pH=11.5 to pH=7, it is obvious that at pH=11.5 the fluorescence intensity is much lower (almost no fluorescence), providing evidence of less adsorption of the polymer on the beads as expected, due to the less charge density on the neutralized PVAm-GAL polymer at elevated pH values.

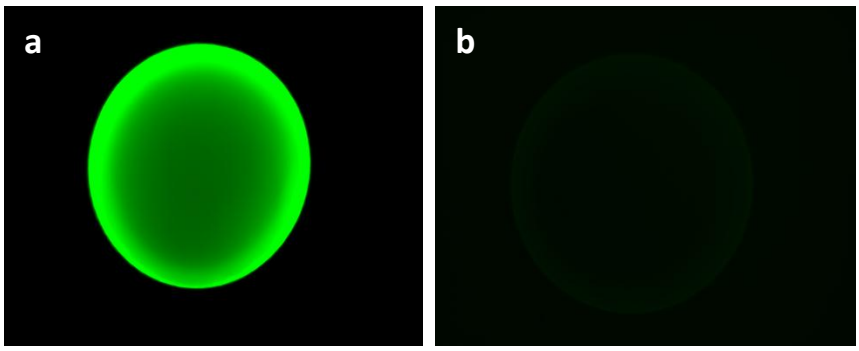

Figure 5.4 Fluorescence intensities of PVAm-GAL coated beads at (a) pH=7 and (b) pH=11.5

5.3.3 Effect of sodium citrate on PVAm-GAL/Alginate polyelectrolyte complexation

Uncoated Ca-alginate beads are tiff gels at physiological salt concentration. Upon exposing the beads to sodium citrate solution, crosslinking Ca^{2+} ions are replaced with non-gelling Na^{+} ions, causing the beads to dissolve. To examine the resistance of the coated beads to chemical degradation the capsules are challenged with sodium citrate which can lead to dissolution of the capsule's core, and of the surface coating. First uncoated Ca-Alginate beads were exposed to 170 mM citrate solution and observed under bright field microscope as presented in figure 5.6. The beads dissolved immediately after exposure to citrate solution as expected.

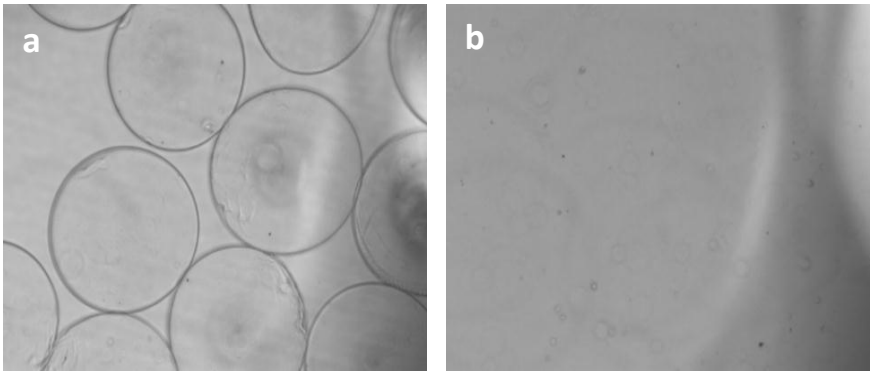

Figure 5.5 Uncoated Ca-alginate beads (a) before and (b) after exposing to 170 mM sodium citrate solution

Same experiment was repeated with PVAm-GAL/Alginate polyelectrolyte beads and the effect was observed over time. The fluorescence images are presented in Figure 5.7. Sodium citrate treatment of PVAm-GAL coated capsules liquefies the core as the Ca^{2+} ion is replaced by Na^{+} ion. The capsules swelled over time as the gel core is liquefying but the polyelectrolyte shell survived. PVAm-GAL acts as a shield and protects the beads from dissolving in presence of sodium citrate. Bright field microscopy images of the swelling phenomenon of the beads with polyelectrolyte coating are also available in Appendix E.

