Highly Shear-Thinning Mucoadhesive Hydrogels for Ophthalmic Applications

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Abstract

Highly shear-thinning polymers that can easily flow upon the application of shear but form gels at rest have multiple potential applications in the eye. In the front of the eye, a formulation that can easily be administered via a conventional eye dropper but form a gel within the tear film once applied would be beneficial for prolonging drug release at the front of the eye, either alone or as a medium for entrapping nanoparticles or nano-objects loaded with drugs. In the back of the eye, vitreous substitutes that can be administered through a narrow gauge needle (and, ideally, removed via the same) may be beneficial for retinal surgeries.

The overall objective of the proposed research is to chemically modify PVP through grafting strategies to improve its viscometric and mucoadhesive properties while maintaining the beneficial properties, which make it useful in ophthalmic applications.

N-vinylpyrrolidone is copolymerized with N-vinylformamide to produce a functionalized grafting platform P(VP-co-VF), which is then grafted with low concentrations of short hydrophobic grafts to introduce non-Newtonian flow profile to the precursor.

For applications at the back of the eye, the hydrophobic grafted PVP can be injected into the vitreous cavity of the eye in a liquid form to form subsequently a gel-like substance and function as a substitute for the vitreous humour. For application at the front

of the eye, the shear thinning properties of hydrophobic-grafted PVP is combined with the mucoadhesive properties of phenylboronic acids (PBA) to improve the bioavailability of the drugs delivered to the front of the eye with eye drops.

Rheological characterization of the solutions has shown the potential to form gel-like materials via hydrophobic associations without sacrificing the facile injectability of the material. Targeted gelation and mucoadhesion properties can be obtained by the synthesis of polymers with desired PBA and hydrophobic graft contents.

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1. Introduction

Dry eye syndrome affects approximately one in five North Americans, while vitreoretinal diseases are the second leading cause of blindness, affecting millions of individuals each year.

Our goal was to design a novel, self-associating hydrogel to be used in the treatment of the two mentioned widespread eye disorders.

Treatment of dry eye typically involves applying a slightly viscous solution in drop form to the eyes to provide a temporary relief before the solution is wiped away by blinking or evaporates. In topical application of eye drops the solution tends to be cleared from the eye rather quickly due to physiological constraints such as the blinking reflex. Therefore, frequent dosing is generally necessary to maintain a therapeutic drug level in the tear film or at the site of action. In order to avoid the frequent instillation of concentrated solutions, which may cause discomfort, or irritation of the ocular surface (Salminen, 1990)(Arici, Arici, Topalkara, & Guler, 2000), the residence time of the preparation in the tear film should be lengthened.

Water-soluble polymers can be used in the preparation of a formulation that can easily be administered via a conventional eyedropper but form a gel within the tear film once applied. Such formulations would be beneficial for prolonging drug release at the front of the eye, either alone or as a medium for entrapping nanoparticles or nano-objects loaded with drugs. Artificial tear formulations that can deliver drugs to the cornea with controlled release kinetics have significant potential to alleviate dry eye uncomfortable conditions. Therefore the first objective our work is to design a novel artificial tear

composition, which addresses many of the problems, associated with current formulations including the short residence time of the preparations in the eye.

Over the last two decades vitreous replacement became a key strategy to treat eye disorders such as vitreous opacification or retinal detachment.

Vitreous substitutes are needed to tamponade the retina or during vitrectomies for treatment of retinal detachments. Current material used to replace the vitreous humour, such as Gases, perfluorocarbon liquids and silicone or fluorosilicone oils cannot be used as long term substitutes due to short retention time in the vitreous cavity or causing cell toxicity (Swindle & Ravi, 2007).The second objective of this project is therefore concerned with synthesis and characterization of PVP-based polymers with a low concentration of short hydrophobic grafts to introduce improved surface activity and non-Newtonian flow profiles to PVP-based systems which make PVP injectable into the vitreous cavity of the eye in a liquid form to form subsequently a gel-like substance and function as a substitute for the vitreous humour.

The hydrophobic interactions between the grafts promote associative thickening to create gel-like substance in the eye but also facilitate significant shear thinning during injection, enabling facile delivery to and removal from the eye via injection. Therefore unlike most other vitreous humour substitute formulations that contain crosslinking agents, our system is designed to create a gel-like substance without the aid of any cross linking agent. The physical gels thus produced will maintain their rheological properties

virtually unchanged before and after injection, displaying suitable characteristics as vitreous substitutes.

Our approach for fabricating such materials is to synthetize a "graft platform" with a high water binding capacity, which also contains reactive functional groups. Therefore –N-vinylpyrrolidone (NVP) is copolymerized with N-vinylformamide (NVF) to produce the grafting platform (P(VP-co-VF)). Stepwise hydrolysis of the NVF residues exposes controlled numbers of reactive amine groups to be available for grafting, as confirmed by potentiometric titration and NMR analysis. The PVP-based graft platform is then grafted with low concentrations of short hydrophobic grafts. The hydrophobic groups can self-associate at low shear to form a transient gel that can be readily streamed by the application of shear.

Short-chain alkyl halides (C_{10}-C_{18}) grafted in low concentrations to the PVP platform can form hydrophobic crosslinks which can be disrupted by shear, imparting higher zero shear viscosities and shear thinning behavior to the polymer solution while still allowing easy injection or delivery via routine eye drop containers.

For applications at the back of the eye, the hydrophobic grafted PVP can be injected into the vitreous cavity of the eye in a liquid form to form subsequently a gel-like substance and function as a substitute for the vitreous humour.

For application at the front of the eye, mucoadhesive phenylboronic acid (PBA) functional groups are sequentially grafted to the hydrophobically-modified P(VP-co-VF) graft platform with the objective of increasing the residence time and thus the

bioavailability of the drugs delivered to the front of the eye with eye drops. Targeted gelation and mucoadhesion properties can be obtained by the synthesis of polymers with the desired PBA and hydrophobic graft contents. Such a material is of significant interest since it can interact selectively with each layer of the tear film: adhesion to the mucous layer, gelation in the aqueous layer, and hydrophobic interactions with the lipid layer. As such, in addition to its application as vitreous humour substitutes, we also believe that this material has potential applications for drug delivery at the front of the eye as well as the treatment of dry eye.

2. Literature Review

2.1 Dry Eye

2.1.1 Introduction

Among the major organs of the body, the eye is unique in its direct exposure to the external environment. Therefore a stable tear film is a necessary component of a healthy ocular surface and a requirement for clear vision (Koh et al., 2010)(Koh et al., 2006)(Albarrán, Pons, Lorente, Montés, & Artigas, 1997).Tears also lubricate the eyelids and the ocular surface to protect them form being damaged by the high blinking shear.

The constant flow of tears across the ocular surface dilutes and flushes exfoliated cells, debris, or foreign bodies from the eye. As such, tears are considered as the first and most important line of defense for the eye against environmental pathogens. Tears also contain a number of antibacterial proteins and glycoproteins (ie. lysozyme, β-lysin, and lactoferrin) which assist in healing corneal inflammations. Tears also hydrate the corneal epithelial cells and cool the ocular surface by evaporation, thus inhibiting microbial growth. Based on these key roles of tears in the eye, insufficient volume, excessive evaporation, or instability of tear film can lead to symptoms of ocular discomfort including dryness, itching, foreign body sensation and acute blurred vision (Fischer & Wiederholt, 1982). These symptoms collectively describe the medical condition known as “dry eye”. Dry eye directly affects approximately 1 in 5 Americansi and even higher percentages of contact lens users and people over the age of 40 (Troyer & Spencer, 2003) On this basis, “dry eye” is one of the most widespread medical problems in the developed

world, and the development of dry eye treatments has become the focus of considerable research.

2.1.2 Definition of Dry Eye

Dry eye is defined as a common source of discomfort and is one of the most frequently encountered ocular conditions that can seriously affect a patient's quality of life, especially in the elderly population (Moss, Klein, & Klein, 2004). The definition of dry eye and classification of dry eye conditions remained confusing for years. The National Eye Institute/Industry workshop in 1995 defined dry eye as "a disorder of the tear film due to deficiency or excessive tear evaporation which causes damage to the interpalpebral ocular surface and is associated with symptoms of ocular discomfort"(Lemp, 1995). Based on this report, dry eye is primarily divided in two groups: aqueous-deficient dry eye and evaporative dry eye.

Over the past 25 years, extensive research on the dry eye disease resulted in improved understanding of the pathophysiological mechanisms, symptoms and effects of dry eye on vision, leading to the proposal of a new definition. The International Dry Eye Workshop conducted in 2007 now defines dry eye as a "multifactorial ocular surface disease diagnosed by symptoms of discomfort and signs of visual disturbance, tear film instability and ocular surface damage, accompanied by increased osmolarity of the tear film and ocular surface inflammation."((DEWS), 2007). The terms "aqueous-deficient

dry eye" and "evaporative dry eye" were removed from the earlier definition, but are still retained in the classifications of dry eye

2.1.3 Structure of The Natural Tear Film

The exposed part of the eye is covered by a thin fluid layer, the so-called precorneal tear film. Design of a successful tear replacement requires an understanding of the structure and composition of the natural tear film and the ocular surface.

Tear films are only temporarily stable. During the short time interval between two blinks, concentration gradients and dispersions forces on the mucous layer result in tear film break-up, which causes the formation of dry spots on the cornea (dewetting). Therefore, an unpleasant sensation as a result of irritation of the corneal nerve endings compels humans to blink after 20 to 40 seconds. A new tear film spreads over the external eye surface as the eyelids open. (Greaves & Wilson, 1993)(Frank J. Holly, 1973).

The tear film was believed to be a trilaminar fluid, with an outermost lipid layer, a thick central aqueous layer, and a pre-epithelial mucous layer (Wolff, 1955).This model has evolved recently and the most currently accepted concept is that the tear film is an aqueous gel gradient with an overlying lipid layer(Dilly, 1994).This inner aqueous layer consists of a dense mucin phase closest to the ocular surface, with the density of mucin decreasing toward the lipid layer. The aqueous layer and the lipid layer are easier to visualize than the mucous layer which has a refractive index that is identical with the aqueous phase of the tear film and hence is difficult to visualize(Dilly, 1994). Each

component of the tear film has a distinct origin and purpose. The secretions of the main and the accessory lacrimal glands mainly form the aqueous layer. The corneal and conjunctival epithelial cells also contribute to the secretion of the aqueous component by secreting water and electrolytes into the tear film(Klyce & Crosson, 1985)(Hamann et al., 1988)(Corfield, Carrington, Hicks, Berry, & Ellingham, 1997). The aqueous portion is made up of water, oxygen, proteins, electrolytes, growth factors, peptides and inflammatory mediators. The electrolytes (sodium, potassium, magnesium, calcium, chloride, and bicarbonate, and phosphate ions) determine the osmolality of the tear film(Gilbard, 1994) and maintain its pH in the range of 7.2-7.6(Fischer & Wiederholt, 1982). The lipid layer is secreted by the meibomian glands and is made up of phospholipids, free fatty acids and cholesterol. The lipid layer assists in the spreading of the tear film over the corneal surface and eliminates the evaporation of the aqueous layer (Gouveia & Tiffany, 2005). The lipid layer also plays a role in preventing contamination of the tear film by the polar liquids secreted by the sebaceous glands of the eyelids (Lemp & Chacko, 1997).

Secretion of the goblet cells and the squamous epithelial cells of the cornea and conjunctiva mainly forms the mucin part of the aqueous-mucin component of the tear film (Ludwig, 2005a). Figure 2-1 shows the structure of the mucin and its subunits. Ocular mucins can be classified in two types, namely secreted mucins and soluble mucins (Mantelli & Argüeso, 2008). The soluble mucins are secreted by the conjunctival goblet cells. The mucin gel remains hydrated due to the interactions between the soluble mucins with the membrane-bound mucins and the aqueous layer which forms a gel that traps

water(Inatomi et al., 1996).

The secreted mucins are produced by the corneal and conjunctival epithelial cells and are associated to the membrane cells. They form a gelatinous layer of secreted mucins (glcocalyx) that provides a lubricating layer, which allows for an even spread of the tear film over the hydrophobic epithelial cells(Govindarajan & Gipson, 2010). The glycocalyx is also proven to have an antimicrobial effect by preventing the adherence of bacteria, viruses and inflammatory cells to the ocular surface (Gipson & Inatomi, 1998).

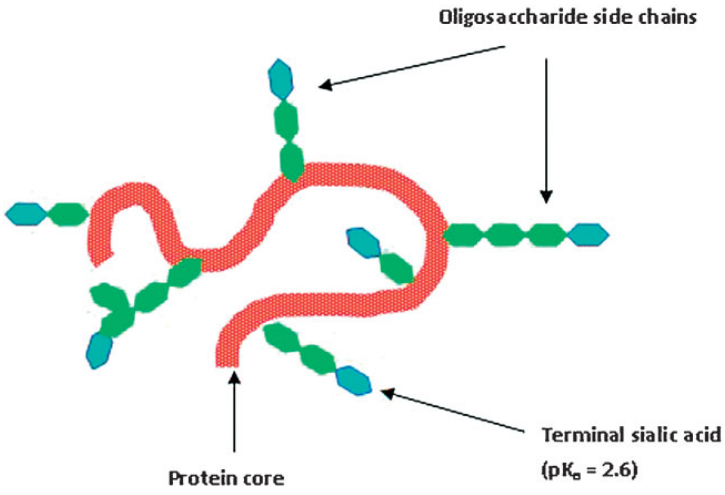


Figure 2-1 Schematic structure of mucin subunits

During blinking the rapid movement of the eyelids across the eye spreads the secreted mucins and some of the membrane bound mucin molecules transfer into the aqueous layer due to the turbulence caused by blinking(J M Tiffany, 1994). Therefore the

mucin gel remains hydrated due to the interactions between the soluble mucins with the membrane-bound mucins and the aqueous layer, which forms a gel that traps water. As such both the gel-forming and the transmembrane mucins play an important role in wetting of the ocular surface

The relative velocity of the lid and globe during blinking is about 15-25 cm/s with a blinking shear rate of about 20,000 s^{-1} at the aqueous-mucous interface.The aqueous mucin component of the tear film shows a non-Newtonian behavior during blinking and therefore helps lubricate the movement of the eyelids(Dilly, 1994). In non-Newtonian fluids the viscosity depends on the shear rate; in the case of shear-thinning fluids, when a shear force is applied as during blinking, the viscosity of the solution decreases (Hamano & Mitsunaga, 1973)(John M. Tiffany, 1991).

The free energy of binding of mucous to itself in water is positive due to its intense electron donor type monopolarity which results in the formation of a highly expanded and hydrated gel that cannot adhere to the underlying epithelium in the presence of aqueous tears. This physical characteristic reduces the shear force of blinking by facilitating the spreading of mucous on the ocular surface (Sharma, 1993). Figure 2-2 shows a schematic of the structure of the natural tear film.(Khutoryanskiy, 2011)

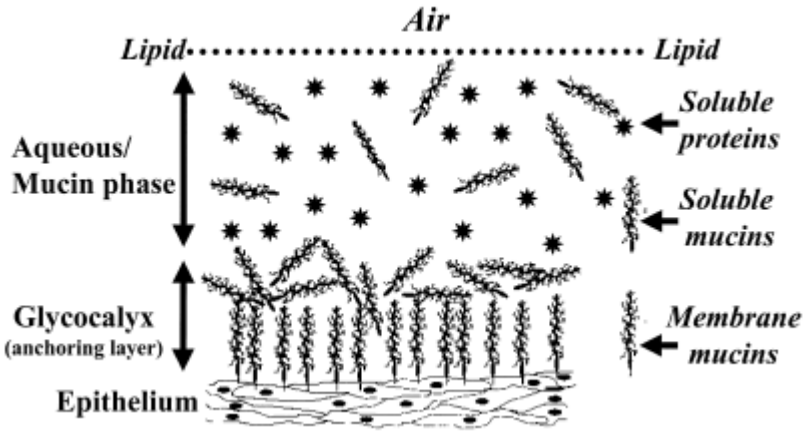


Figure 2-2 Schematic of the structure of the precorneal tear film

2.1.4 Treatments for Dry Eye

The precise etiology of dry eye remains unknown and is likely multi-factorial. As such, despite its high prevalence, dry eye remains a condition without complete cure.(Schaumberg, Sullivan, & Dana, 2002). Current therapies for dry eye including the use of lubricants and biological tear substitutes are designed to provide ocular comfort but do not yet eliminate the underlying pathology of the disease((DEWS), 2007).

Despite the short residence time of artificial tear solutions within the eye, they still remain to be the preferred treatment for the dry eye due to their causing less optical blurring, inflammation and discomfort compared to other methods of treatment such as ocular inserts and lubricant ointments (Govindarajan & Gipson, 2010)(Gipson & Inatomi, 1998). However, the protective mechanisms of the eye such as blinking and drainage rapidly remove foreign substances including drugs from the surface of the eye, making

the bioavailability of ophthalmic drugs and the residence time of polymers in the eye relatively poor (V. H. L. Lee & Robinson, 1986).

In order to maintain a therapeutic drug level in the tear film, frequent instillation of eye drops is necessary. This frequent use of highly concentrated solutions may cause toxic side effects and cellular damage at the ocular surface(Salminen, 1990)(Baudouin, 1996)(Arici et al., 2000). Also, the preservatives used in artificial tear solutions such as benzalkonium chloride, may be toxic to the ocular surface ephithelium (Gobbels & Spitznas, 1992), although unit-dose nonpreserved artificial tears have assisted with this challenge(Pflugfelder, Solomon, & Stern, 2000). In order to avoid the frequent instillation of eye drops, efforts have been made into the development of once-a-day formulations that increase the residence time of drugs in the tear film (Robinson & Mlynek, 1995a).

Numerous strategies have been developed to prolong the contact time between the artificial tears (and, by extension, any drugs contained within the tears) and the ocular surface. The use of a water-soluble polymer to enhance the contact time and possibly also the penetration of the drug was first proposed by Swan (Swan, 1945).Viscous semi-solid preparations, such as gels and ointments, provide a sustained contact with the eye, but they cause a sticky sensation, blurred vision and induce reflex blinking due to discomfort or even irritation(Sintzel, Bernatchez, Tabatabay, & Gurny, 1996).An alternative approach has been the application of *in situ* gelling systems or phase transition systems, which are instilled in a liquid form and shift to a gel or solid phase in the cul-de-

sac. The phase transition is triggered by the pH of the tears, the temperature at the eye surface or the electrolytes present in the tear film(Ibrahim, Bindschaedler, Doelker, Buri, & Gurny, 1991)(Robinson & Mlynek, 1995b).

During the past 25 years there has been progress in our understanding of the pathophysiology of dry eye that has allowed a shift in dry eye management from simply lubricating and hydrating the ocular surface to treatments that stimulate natural production of tears. Current therapies focus on replacing or providing specific tear components that have an important role in maintaining ocular surface integrity((DEWS), 2007). Given the complex role of mucin in tear film wetting (as previously discussed) and the fact that most clinical cases of dry eye are traced to mucin deficiencies instead of a dramatic decrease in aqueous tear volume, one major approach to optimize the ocular dosage form and the treatment of dry eye is the implementation of the mucoadhesive materials, shown to be successful in buccal and oral applications (Bansil & Turner, 2006)(S. K. Lai, Wang, & Hanes, 2009).

Mucoadhesion has been a dominant focus of drug delivery research in recent years as a strategy to increase the bioavailability of pharmaceuticals by direct delivery across the oral, nasal, gastric, intestinal, and vaginal mucous surfaces(F J Holly, 1992) and will be further discussed in the next section.

2.2 Mucoadhesion

2.2.1 Mucoadhesion and Mucoadhesive Polymers

Mucoadhesion is the ability of materials to adhere to mucosal membranes to provide a temporary retention. Alternately, mucoadhesion can be defined as an attractive interaction at between a pharmaceutical dosage form and a mucosal membrane. Mucoadhesion is accepted as a promising strategy to prolong the residence time and to improve the localization of drug delivery systems on various mucous membranes (Grabovac, Guggi, & Bernkop-Schnürch, 2005). From a drug delivery perspective, mucoadhesion has been linked to improved drug bioavailability, increased dosage form residence time, reduced administration frequency and the possibility of targeting particular body sites and tissues (Yuan et al., 1994).

Mucoadhesive polymers are synthetic or natural macromolecules capable of attaching to mucosal surfaces through a variety of both chemical and mechanical interlocking interactions. Hydrophilic polymers possessing strong hydrogen bonding (carboxyl, hydroxyl, amino- and sulfate groups) or charged groups are capable of adhering to mucosal surfaces. High molecular weight, high chain flexibility and surface energy properties favoring spreading onto mucosal surfaces are the other necessary characteristics of mucoadhesive polymers (Peppas & Buri, 1985)(J. W. Lee, Park, & Robinson, 2000). Some mucoadhesive polymers are also able to modify the tight junctions between the cells and therefore enhance drug permeability through the

epithelium in addition to increasing the dosage form residence time (S. K. Lai et al., 2007).

Mucoadhesivity of a polymer is primarily determined by its molecular weight and type of functional groups, both of which influence the degree of swelling (hydration) of the water-soluble polymer in the tear(J. W. Lee et al., 2000). Polymer hydration results in the relaxation of stretched, entangled or twisted macromolecules, exposing the adhesive functional groups and enabling stronger mucoadhesive interactions. Furthermore, chain interdiffusion is favoured by polymer–water interactions dominating the corresponding polymer–polymer interactions (Ludwig, 2005b).The minimum molecular weight needed to obtain adequate molecular entanglement between the polymer and the mucous layer is reported to be100,000Da (Ludwig, 2005a). Excessive cross-linking in the polymer, however, decreases the chain length available for interfacial penetration. Also, excessive formation of hydrogen bonding within the polymer itself and interchain physical entanglements hinders the polymer chains from diffusing into the mucous network (Madsen, Eberth, & Smart, 1998)(Robinson & Mlynek, 1995a). As a result, chain flexibility is critical for interpenetration and entanglement with the mucous gel. Higher mobility of polymer chains results in greater interdiffusion within the mucous layer(Imam, Hornof, Valenta, Reznicek, & Bernkop-Schnurch, 2003) . Coiling of polymer chains, due to pH or osmolality of the medium, can result in the shielding of active groups necessary for the adhesion process (Robinson & Mlynek, 1995b)(Madsen et al., 1998).