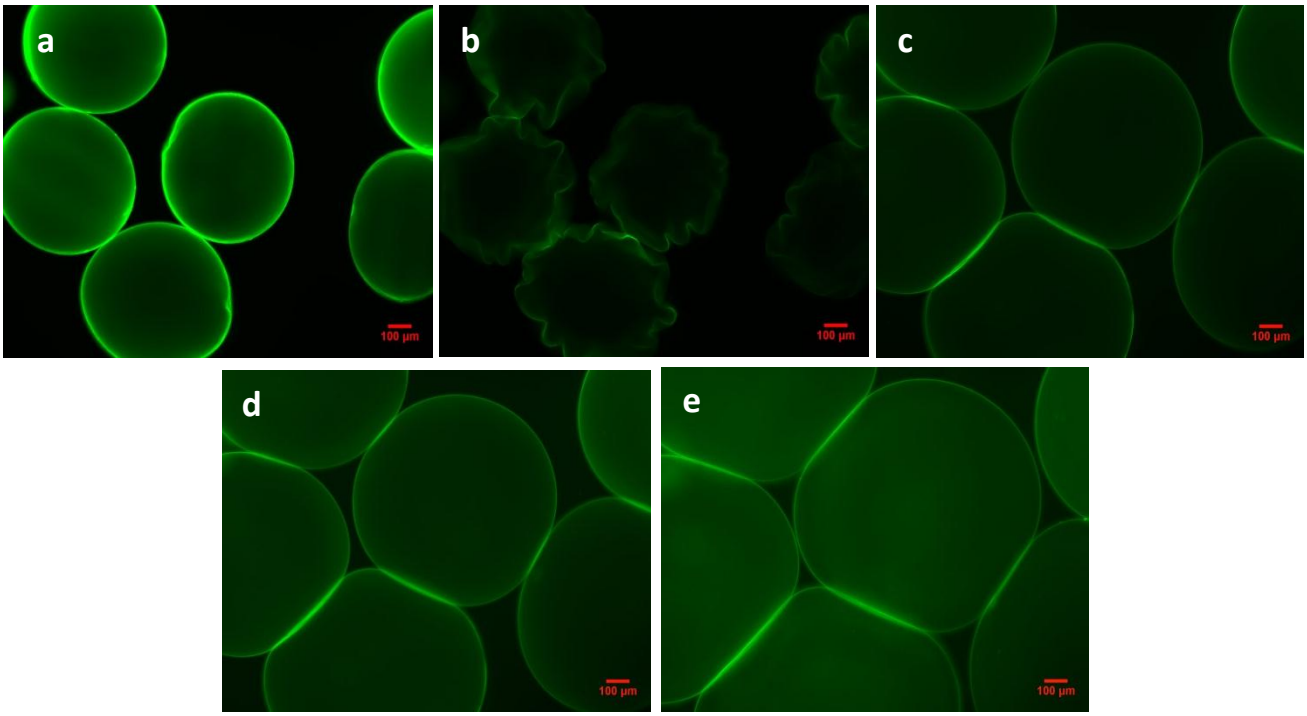

Figure 5.6 Fluorescence images of Ca-alginate beads coated with FITC labeled PVAm10-GAL2 (a) before and after exposure to sodium citrate solution for (b) 1 min (c) 5 min (d) 30 min (e) 5 hours

5.3.4 Effect of exposure time to the polymer solution

The nature of coating formed by interaction of polycation with Ca-algiante beads depends on many factors such as polycation concentration, MW, and exposure time (King et al., 1987; B. Thu et al., 1996; Beate Thu et al., 1996). It has been reported that longer exposure time to the polymer can cause the surface of the beads to become wrinkled. This usually happens for higher molecular weight polymers that form a strong polyelectrolyte complex which cannot redistribute itself on the surface, as well as in cases where the osmotic pressure due to the concentration of polycation is high (Gåserød, Smidsrød, & Skjåk-Bræk, 1998; Mazumder, Shen, Burke, Potter, & Stöver, 2008). Effect of exposure time to PVAm-GAL solution was examined under the microscope and the results are present in Figure 5.7.