The concept of polymers adhering to mucosal membranes dates back to the

invention of an oleaginous pectin-based gel that adheres to the mouth tissues (Orabese gel) in 1947 (Khutoryanskiy, 2011). More recently mucoadhesive polymers have become commercially available for vaginal, oral, nasal and ocular use(Robinson & Mlynek, 1995c). Many high molecular weight polymers with different functional groups (such as carboxyl, hydroxyl, amino, and sulfate) capable of forming hydrogen bonds, and not crossing biological membranes, have been screened as a possible excipient in ocular delivery systems. A general conclusion is that charged polymers both anionic and cationic demonstrate a better mucoadhesive capacity in comparison to non-ionic cellulose-ethers or polyvinyl alcohol (PVA)(Meseguer et al., 1993)(Séchoy et al., 2000)(Ludwig, 2005b).

Hui and Robinson were the first to use the mucoadhesion concept in ocular drug delivery (HUI & ROBINSON, 1985). They used polycarbophil, an acrylic acid based polymer lightly cross-linked which is able to absorb up approximately 100 times its weight in water at neutral pH, enabling high entanglement with the native mucin.

2.2.2 The Use of Mucoadhesive Polymers in Ocular Drug Delivery

As previously described, the bioavailability of ophthalmic drugs is very poor due to efficient protective mechanisms of blinking which rapidly removes foreign substances. Increasing the viscosity of artificial tear formulations by adding natural or synthetic

viscosifying agents prolongs their residence time on the corneal surface and increases the break-up time of the tear film(Ludwig, 2005a). On the other hand, results of a study on high-viscosity artificial tear solutions done by Diebold et al. showed that highly viscous solutions increase the friction coefficient between the eyelids and the tear film upon blinking, decreasing the lubricity between the surfaces and potentially damaging the ocular surface. Artificial tear solutions with high viscosity may also cause a sticky sensation, blurred vision or even irritation(Sintzel et al., 1996)(Ludwig, 2005b).

Polymer solution dropped in the eye will first encounter mucin at the cornea and conjunctival surface. If the polymer attaches to the mucin, the interaction is referred to as mucoadhesion. A volume of about 2 to 3 µLof mucous is secreted from the eyes daily (Mantelli & Argüeso, 2008).The turnover of the mucous layer in reported to be approximately 15 to 20 h, which is orders of magnitude slower than the tear turnover rate. Therefore the interactions between the mucoadhesive polymers and the mucous layer results in an increase in the precorneal residence time of the artificial tear solution. Mucoadhesive polymers are able to strengthen the mucous layer and therefore have been used in the development of efficient artificial tears(Calonge, 2001)(Ludwig, 2005b). Some mucoadhesive polymers showed protective and healing properties to epithelial cells in addition to increasing the bioavailability of the drugs (Calonge, 2001).

2.2.3 Mucoadhesivity of Boronic Acid Copolymers

Phenylboronate-functionalized water-soluble polymers are able to form complexes with carbohydrates(Khutoryanskiy, 2011). Specifically, phenyl boronic acid (PBA) is

known to form reversible covalent complexes with *cis*-diols and poly(hydroxlyated) polymers such as poly(vinyl alcohol)(Winblade, Nikolic, Hoffman, & Hubbell, 2000). Complexation of boronic acid copolymers with mucin is most assured at weakly basic pH values of ~7-9; as such,Ivanov et al. discussed the capability of boronic acid copolymers as mucoadhesives in nasal, ocular and buccal drug delivery(Ivanov, Nilsson, Galaev, & Mattiasson, 2008). In particular, results of a study done by Ivanov et al. showed that the copolymers of N-acryloyl-m-aminophenylboronic acid with N,N-dimethyl- acrylamide are capable of forming insoluble complexes with porcine stomach mucin at pH=9(Ivanov et al., 2008). The use of PBA grafts is especially attractive for promoting ocular mucoadhesion applications since sialic acid, the most prevalent carbohydrate residue found in the mucopolysaccharides comprising the mucin layer, contains *cis*-diol groups and thus should adhere strongly to PBA-containing polymers. Boric acid-based chemicals are also proven to be ocularly compatibile, as boric acid is commonly used as a buffer in artificial tears.

The potential drawback of strategies involving the conjugation of phenylboronic acids to improve mucoadhesion is that boronate must be in the tetrahedral ionic form to form a covalent complex with *cis*-diol-containing compounds. Phenylboronic acid groups alone have a pK_a of ~8.8(Kitano, Koyama, Kataoka, Okano, & Sakurai, 1991), which is significantly higher than the target ocular pH of 7.4. Thus only a small fraction of the phenylboronic acid moieties is expected to be ionized under physiological conditions (pH 7.4), resulting in poor mucoadhesion.

There is evidence that presence of amino groups coordinates to boronate to stabilize its ionized form (W. Chen, Lu, & Pelton, 2006)(Soundararajan, Badawi, Kohlrust, & Hageman, 1989) (Wulff & Vesper, 1978), leading to a reduction in the apparent pK_a of boronate groups. Therefore, conjugating the PBA moiety to the polymer through an amine linkage (and thus surrounding PBA with a highly basic environment) lowers the pK_a of boronic acid, facilitating the formation of covalent complexes with diol compounds at physiological pH. . The P(VP-co-VF) grafting platform is ideal in this respect since free amines are available as the conjugation sites and the adjacent NVP residues have highly basic tertiary nitrogen centres. The presence of nearby amine groups promotes bond formation at much lower pH values due to the interaction between nitrogen and boron.(System et al., 2006)(W. Chen, Leung, Kroener, & Pelton, 2009).

2.3 The Vitreous Humour

The vitreous humour is the major component behind the lens, which occupies two thirds of the volume of the eye (Swindle & Ravi, 2007). The human vitreous is a gelatinous substance composed of 98-99% water with a pH of 7-7.4, an intrinsic viscosity of ~ 4 cm^3/g and a refractive index of 1.3345-1.3348 (Gloor, 1987)(Swindle & Ravi, 2007). The vitreous has no blood vessels and derives its nutrition from the surrounding structures like the choroid, the ciliary body and the retina. The vitreous is probably never regenerated (Chatterjee & Agarwal, 1997)(Soman & Banerjee, 2003).Vitreous humour acts as a shock absorber, supporting the shape of the eye and the posterior surface of the lens. Its other roles are to hold the neural and the pigmented parts of the retina together

and to allow the circulation of metabolic solutes and nutrients(Snell, 1995)(Soman & Banerjee, 2003). The gelatinous nature of the vitreous body is the result of long collagen fibrils suspended in a pattern of hyaluronan macromolecules, which surrounds and stabilizes water molecules(Sebag & Balazs, 1989)(Yurchenco, 1994).The presence of both human vitreous hyaluronan (HA) and collagen together in their natural molecular architecture determine the viscoelastic properties of the vitreous (Swann & Constable, 1972).

2.3.1 Vitreous Humour Replacements

Vitreous and retinal pathologies (i.e. posterior segment eye disorders) are related as retinal tears can lead to vitreous displacement and vitreous scarring causes retinal detachment. Therefore successful treatment of vitreous pathologies such as vitreous opacities(Nema, 2012)(Snell, 1995), vitreous hemorrhage, and vitreous detachment as well as retinal pathologies such as retinal detachment, diabetic rethinopathy(Chatterjee & Agarwal, 1997) and age-related macular degeneration (ARMD) require partial or complete removal and replacement of the vitreous humour. Therefore vitreous substitutes may be required as a short-term temponade agent in treatment of pneumatic retinopexy or long-term temponade agent in cases of retinal detachment and degenerative changes in the vitreous. Vitreous substitutes may also allow for (or be incorporated in conjunction with) sustained release systems that could maintain therapeutic drug levels in the posterior segment of the eye over long periods (Colthurst, Williams, Hiscott, & Grierson, 2000).

Besides several physicochemical and optical properties, an ideal substance for a vitreous humour substitute should not cause inflammatory or foreign body reactions and should be inert and well tolerated by the tissues with which it is in contact (adjacent ocular tissues). These properties are generally referred to as ‘‘biocompatibility’’, which is generally assessed by performing *in vitro* and *in vivo* tests (Matteucci et al., 2007).

Currently available vitreous substitutes can be classified into three major categories: gases (air, perfluorocarbon gases), liquids (balanced salt solutions, perfluorocarbon liquids, semifluorinated alkanes, silicone oils, etc.) and gels. Gels used as vitreous substitutes are mainly polymeric materials and can be further grouped as natural (or semi-synthetic) polymers (such as methylated collagen, sodium hyaluronate, hyaluronic acid/collagen, and sodium carboxymethylcellulose) and synthetic polymers (such as poly(vinyl alcohol), poly(1-vinyl-2-pyrrolidone),(Hong, Chirila, Vijayasekaran, et al., 1996a; Hong et al., 1998), poly(acrylamide)(Swindle-Reilly et al., 2009), polyvinyl alcoholmethacrylate,(Cavalieri, Miano, D’Antona, & Paradossi, 2004), poly(glyceryl methacrylate), poly(2-hydroxythylacrylate), and poly(methyl-2-acrylamido-2-methoxyacetate).(Maruoka et al., 2006)(Chirila, Tahija, Hong, Vijayasekaran, & Constable, 1994). These synthetic polymers can be used in the synthesis of hydrogels to be used as retinal tamponade with the possibility of drug delivery by controlling network pore size(Kleinberg, Tzekov, Stein, Ravi, & Kaushal, 2011).

An ideal vitreous replacement would be a transparent, inert, hydrophilic material, which would be liquid outside of the eye (for ease of injectability) but gel once inside the

vitreous cavity with a high interfacial tension and high viscosity to serve as an effective tamponade agent. Currently available vitreous replacements satisfy only the biomechanical aspects of an ideal vitreous substitute; however, the generation of long-term nontoxic replacements remains a goal still to be achieved (Kleinberg et al., 2011).

2.3.2 Polymeric Vitreous Substitutes

The use of synthetic polymers as a replacement for the vitreous body began in the mid 1950's. Polyvinyl pyrrolidone was the first synthetic polymer to be experimentally used as a vitreous substitute in rabbits in 1954. PVP was used as solutions in water or aqueous salts and therefore had a short intravitreal time not suitable for vitreous substitutes(Chirila, Hong, Dalton, Constable, & Refojo, 1998). Injectable polymers of vinyl pyrrolidone were selected for clinical study to evaluate the effect of cross-linking on enhancing the retention time of vitreous substitutes (Hong, Chirila, Cuypers, & Constable, 1996).While PVP showed no significant inflammatory reactions, considerable biodegradation was observed for PVP hydrogels in short periods of about four weeks, which does not warrant its use as a longer-term vitreous substitute (although may be useful in the design of drug delivery devices for the posterior chamber (Hong, Chirila, Vijayasekaran, et al., 1996b; Hong et al., 1998)(Vijayasekaran et al., 1996).

Polyvinyl alcohol is another synthetic polymer that has successfully been used as vitreous replacement(Hara et al., 1998). However the users should be fully aware of the technology used for its manufacture. It has been shown that commercial PVA obtained by methanolysis is far more toxic to the ocular tissues than is the one obtained by

ethanolysis, which is virtually non-toxic (Snell, 1995).

2.4 Hydrophobic modified water-soluble polymers

Hydrophobic associating water-soluble polymers are water-soluble polymers that contain a small number of hydrophobic groups attached directly to the polymer backbone. The hydrophobic substituents (Tanaka, Meadows, Phillips, & Williams, 1990) are incorporated into the polymer molecule through proper copolymerization techniques(Schulz et al., 1987a) or chemical grafting (Landoll, 1982). The intermolecular association of neighbouring hydrophobic substituents gives rise to a three-dimensional polymer network and causes unusual rheological characteristics (Tanaka et al., 1990). As such, hydrophobic modified water-soluble polymers have achieved large-scale commercial acceptance since early 1980's, primarily as rheology modifiers (Schulz & Bock, 1991a)(J. Bock, Siano, Valint Jr, & Pace, 1989).

In aqueous solutions, the hydrophobic groups of these polymers associate to minimize their exposure to the solvent, analogous to the formation of micelles by surfactants above their critical micelle concentration. Therefore, the hydrodynamic size of the polymer increases which results in an increase in the solution viscosity. Amphiphilic polymers were prepared by hydrophobic substitution of various hydrophilic backbones such as biopolymers or synthetic charged or non-ionic polymers. Polysaccharides substituted with long hydrophobic alkyl chains have been used in different applications such as biologically active polymers(Suzuki, Mikami, Matsumoto,

& Suzuki, 1977), materials for immobilizing enzymes (Sandberg, Lundahl, Greijer, & Belew, 1987) or carrier gels (Butler, 1975).

The molecular weight of the polymer backbone and the degree of substitution and functionality of the grafts control the aqueous solution properties of the hydrophobic substituted derivatives. High molecular weight polymers normally show mechanical degradation at high deformation rates as well as permanent loss of high viscosity at low deformation rates. Therefore, the achievement of high viscosities at low shear rates without the need to increase the molecular weight (and therefore retain ability to regain high viscosities at low shear rates) is the major driving force in making hydrophobic associating water-soluble polymers (Taylor & Nasr-El-Din, 1998).

Hydrophobic interactions are not directly due to cohesive interactions between molecules and therefore differ from other interactions such as van der Waals interactions, hydrogen bonds, and electrostatic interactions. Instead, the driving forces for hydrophobic associations results from the desire to minimize the structuring of the water molecules surrounding the hydrophobic sites. As such, the magnitude of the hydrophobic interaction is determined by a number of factors including the nature of the hydrophobic chain and the degree of substitution, temperature, the ionic content of the polymer solution and polymer concentration.

Similar to micellization, which occurs above a critical concentration, intermolecular hydrophobic association only occurs above a critical polymer concentration(Tanaka et al., 1990). Therefore, interesting physicochemical properties in aqueous solutions within specific concentration ranges are observed (Sinquin, Hubert, & Dellacherie, 1993). At

very low concentrations, hydrophobic interactions may exist but they can only have a small effect on the overall mechanical properties of the solutions. In solutions with polymer concentrations lower than the critical one, If the reduced viscosity is plotted versus polymer concentration for associating and non-associating polymers, the critical concentration above which the associating polymer shows enhanced viscosity is known as the overlap concentration, or the critical aggregation concentration(*C**)(Schulz & Bock, 1991b). Below *C**, the introduction of hydrophobic groups results in a solution viscosity that is either lower (Magny, Iliopoulos, & Audebert, 1991)(Tanaka et al., 1990) or slightly higher (Charles L. McCormick, Nonaka, & Johnson, 1988) than that of the precursor, as intrachain aggregation competes with interchain crosslinking. Typically, intramolecular associations leads to the contraction of the polymer chains, which also results in a decrease in the intrinsic viscosity and an increase in the Huggins constant (Magny et al., 1991). Above *C**, interchain interactions become more efficient and a large increase in viscosity is observed. As such, it is meaningful to examine the viscosity of associating polymers in two concentration regimes: a dilute regime, where polymer concentration is less than the critical overlap concentration and a semi-dilute regime, where polymer concentration is higher than the overlap concentration.

The length of the hydrophobic graft also plays a significant role in determining the solution viscosity. The longer the alkyl chain or the larger the degree of substitution of hydrophobic groups on the backbone polymer, the larger the viscosity increase is observed. Desbrieres et al. showed that in hydrophobic modification of chitosan a minimum of six C atoms along the alkyl chain are necessary to observe the hydrophobic

interactions. Annable et al. also showed that in a solution of end-capped PEG polymers the length of the hydrophobic tail must exceed 6 carbons to influence the viscosity (Clinton, 1975). It is also known that there is an upper limit in the degree of hydrophobic modification. A too high modification degree and/or the selection of hydrophobic tails that are too long will limit the solubility of the polymer material.

Hydrophobic graft concentration also strongly affects the associative interactions observed. In order for the polymer chains to form networks, each polymer chain must contain at least two hydrophobic tails on average, which means that the modification degree must exceed a certain threshold value. Bock et al. examined the variation of the reduced viscosity with polymer concentration for polyacrylamide/*N*-octylacrylamide copolymers having hydrophobe contents of 0.75 and 1 mol%(Jan Bock & Valint Jr., 1989). At a hydrophobe content of 0.75 mol%, they found that the viscosity significantly increased as polymer concentration was increased because of intermolecular association. Higher viscosities were observed for polymers with higher hydrophobic contents (1 mol%). Results of their study also showed that at high polymer concentrations low amounts of the hydrophobic substitutes are able to increase the viscosity by orders of magnitude, showing the power of this approach.

Bock et al. also examined the effect of polymer molecular weight on hydrophobic associations in *N*- octylacrylamide/acrylamide copolymers(J Bock, 1989). Results showed that at a given polymer concentration, increasing the molecular weight resulted in higher viscosity. However, an increase in the polymer molecular weight or hydrophobic chain length also decreases the required hydrophobic content to make the polymer

insoluble. Obviously, this will limit the maximum hydrophobic content that can be introduced into an associating polymer. Introducing ionic character on the polymer backbone leads to an increase in the solubility of associating polymers in water (C.L; McCormick, Bock, & Schulz, 1989) as well as enables modification of the rheological properties of the associating polymers**.**

Hydrophobic molecules tend to dissolve in the polymer hydrophobic domains, with surfactants particularly prone to binding to these regions.(Butler, 1975).. It is well known that the viscosity of semidilute solutions of associative polymers is sensitive to the presence of surfactants.

If hydrophobic associations are sufficiently strong in such systems, the polymer solution is considered to convert to a physical gel. In contrast to chemical gels, in which covalent bonds form the crosslinking points of the network and the network is thus (at least at a specific time) functionally irreversible, physical gels are reversible and formed by cooperative physical bonds such as hydrogen or electrostatic bonds and hydrophobic interactions. The 'gel' term can be used in different conditions. Based on Almdal and Kramer studies (Almdal, Dyre, Hvidt, & Kramer, 1993),the systems characterized by specific mechanical properties in which the storage modulus G'(ω) presents a plateau with frequency (ω) and the loss modulus G "(ω) is smaller than G'(ω) (with a factor 5 or 10) will be considered as gels.

2.4.1 Synthesis of associating polymers

Associating polymers have been prepared by two general methods. The first

method is the copolymerization of water-soluble and hydrophobic monomers. In these copolymers, the hydrophobic group is a segment of the polymer backbone. The second method is the modification of polymers after polymerization to introduce hydrophobic or hydrophilic groups via grafting.

A large fraction of reported associating polymers have been prepared using acrylamide as the base monomer via free radical polymerization. Most of the preparations of associating polymers have used a micellar radical polymerization technique, in which a surfactant such as sodium dodecyl sulfate (SDS) is used in an aqueous solution to solubilize the hydrophobic monomer. Micelles of SDS may then contain molecules of the hydrophobic monomer. Impurities such as alcohols or heavy metal cations could interfere with the polymerization, resulting in polymers of reduced molecular weight. Acrylamide is among the monomers most suited for the manufacture of high molecular weight water-soluble polymers; as such, although other monomers have been used to prepare associating polymers, acrylamide has been the most successful at producing water-soluble associating copolymers that are effective at polymer concentrations below 1 mass%. (Taylor & Nasr-El-Din, 1998)

Other radical-based techniques have also been reported to be useful in synthesizing associative polymers. Polymerization in a microemulsion containing water, monomers, surfactant and oil has been reported (Turner et al., 1985c), although this technique was largely superseded by the introduction of micellar polymerization. Associating polymers have also been prepared using hydrophobic monomers that are surfactants. These

hydrophobic monomers (Schulz et al., 1987b)(Peiffer, 1990) have been called 'surfomers'.

The second method to make hydrophobic associating polymers is incorporating hydrophobic groups into the polymer *after* the polymerization process. The advantage of this approach is that commercially available polymers can be used as starting material. (Taylor & Nasr-El-Din, 1998) , with grafting occurring via a range of available functional group chemistries. In this method, the polymerization and grafting steps can be fully decoupled in terms of solvents, surfactants, etc., allowing for more control over the process. However, grafting yields can be somewhat lower than monomer incorporation via polymerization, demanding a balance when selecting the most appropriate technique.

2.5 Materials Used In This Study

2.5.1 Poly vinylpyrrolidone

A class of water soluble polymers widely used in medical practice is poly(*N-vinylamides*), a family of polymers that differ in solution properties depending on their side group substituents (Kirsh, 1998)**.**

Polyvinylpyrrolidone, also called by the trade name Povidone or the acronyms PNVP or PVP, is the most commercially-used poly (*N-vinylamide*) due to its unique combination of chemical, physicochemical and biological properties which makes it widely used in various biomedical applications. PVP is one of the numerous products of

the acetylene chemistry and was first synthesizd by Reppe **et al** in 1930s (Haaf, Sanner, & Straub, 1985). Acetylene and formaldehyde react to form 1,4-butine dioal, which is then hydrogenated to butane diol. Butyrolactone is formed by oxidative cyclization and then reacted with ammonia; following to which water is removed and pyrrolidone is formed. Finally the vinyl group is introduced to form N-vinylpyrrolidone. The course of these reactions is shown in Figure 2-3.

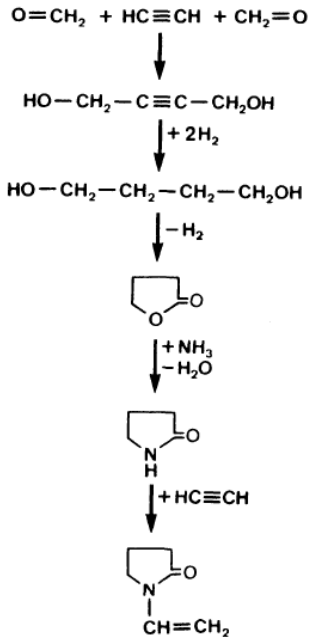


Figure 2-3 Synthesis of N-vinylpyrrolidone from acetylene and formaldehyde

N-vinylpyrrolidone (NVP) is a yellowish, hydrophilic, nonionic monomer that readily polymerizes thermally (Bork & Coleman, 1960) or photolytically to poly(N-vinylpyrrolidone) (PNVP). Unlike the building block N-vinylpyrrolidone, the polymer (PNVP) is non-toxic. Figure 2-4 shows the polymerization of vinylpyrrolidone.