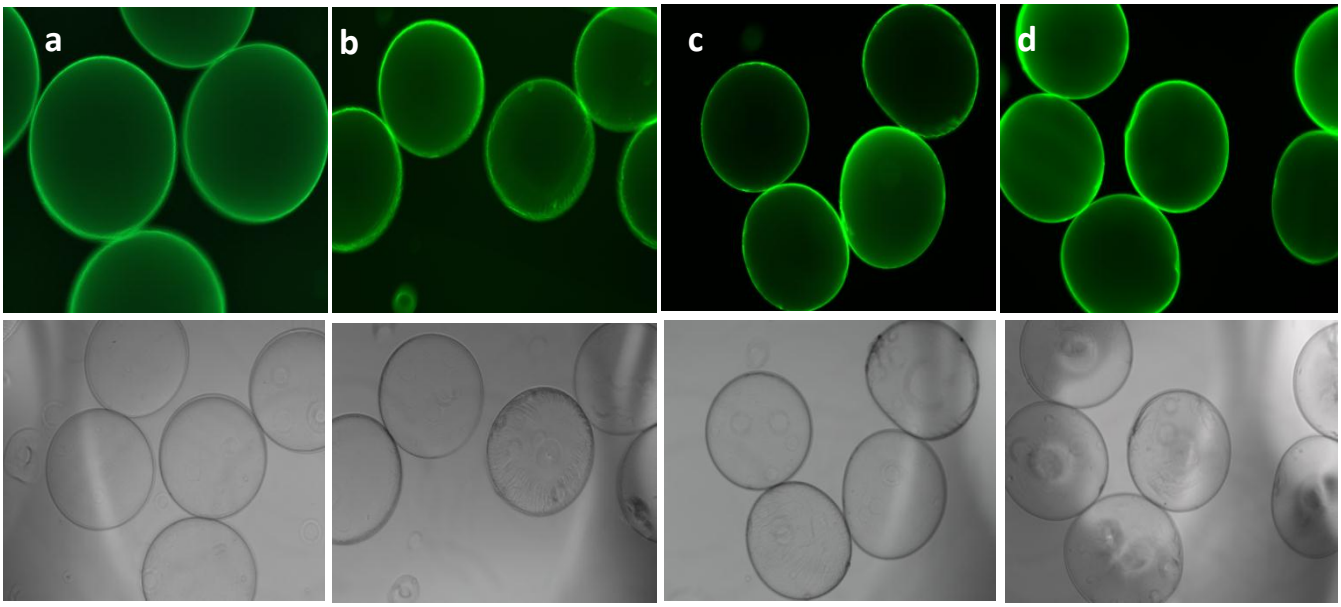

Figure 5.7 Fluorescence (top) and bright field (bottom) images of Ca-alginate capsules exposed to 0.05 wt% FITC labeled PVAm10-GAL2 for (a) 1 min (b) 30 min (c) 60 min (d) 120 min

There was no wrinkling of the surface with exposure times as high as 2 hours. It is expected as the molecular weight of the PVAm-GAL is small enough to redistribute itself on the surface upon complexation with the alginate beads.

5.3.5 Effect of saline concentration

Another test to measure the stability of the coating on the microcapsules is treatment with high salt concentrations. In the case of polyelectrolyte complexes which are held together by ionic interactions alone, the capsules fall apart when exposed to 2 M NaCl. This is normally observed for PLL coated alginate beads (Mazumder et al., 2008). Electrostatic interactions are screened by presence of salt ions, reducing the strength of the interaction. The PVAm-GAL coated beads were challenged against 2 M and 4 M NaCl solutions after liquefying the gel core with sodium citrate treatment. The results are presented in figure 5.8.