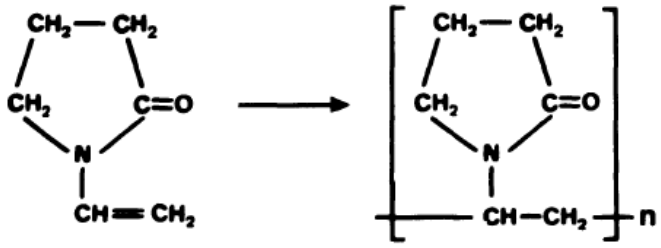


Figure 2-4 Polymerization of vinylpyrrolidone

PVP is soluble in water and in a range of organic solvents due to the presence of non-polar (methane and methylene) and polar (cyclic amide) groups in its backbone(Kirsh, 1998).Hence, PVP can be used for its amphiphilic properties as a surfactant for the stabilization of heterogeneous systems.(Nguyen, Eagles, Davis, Barner-Kowollik, & Stenzel, 2006). However, of particular relevance to this application, PVP is among the most highly water-binding non-ionic synthetic polymers available, with a water content of 1.37 g/g polymer capable of being bound. As such, PVP can tolerate significant hydrophobic grafting without becoming insoluble, key to the formation of associative networks.

The most common method for the industrial preparation of PVP-based polymers is free radical polymerization, in large part due to its tolerance to impurities. High molecular weight polymers can be obtained by free radical polymerization at moderate temperatures (20 – 150 ºC) and atmospheric pressure within short reaction times. The three principal steps of a free radical polymerization are: (1) the initiation of the active monomer; (2) propagation or growth of the active (free-radical) chain by sequential

addition of monomers; (3) termination of the active chain to give the final polymer product. In general, free radical polymerization method has a very rapid propagation rate due to the extremely high k_p /k_t ratio (where k_p is the propagation rate constant and k_t is the termination rate constant). One drawback of the free radical polymerization method is that high reactivity of radical species results in high polymerization rates, enabling the occurrence of side-reactions, resulting in broad molecular weight distributions, and leading to a lack of control over the chain end compositions.

Polymers of vinylpyrrolidone with degrees of polymerization of 10-100,000 (corresponding to molecular weights of 1000-10 million) are easily obtained by free radical polymerization. Polymerization of vinylpyrrolidone can be done either in bulk, solution or suspension polymerization in aqueous solution using a variety of initiators that can lead to a variety of end groups of interest (Francois, 1996). More controllable end groups may be obtained by solution polymerization in organic solvents, since the solvents may act as chain transfer agents to produces low molecular weight products with well-defined terminal chemistries. Figure 2-4 shows a scheme of polymerization mechanism of vinylpyrrolidone in organic solvents.

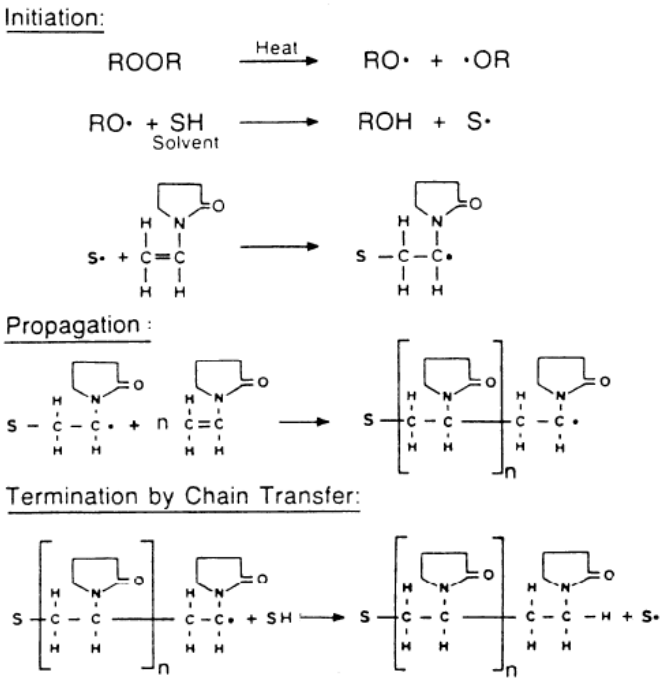


Figure 2-5 Polymerization mechanism of VP in organic solution

2.5.1.1 Applications of PVP

PVP is a good example of a polymer used for its properties in solution. These properties include solubility in water and polar organic solvents and even in blood plasma. PVP is a nontoxic polymer which crosslinks into hydrogels with high water uptake. It also possesses adhesive properties and is able to complex with a number of salts and acids. As such, PVP is one of the most important water-soluble polymers used in a variety of fields such as medicine, pharmaceuticals, cosmetics and food (Kirsh, 1998).

PVP has been widely reported to be biocompatible in a variety of biological environments due to its reduced protein and bacterial adhesion,(Kristinsson, 1989), anti-thrombopoietic activity(Francois, 1996)(Abraham, de Queiroz, & Román, 2001) and ability to stimulate the growth of endothelial cells(Källrot, Edlund, & Albertsson, 2006)(Smith, Rimmer, & MacNeil, 2006). As a result, medical applications of PNVP-based hydrogels are now wide-spread, including drug delivery systems, artificial muscles, wound dressing and tissue engineering (Atta & Arndt, 2004) (Jeong, Kim, & Bae, 2012).

PVP has also been applied in other environmental and pharmaceutical applications. Complexion of iodine with linear PVP solubilizes small molecules via interactions with the polymer and leads to an effective disinfectant of very low toxicity (Sneader, 2005). PVP is used as a binding or film forming agent for tablets, a solubilizing agent for injections,a disintegration agent for drug tablets, and an excipient for proteins, peptides and drug molecules, as it reduces the toxicity and increases the solubility of these compounds (Kamada et al., 2000)(Tsunoda et al., 2000). PVP can also be used to tune the viscosity and isotonicity of aqueous solutions (e.g. use as plasma expander during the Second World War(Haaf et al., 1985). As such, PVP is one of the most important water-soluble polymers used in a variety of fields (Kirsh, 1998).

2.5.1.2 PVP Copolymers

One possible way to tune the properties of a polymer for a particular application is

to copolymerize it with another comonomer. Hydrogels formed from copolymerization of PNVP and methacrylates are used in many applications in biomedicine, especially in contact lenses (Y. Lai, 1997). Incorporation of vinyl acetate comonomer in PVP results in a poly(vinylacetate – co – N-vinyl pyrrolidone) copolymer with reduced hygroscopy (industrialized under the name copovidone by BASF) that is used in the dry state as an excipient for therapeutic tablets. PVP can be used to make hydrogel scaffolds for tissue engineering and polymeric micelles for the solubilization of hydrophobic drugs(Kamada et al., 2000; Le Garrec et al., 2004)(Kuskov et al., 2007). PVP and NVP copolymers can also be used as polymeric carriers for tumor targeted protein therapy and diagnosis.(Chin, Heng, Bhuvaneswari, Lau, & Olivo, 2006; Kamada et al., 2000; Kishida, 2003), fuel cell membranes,(Qiao, Hamaya, & Okada, 2005; Smitha, Sridhar, & Khan, 2006), and asymmetric membranes for gas separation (Koonjul, Brandt, Lindsey, & Farrant, 1999; Zhang, Liang, Meng, Lu, & Liu, 2007).

2.5.2 Poly vinylformamide

Other important poly(*N*-vinyl amides) include poly(vinylformamide) (PVF) and its copolymers. N-vinylformamide (NVF) is an isomer of acrylamide, which readily polymerizes to poly (N-vinylformamide). NVF is a water-soluble monomer and the key compound in the synthesis of linear cationic polymers with primary amine groups. Polymers incorporating NVF can be used in many of the same applications as acrylamide polymers. Since NVF shows higher reactivity and lower toxicity than the more common acrylamide monomer, it provides a wider range of applications. NVF is a liquid monomer

with a molecular weight of 71 g/mol and a high boiling point of 80°C at 10mbar. NVF is also soluble in a large variety of organic solvents. NVF is neither a carcinogen nor a neurotoxin.

Polymerization of NVF is carried out by free radical polymerization, facilitating facile copolymerization of NVF with a large number of other monomers to form products of various molar masses. The presence of a weakly acidic proton on the nitrogen flanked by the formyl and vinyl groups(Tzeng & Hou, 2008) facilitates the hydrolysis of the formate to form an amine, providing one of the few industrially-relevant pathways to the generation of amine-containing polymers using a free radical process. Polymers with primary amine functionalities show cationic nature in appropriate pH regimes(Gu, Zhu, & Hrymak, 2002a) and high reactivity for various post derivatization reactions and are therefore of great interest. Unfortunately, the simplest precursor monomer to PVAm (vinylamine) is unavailable because it tautomerizes to acetaldehyde imine(Mishra, 2008). Strongly basic polyvinylamines (PVAm) can instead be easily synthesized by cleaving the formyl group by hydrolysis, achievable with NVF under relatively mild hydrolysis conditions (Tzeng & Hou, 2008). Figure 2.5 shows the process of hydrolysis.

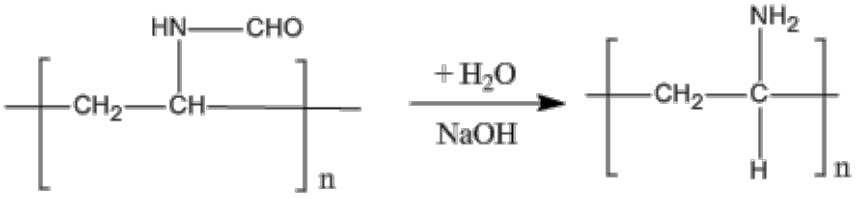


Figure 2-6 Hydrolysis of the amide groups

High charge density and high reactivity of the primary amine groups makes PVAm-based polymers and copolymers relevant in various fields. PVAm shows excellent adhesion to anionically charged biological surfaces, such as cellulose, skin, and hair(Odian, 2004), leading to use as paper modifiers and personal care products. They are also used for chromatographic support, heterogeneous catalysis and biocompatible implant layers.

2.6 Objectives

To generate highly flexible, shear-thinning polymers appropriate for use as dry eye treatments and vitreal replacements, this thesis applies the principles outlined in the literature review to produce a novel dual-grafted polymer based on a poly(NVP-co-NVF) backbone. The random copolymerization kinetics of NVF and NVP allows for incorporation of functional groups at statistically random points along the polymer chain, while selective (and sequential, if desired) hydrolysis can be used to expose the reactive amine groups for subsequent grafting reactions. Hydrophobes of length C_{10}-C_{18} will then be grafted to this reactive platform to create shear-associative physical hydrogels, taking advantage of the high hygroscopicity of the PVP backbone to maintain highly hydrated systems even at higher degrees of grafting and/or grafting of longer hydrophobes. This shear thinning material is targeted for use as a vitreal humour replacement. For dry eye treatment, a second grafting reaction will be conducted to attach phenylboronic acid groups to the polymer, using reductive amination to produce a secondary amine linkage,

reducing the pK_a of the PBA group to an ocularly-appropriate range. This dual grafted polymer (hydrophobic and PBA grafts) can interact specifically with each layer of the tear film: mucoadhesion with the mucous layer (to prolong the residence time of the material on the eye), hydrophobe associative gel formation (as well as association with the aqueous mucous) in the aqueous layer (further prolonging the residence time), and hydrophobe-lipid interactions in the lipid layer (assisting to stabilize the lipid layer). Such a design is anticipated to have significant advantages in the alleviation of dry eye conditions and/or for delivery of drugs to the front of the eye.

3. Design and Synthesis of Dual Hydrophobic-Boronic Acid Graft Copolymers

3.1 Design and Synthesis of a Poly(vinyl pyrrolidone)-Based Grafting Platform

3.1.1 Organic-Phase Free Radical Copolymerization of N-vinylpyrrolidone (NVP) and N- vinylformamide (NVF)

In copolymerization of two monomers when the reactivity ratios of both monomers equal unity, there is no preferential monomer incorporation into the propagating chain. This results in a random copolymerization with tailored composition of the monomers.(Kirsh, 1998) N-vinylpyrrolidone (NVP) and N-vinylformamide (NVF), copolymerized at 65°C in isopropanol, undergo such an azeotropic copolymerization to produce a fully random polymer (P(VP-Co-VF). The favorable random copolymerization kinetics is based on the conformational structure of the amide groups in VP and the formation of hydrogen bonding conformers between the monomers during the polymerization (Kirsh, 1998).

3.1.2 Experimental

N-Vinylpyrrolidone (NVP) (>99%, Sigma Aldrich) and N-vinyl formamide (NVF)(98%, Sigma Aldrich) were purified prior to use. Monomers were mixed and stirred with Dowex 50W X8 (Hydrogen form, Sigma Aldrich) for 24 hours and then passed through an alumina basic column to remove inhibitors. Dimethyl 2,2'-azobis(2-methylpropionate) (AIBMe) (Wako Chemicals, U.S.A) was used as received.

Copolymerization was conducted in isopropanol as both a solvent and a chain

transfer agent to control the molecular weight of the product. Dimethyl 2, 2'-azobis(2-methylpropionate) (AIBMe) was used as the polymerization initiator. AIBMe is a non-nitrile azo initiator used as an alternative to AIBN, which decomposes at relatively low temperatures (66°C in toluene) and has no potential to prematurely hydrolyze the amide side-chains on NVP and NVF, unlike peroxide-based initiators.

Copolymers of NVP with NVF with different molecular weights were prepared by free-radical polymerization. Copolymerization reactions were carried out in 3-neck glass round bottom flasks under nitrogen purge and continuously stirred using magnetic stirrers. The monomer-isopropanol mixtures was pre-mixed, purged with nitrogen and heated to reaction temperature of 75 °C before the addition of the initiator solution (AIBMe in isopropanol). Copolymer samples with different molecular weights were made by varying the concentration of monomers and initiator in the isopropanol solution. Recipes of the P(VP(90%)-VF(10%) copolymers produced are shown in Table 3-1.

Table 3-1 Recipes used to make the P(VP(90%)-VF(10%)) samples

Recipe number	Monomer Vol%	Initiator wt%	Solvent/chain transfer agent	Initiator
1	15	0.015	Isopropanol	AIBMe
2	20	0.03	Isopropanol	AIBMe
3	30	0.05	Isopropanol	AIBMe
4	30	0.1	Isopropanol	AIBMe
5	50	0.1	Isopropanol	AIBMe
6	50	0.08	Isopropanol	AIBMe

Upon completion of the reactions after 24 hours, copolymers were purified by evaporating the isopropanol solvent, dissolving the copolymer in water, and dialyzing against Milli-Q water (using MWCO 1000 membranes) followed by lyophilizing and storing at room temperature in sealed containers. Figure 3-1 shows a schematic of the copolymerization reaction.

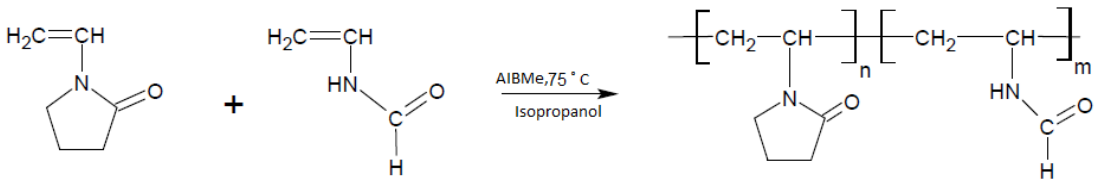


Figure 3-1 Synthesis of the poly (vinylpyrrolidone-co-vinylformamide) graft platform

3.1.3 Characterization of the P(VP-VF) Copolymer

3.1.3.1 Copolymer Composition by Nuclear Magnetic Resonance Spectroscopy

Composition analysis was done by ^{1}H-NMR (Bruker 200 MHz spectrometer) in deuterated water. The ^{1}H-NMR spectrum of a P(VP-VF) sample with 10 mole % NVF and 90 mole% NVP in the monomer feed is shown in Figure 3-2. The copolymer composition was determined based on the areas of the peaks representing the CH_2 in the pyrrolidone ring of NVP (~2.9-3.4 ppm) and CH in the formamide group of NVF appearing at ~7.4-8.1 ppm. Figure 3-3 shows the ^{13}C-NMR spectra for the P(VP-Co-VF). ^{1}H-NMR peak assignments for the whole structure are explained in detail in Appendix A.

As explained earlier, the ratio of the NVP and NVF residues in the copolymer chains was found to be the same as that in the monomer mixture. The molar composition of the NVF residues in the copolymer chains can be calculated from Equation 3-1.

$$\text{NVF\%} = \frac{\text{Foramide grouppeak area}}{\text{Formamide group peak area} + \frac{\text{CH2 peak area}}{2}} \qquad \text{(Equation 3-1)}$$

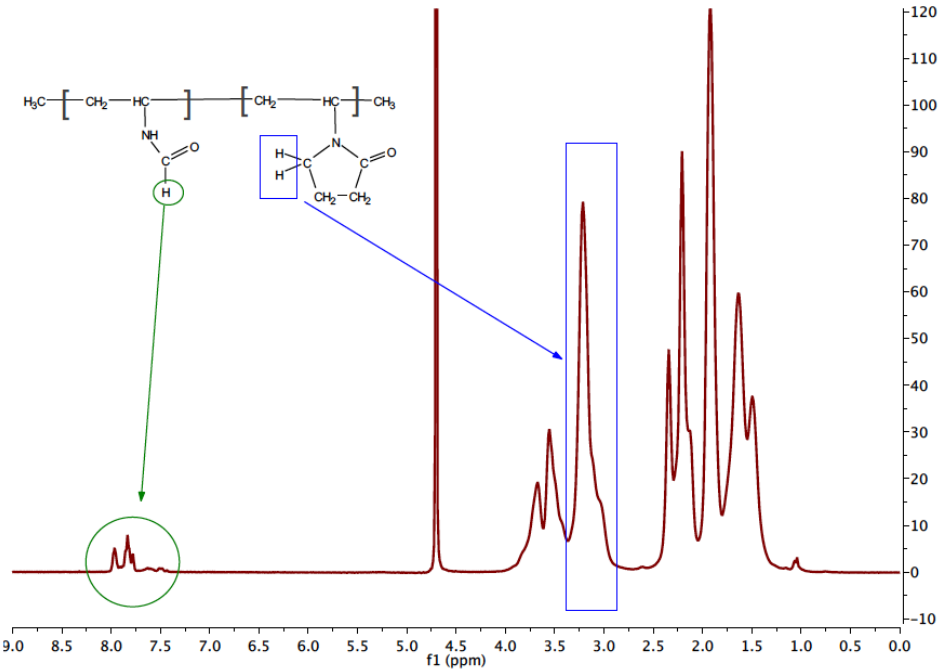


Figure 3-2 ^{1}H-NMR spectrum of the P(VP(90%)- VF(10%)) copolymer

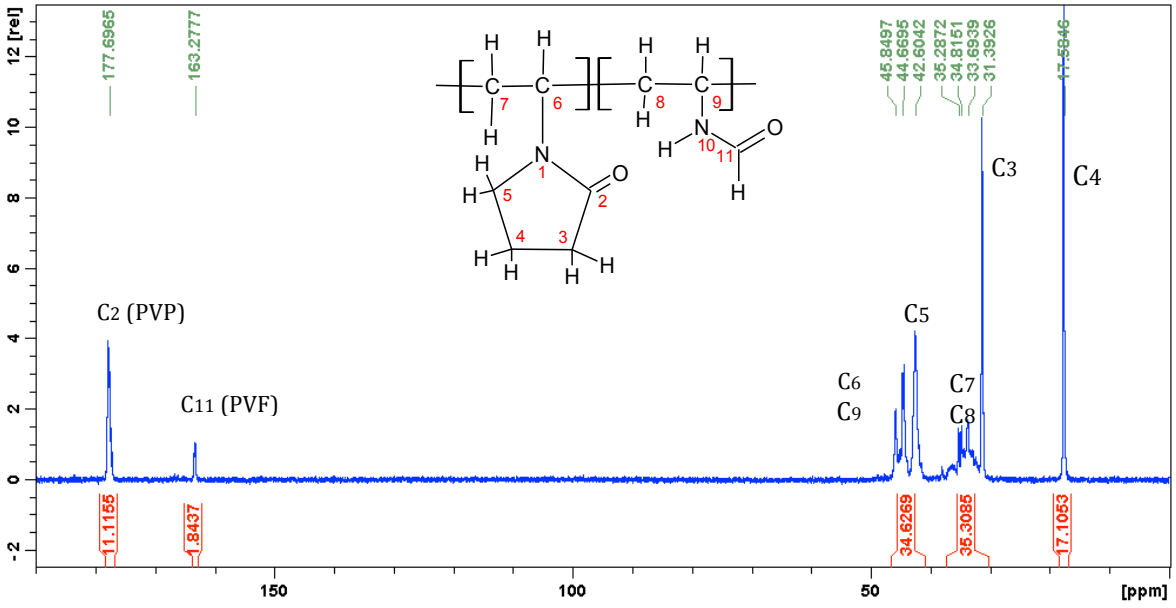


Figure 3-3 ^{13}C-NMR spectrum of P(VP(90%)-VF(10%))

3.1.3.2 Gel Permeation Chromatography

DMF GPC was used to determine the molecular weights of the P(VP-VF) copolymers. PEG standards were used as a reference polymer to calculate M_n, M_w, and PDI values. However the observed retention times yielded much lower molecular weight values than anticipated compared to commercial polyvinylpyrrolidone samples with known average molecular weights (10 kDa, 40 kDa, 55kDa and 360 kDa) (Sigma Aldrich) run under the same conditions. This suggested that the PEG standards were may not be appropriate for molecular weight determinations of the P(VP-VF) polymer. Values of M_w predicted by the PEG calibration for the commercial samples were consistently approximately one third of the M_w reported by the supplier for the PVP polymers; therefore, in order to obtain the most accurate values possible, the values of M_w calculated from the raw GPC chromatogram were multiplied by 3 to estimate the actual molecular weights. M_n and M_w were calculated as follows from the raw GPC chromatogram:

$$M_N = \frac{\Sigma N_i M_i}{\Sigma N_i} \qquad \text{Equation 3-2}$$

$$M_W = \frac{\Sigma N_i {M_i}^2}{\Sigma N_i} \qquad \text{Equation 3-3}$$

Table 3-2 shows M_n, M_w, and PDI for the P(VP(90%)-VF(10%)) samples made by varying the composition of the monomers and the initiator. The corresponding GPC chromatograms for polymers with different molecular weights are shown in Figure 3-4 and are compared to the GPC chromatograms for commercial PVP samples with known molecular weights in Figure 3-5.