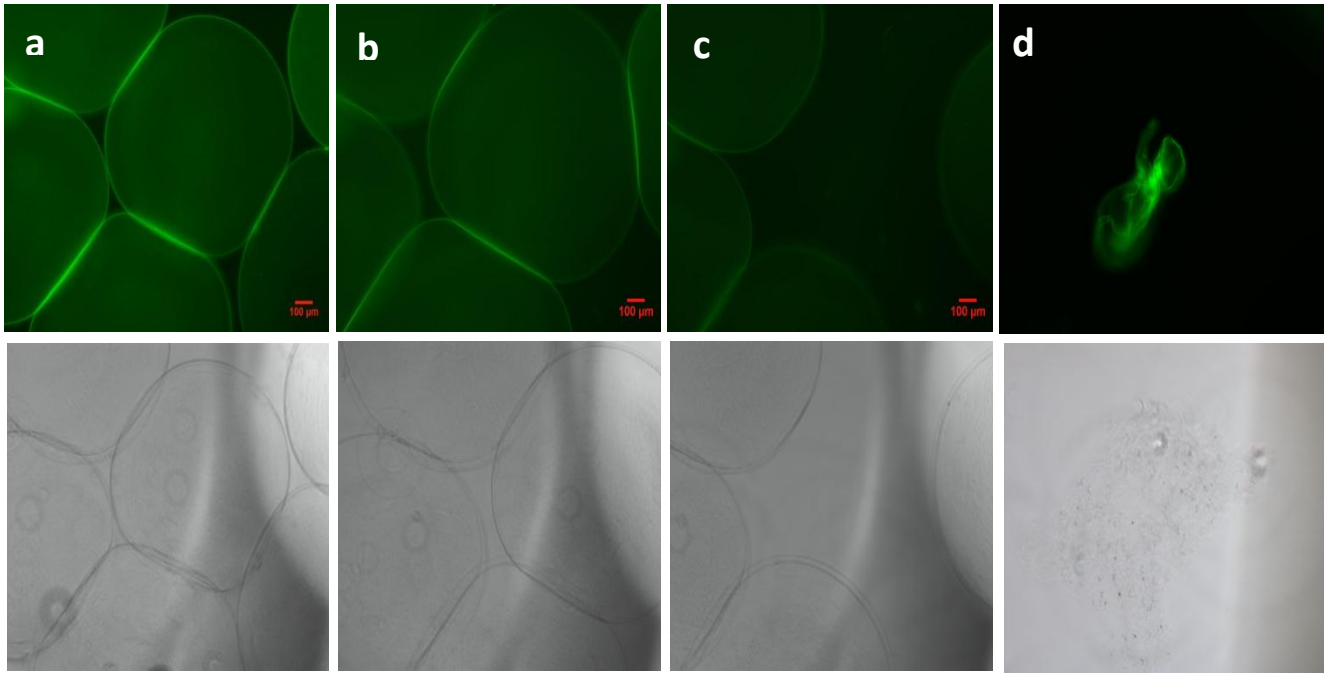

Figure 5.8 PVAm10-GAL2 coated alginate beads (a) before and after treatment with (b) 2 M NaCl for 30 min (c) 4 M NaCl for 60 min (d) 2 M NaCl overnight

Upon exposure of the beads to high concentration saline for up to 60 min, no degradation of the beads occurred. The PVAm-GAL shell held together preventing the beads from dissolving. Therefore the beads were left over night in the 4 M saline solution and observed under microscope as presented in image (d) in figure 5.8. The beads seem to have fallen apart and the shell had collapsed. The reason that the coated capsules did not dissolve immediately could be due to the higher charge density of PVAm-GAL as compared to PLL. According to titration results for PLL with molecular weight of 15-30 kDa, the amine mole content for this polymer is 3.4 mmol/g which is much lower than that of PVAm10-GAL2 (6.5 mmol/g). Therefore the energy of binding of cationic polymer to anionic alginate would be less for PLL resulting in a rapid dissolving of the beads. Moreover, low molecular weight PVAm10-GAL2 penetrates inside the alginate beads (as observed from the fluorescence intensity profile across the bead). As a result the mass transport of PVAm-GAL and collapsing of the beads would be more time demanding.

5.4 Conclusions

1. PVAm-GAL successfully formed a polyelectrolyte complex with Ca-Alginate beads through electrostatic interactions.
2. The complex formation is pH dependent as at elevated pH there was little adsorption of PVAm-GAL on the surface of alginate beads.
3. The PVAm-GAL/Alginate capsules swelled when treated with sodium citrate but the polyelectrolyte shell survived, protecting the alginate from chemical degradation.
4. Exposure of the polymer solution does not cause any deformation or wrinkling of the beads with time.
5. PVAm-GAL coated beads survived treatment to high salt concentration and only collapsed over longer exposure time. This is attributed to higher charge density of polycation which results in stronger polyelectrolyte complexes, as well as penetration of polycation inside the bead.

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Appendices

Appendix A: Calculation of degree of galactosylation from conductometric titration:

Titration of 20 mg PVAm 340 kDa against 0.1 M NaOH in 50 mL 0.001 KCL solution:

ΔV_{NaOH} = 2.132 mL

$$Amine\ content = 2.13\ \times 10^{-3}\ L\ \times 0.1 \frac{mol}{L} = 2.13\ \times 10^{-4}\ mol$$

Titration of 20 mg PVAm340-GAL2 against 0.1 M NaOH in 50 mL 0.001 KCL solution:

ΔV_{NaOH}=1.648 mL

$$Amine\ content = 1.65\ \times 10^{-3}\ L\ \times 0.1 \frac{mol}{L} = 1.65\ \times 10^{-4}\ mol$$

$$\text{Degree of substitution} = \frac{2.13 \times 10^{-4} - 1.65 \times 10^{-4}}{2.13 \times 10^{-4}\ mol} \times 100\% = 22.6\%$$

Appendix B: Sample calculation for N/P adjustment of PVAm-GAL/DNA polyplexes

Properties of DNA 84605406 (T100) sample:

5'-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT T-3'

Molecular weight: 30357.6 g/mol

Number of moles in the sample: 149.9 nMoles

Number of DNA bases: 100

Weight of the sample: 4.55 mg

Calculating the phosphate content of DNA:

Each individual base in the DNA strand has one phosphate group. Therefore the number of phosphate groups for a DNA sample that has 100 repeating bases is 100 in each strand.

Total PO_4^{3-} molar content of the DNA sample is calculated based on number of moles of DNA and number of bases :

Total number of moles of PO_4^{3-} = Number of bases × Total number of DNA moles in the sample

$$= 100 \times 149.9 \times 10^{-9} = 1.499 \times 10^{-5}\ \text{mol}$$

$$PO_4^{3-} molar\ content = \frac{1.499 \times 10^{-5}\ \text{mol}}{4.55\ mg} = 0.33 \times 10^{-5}\ mol/mg$$

The entire DNA sample was dissolved in 1 ml of solvent resulting in a 4.55 mg/ml solution.

Calculating the amine content of PVAm-GAL:

From Titration results on PVAm10-GAL2, the volume of NaOH 1 N used to neutralize the amine groups on PVAm-GAL polymer was calculated to be 1.3063 ml

Total number of moles of NH_3^+=1.3063 × 10^{-3} L × 0.1 mol/L = 1.3063 × 10^{-4} mol

$$NH_3{}^+ molar\ content = \frac{1.3063 \times 10^{-4}\ \text{mol}}{20\ mg} = 0.65 \times 10^{-5}\ mol/mg$$

A PVA-GAL solution with concentration of 1 mg/ml was prepared by dissolving 10 mg of the polymer in 10 ml of solvent.