Table 3-2 Molecular weights and molecular weight distributions of the P(VP(90%)-VF(10%)) polymers prepared for this work

Sample Code	Recipe number	Mn (Da)	Mw (Da)	PDI	Corrected Mw (Da)
P(VP(90%)-VF)-55kDa	1	8600	18800	2.2	56500
P(VP(90%)-VF)-170kDa	2	17400	56200	3.2	168000
P(VP(90%)-VF)-185 kDa	3	13000	62000	4.8	186000
P(VP(90%)-VF)-255 kDa	4	16000	85000	5.3	255000
P(VP(90%)-VF)-295 kDa	5	23000	98000	4.3	294000
P(VP(90%)-VF)-410 kDa	6	29000	137000	4.7	411000

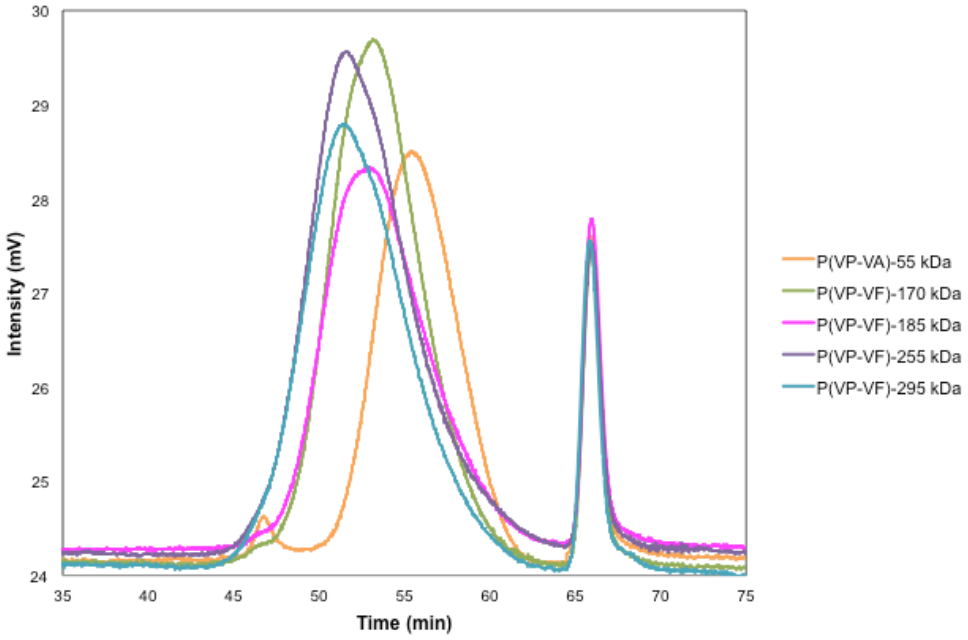


Figure 3-4 Raw GPC chromatograms of P(VP-VF) samples with different molecular weights

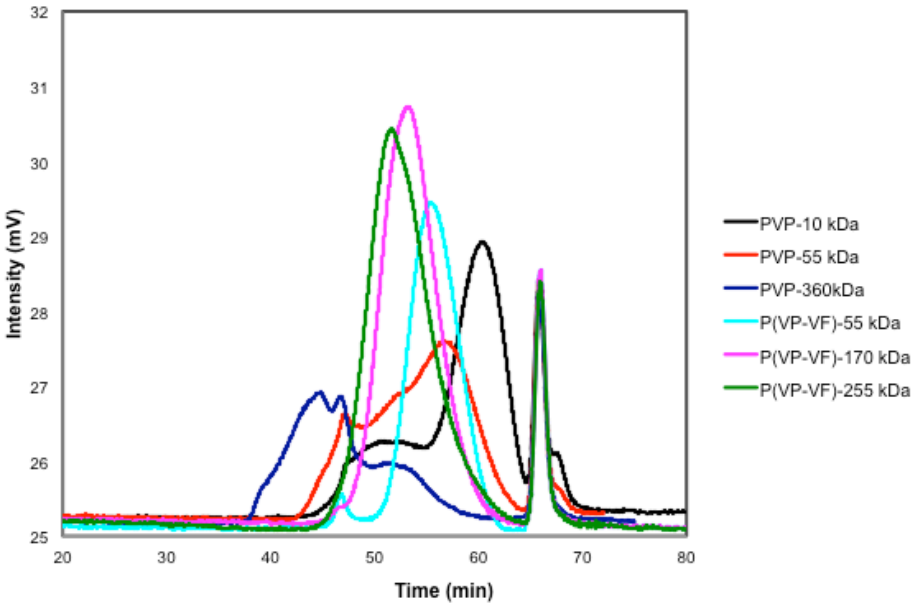


Figure 3-5 GPC chromatogram of P(VP-VF) samples compared to commercial PVP samples with known M_w; PVP-10 kDa, PVP-55 kDa, and PVP-360 kDa are the commercial samples

3.2 Synthesis of P(VP-VA) through hydrolysis of the NVF residues

3.2.1 Hydrolysis of Poly(N-vinylformamide)

Hydrolysis of the randomly-distributed and selectively-hydrolyzable NVF residues within the P(VP-VF) chains produces functional primary amine groups which are highly reactive toward subsequent grafting reactions.

Poly(N-vinylformamide) (PNVF) has no alkyl group on its side chain; therefore, hydrolysis of NVF polymers and copolymers to amino-functionalized polymers under acidic or basic conditions is relatively faster and requires milder conditions than other poly(vinylamine) precursors (Yamamoto et al., 2003). Furthermore, NVF has a much lower hydrolytic stability than NVP; NVP slightly hydrolyzes when subjected to aggressive hydrolysis conditions (Conix & Smets, 1955; Frank, 1954).

PNVF hydrolysis under acidic conditions exhibits limited conversions (about 60-70mol% conversion) because of the charge repulsion effects among the cationic amine groups generated during hydrolysis and proton hydrates(Gu, Zhu, & Hrymak, 2002b). In comparison, it has been reported that nearly complete amide conversions can be achieved by basic hydrolysis(Badesso, Nordquist, Pinschmidt, & Sagl, 1996; Badesso, Pinschmidt, & Sagl, 1993) However, opinions presented in the literature on conversion limits achieved by basic hydrolysis are ambiguous. Some studies have shown that both acidic

and basic hydrolysis of PVF under proper conditions can potentially achieve almost 100% conversion. (Yamamoto et al., 2003), while others argue that complete conversion only occurs under basic hydrolysis conditions. (Pinschmidt, Wasowski, Orphanides, & Yacoub, 1996) and others report that neither basic nor acidic hydrolysis can achieve full conversion. (Witek, Pazdro, & Bortel, 2007) Of particular significance, Witek et al. showed by ^{13}C-NMR analysis that basic hydrolysis of poly(vinylformamide) results in some formamide groups being converted into OH groups, resulting in a copolymer consisting of vinylamine and a significant share of vinyl alcohol units.

Figure 3-6 shows a schematic of basic hydrolysis of the P(VP-VF) polymer to form the P(VP-VA) graft platform.

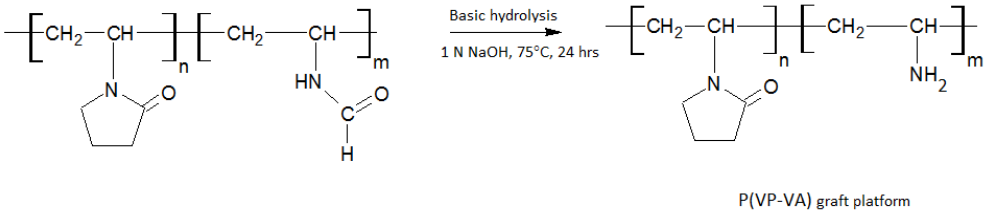


Figure 3-6 Basic hydrolysis of the P(VP-VF) copolymer to obtain the P(VP-VA) graft platform

3.2.2 Experimental

A three-necked round bottom flask, equipped with a reflux condenser and a magnetic stirrer, was charged with 500 mg of P(VP-VF) dissolved in 250 mL of 1N NaOH (polymer concentration 0.2 wt%) and heated up to 75°C. In order to investigate

the effect of reaction time on the degree of hydrolysis, 25 mL of the solution was taken out of the flask at time intervals of 2,4,6,8,10,12 and 24 hours and neutralized with concentrated HCl solution to pH 6 in order to stop further hydrolysis. Solutions were dialyzed against Milli Q water for 3 days, changing the dialysis water every 6 hours, and lyophilized. Potentiometric-conductometric titration and ^{1}H-NMR were conducted to determine the degree of hydrolysis for each sample. Figure 3-7 shows hydrolysis conversion versus reaction time. The highest conversion achieved by basic hydrolysis (1N NaOH , 75 °C) was around 87%. Tests were replicated 4 times at reaction durations of 12 hours and 24hours.

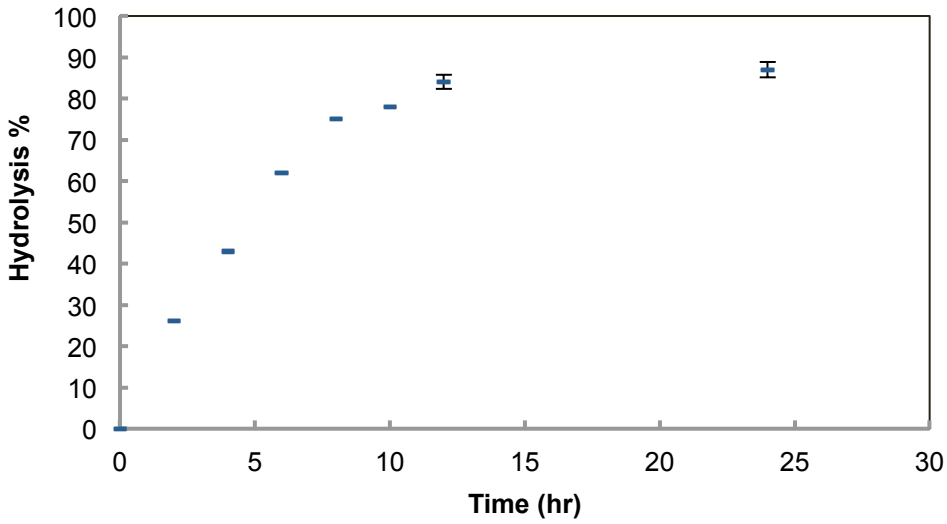


Figure 3-7 Effect of reaction time on the degree on hydrolysis achieved

3.2.3 Characterization of The P(VP-VA) Grafting Platform

3.2.3.1 Amine Content by Nuclear Magnetic Resonance Spectroscopy

The amine content of the hydrolyzed P(VP-VF) samples were determined by ^{1}H-NMR. With the progress of the hydrolysis reaction, the amide groups convert into amine groups and therefore the area of the amide group peak (7.4-8.1 ppm) diminishes. The molar conversion of hydrolysis can be calculated by monitoring the change in the amide group peak area. Figure 3-8 shows the decay in the formamide peaks with the progress of the hydrolysis reaction.