Adjusting the N/P ratio

For $N/P = 1$ the calculation is as follows:

Number moles of NH_3^+= Number moles of PO_4^{3-}

$C_{NH_3{}^+} \times V_{NH_3{}^+} = C_{PO_4{}^{3-}} \times V_{PO_4{}^{3-}}$

Assume 100 µL of DNA solution: $V_{PO_4{}^{3-}} = 0.1\ mL$

$$0.65 \times 10^{-5}\ \frac{mol}{mg} \times 1 \frac{mg}{mL} \times V_{NH_3{}^+} = 0.33 \times 10^{-5} \frac{mol}{mg} \times 4.55 \frac{mg}{ml} \times 0.1\ m\text{L}$$

$V_{NH_3{}^+} = 0.231\ mL = 231\ \mu L$

For other N/P ratios the concentration of PVAm-GAL was adjusted:

N/P	1	2	3	4	6
$C_{PVAm-GAL}$ (mg/mL)	1	2	3	4	6

Appendix C: Cell culture procedures

Cell thawing:

1. Thaw cryovial by dipping in 37 °C water bath and gently agitating it for approximately 2 minutes. Remove the vial from the bath as soon as the contents are thawed.
2. In the biological cabinet, transfer the vial content to a 15 ml Falcon centrifuge tube and add 10 ml of complete EMEM media.
3. Centrifuge at 1000 rpm for 5 minutes, remove the supernatant and replace with freshly warmed media.
4. Pipette gently to re-suspend cell pellet
5. Transfer cell suspension to a 25 cm^2 culture flask
6. Incubate at 37 °C with 5% CO_2
7. Change medium every 2-3 days
8. Split culture flask when 90% confluent

Cell splitting

1. Remove and discard culture medium
2. Add 5 ml 0.25% (w/v) Trypsin-0.53 mM EDTA into the flask and shake it gently for 5-15 minutes to detach cells and observe under the microscope until the cell layer is dispersed.
3. Add 6 ml of media and transfer cell suspension to a 15 ml Falcon centrifuge tube.
4. Centrifuge at 1000 rpm for 5 minutes.
5. Remove the supernatant and replace with 12 ml of fresh media.
6. Pipette to re-suspend the cells and split as required.
7. Incubate at 37 °C with CO_2
8. Feed with new media every 2-3 days

Cell counting

1. Remove old media
2. Add 5 ml trypsin-EDTA to the flask and shake gently until the cells are detached from the bottom (5-15 min).
3. Add 6 ml of complete EMEM media into the flask and transfer the content to a 15 ml centrifuge tube.
4. Centrifuge for 5 minutes at 1000 rpm
5. Remove supernatant and replace with 15 ml freshly warmed EMEM media
6. Remove 100 µL of cell solution and place in hemocytometer.

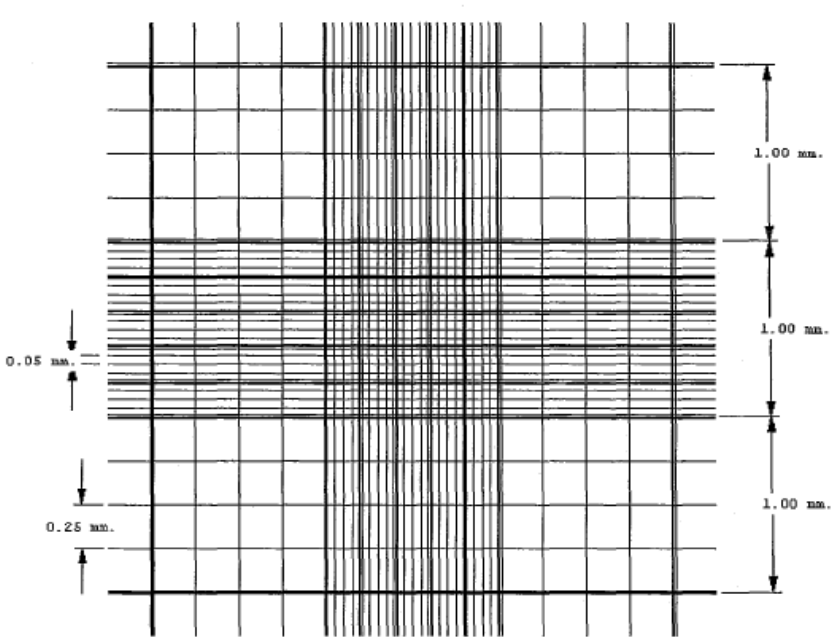

Figure C.1 Hemocytometer Grid

7. ount number of cells in outer quadrants (single quadrant area= 0.l mm^3) and calculate cells per quadrant (see Figure C-1)
8. Number of cells is obtained from the following formula:

$$Number\ of\ cells = Cell\ Count\ (cells/0.1mm^3) \times 10000 \left(\frac{0.1mm^3}{mL}\right) \times 15mL$$

Appendix D: Chemical structure of alginate polymer:

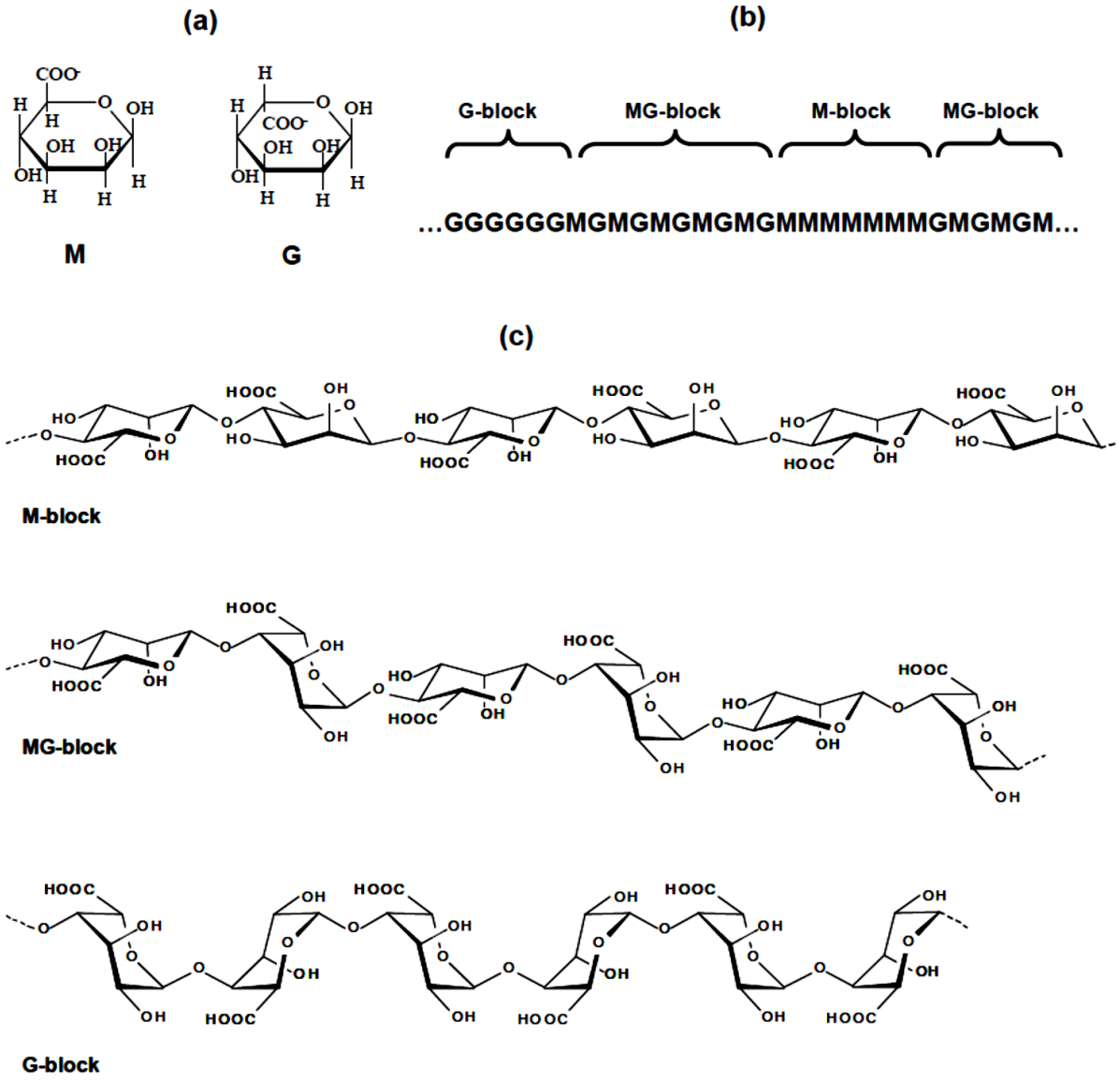

Figure D.1 Alginate chemical structure (a) illustration of the M and G monomers (b) block composition in alginate (c) ring conformation in alginate chains, cavities in the structure provide favorable binding site for cations (Mørch, 2008)

Appendix E: Bright field microscopy images of PVAm-GAL coated alginate beads exposed to sodium citrate solution

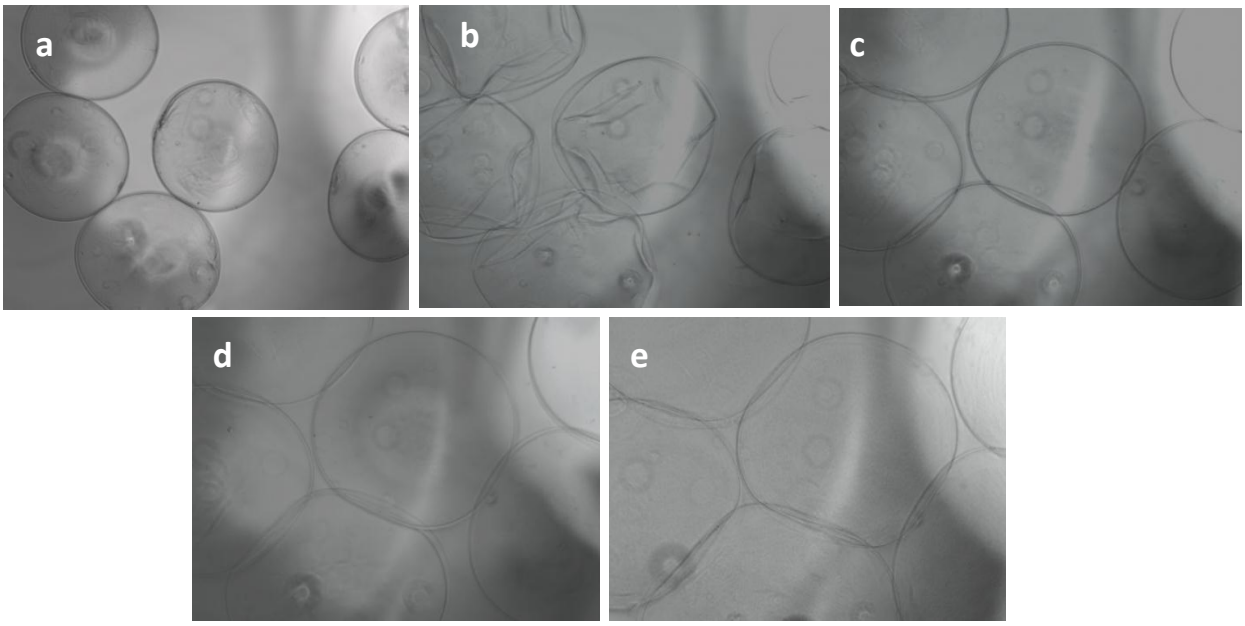

Figure E.1 Bright field spectroscopy images of PVAm10-GAL2 coated alginate beads (a) before (b) 1 min (c) 5 min (d) 10 min (e) 5 hours after exposure to sodium citrate solution