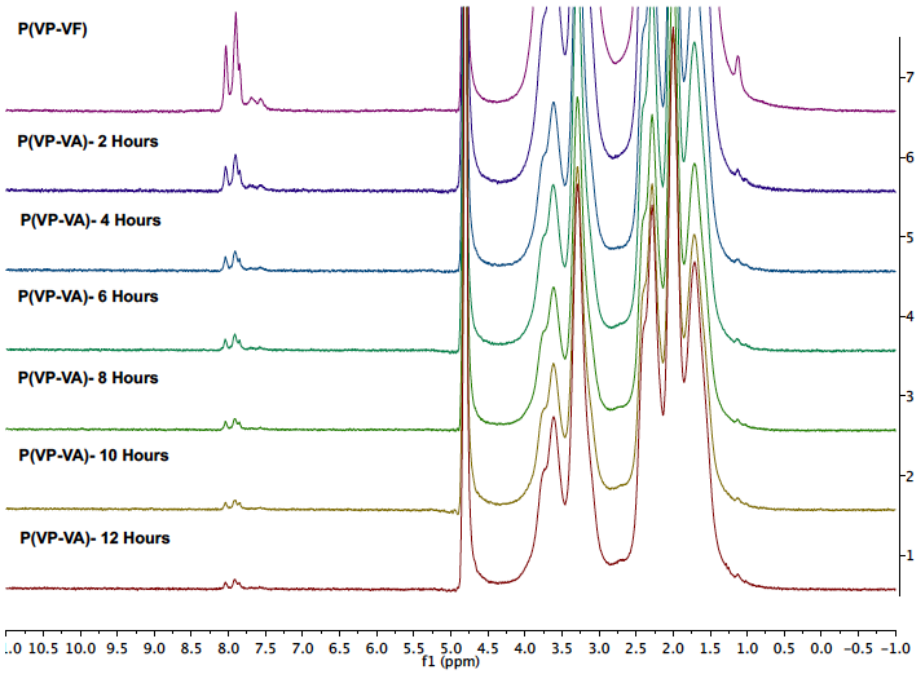


Figure 3-8 Decay of the formamide peaks (chemical shift ~7.8) with the progress of the hydrolysis reaction

Figure 3-9 shows the ^{1}H-NMR spectra for the P(VP-VF) copolymer and the corresponding P(VP-VA) polymer hydrolyzed under basic conditions. The degree of hydrolysis can be calculated from Equation 3-4.

Equation 3-4

$$Hydrolysis\% = \frac{Formamide\ peak\ area_{P(VP-VF)} - Formamide\ peak\ area_{P(VP-VA)}}{Formamide\ peak\ area_{P(VP-VF)}}$$

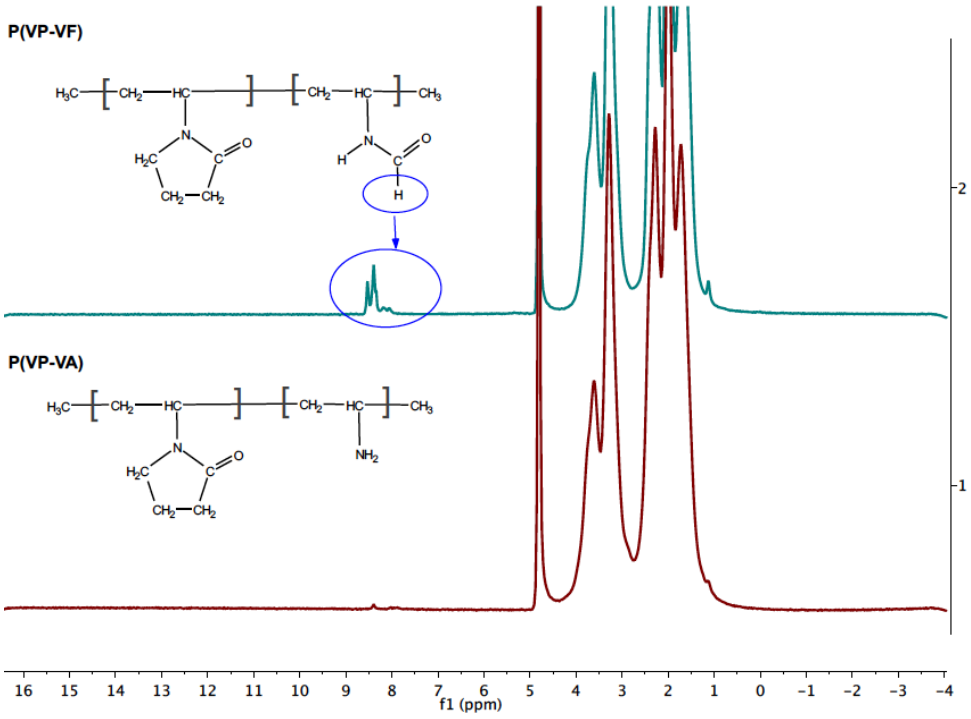


Figure 3-9 NMR spectra of P(VP(90%)-VF) and the corresponding P(VP(90%)-VA)

3.2.3.2 Amine Content by Potentiometric-Conductometric Titration

Degree of hydrolysis can also be quantitatively measured by potentiometric-conductometric titrations of the amine groups by dissolving 50mg of the P(VP-VA) polymer into 50mL water. HCl was added drop-wise to adjust the initial solution to pH~2.5. Samples were then titrated with NaOH (0.1M) using a delta pH/mV of 80, minimum single injection of 0.0010mL to a maximum single injection of 0.04mL with 45 seconds between each injection. Both pH and conductivity were recorded as a function of the volume base added (Mandel PC Titrator).

For all samples, degree of hydrolysis obtained by titration of the amine groups was lower than the degree of hydrolysis obtained from the ^{1}H-NMR spectra. Figure 3-10 compares the ^{1}H-NMR spectra of P(VP(90%)-VF)-55 kDa and the corresponding P(VP-VA) hydrolyzed for 24 hours. The intensity of the signals representing the formamide groups decreases to zero in the P(VP-VA) spectrum, indicating complete loss of amide groups. In comparison, the titration curves shown in Figure 3-11 indicate that the number of titratable amine groups present in 25 mg of the P(VP-VA) sample was 1.91×10^{-5} indicating that only 87% of the formamide groups were hydrolyzed into amines. Therefore it can be concluded that the diminished formamide groups were not all converted into amine groups, presumably leading to the generation of some residual –OH groups.

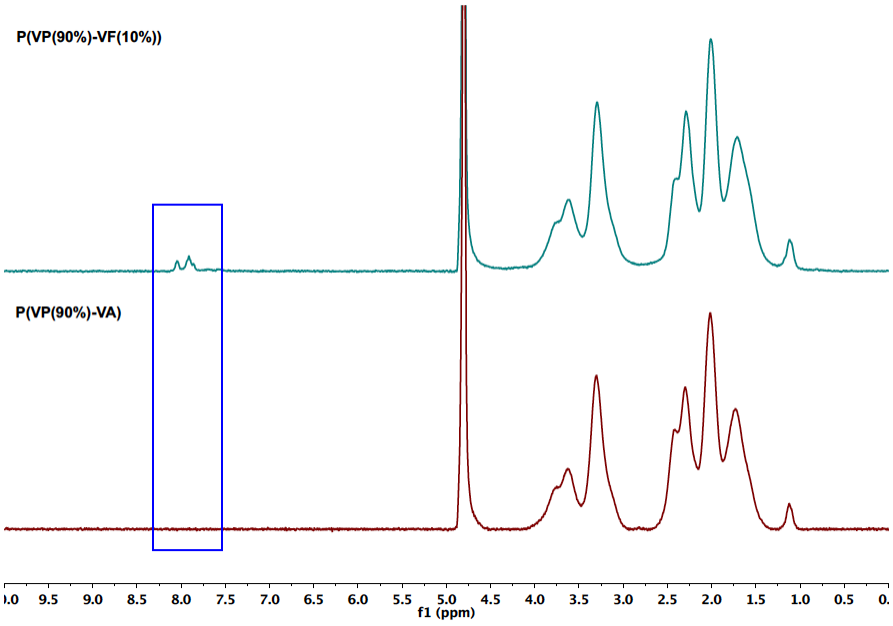


Figure 3-10 ^{1}H-NMR spectra for P(VP(90%)-VF)- 55 kDa and the corresponding P(VP-VA)

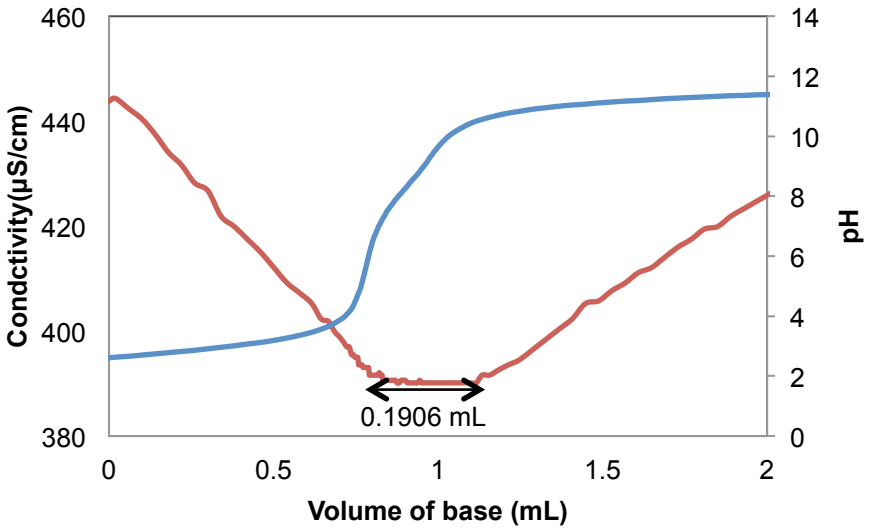


Figure 3-11 Conductometric-potentiometric titration curves for P(VP(90%-VA)-55 kDa

The potentiometric titration curves of the P(VP-VA) samples obtained by

hydrolyzing the P(VP-VF) precursor for different time periods are shown in Figure 3-12. A clear increase in titratable functional groups is observed as the time of the hydrolysis is extended.

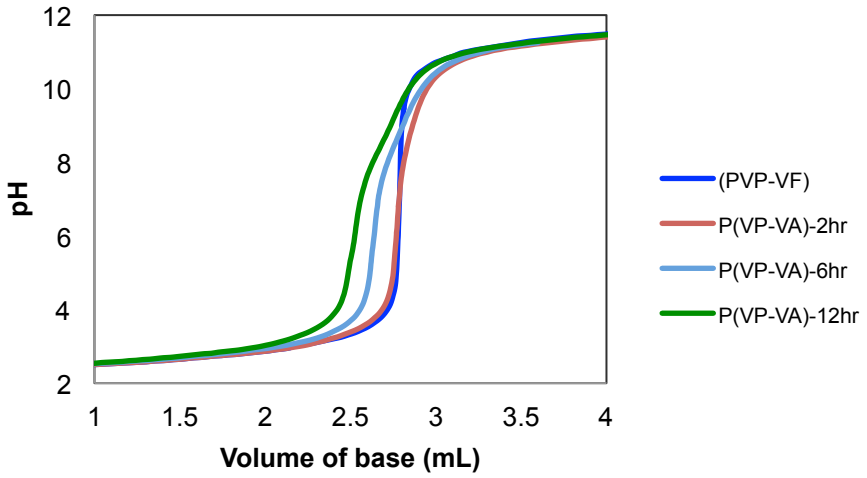


Figure 3-12 Potentiometric titration of the P(VP-VA) samples hydrolyzed for different time periods

Figure 3-13 compares the potentiometric titration curves for 50 mg of a P(VP(90%)-VF)- 55 kDa polymer and the corresponding 85% hydrolyzed P(VP-VA). The generation of amine groups is also obvious here, with titratable functional groups clearly present in the pH range $5 < pH < 9$ that roughly corresponds to the pK_a of a primary amine group.

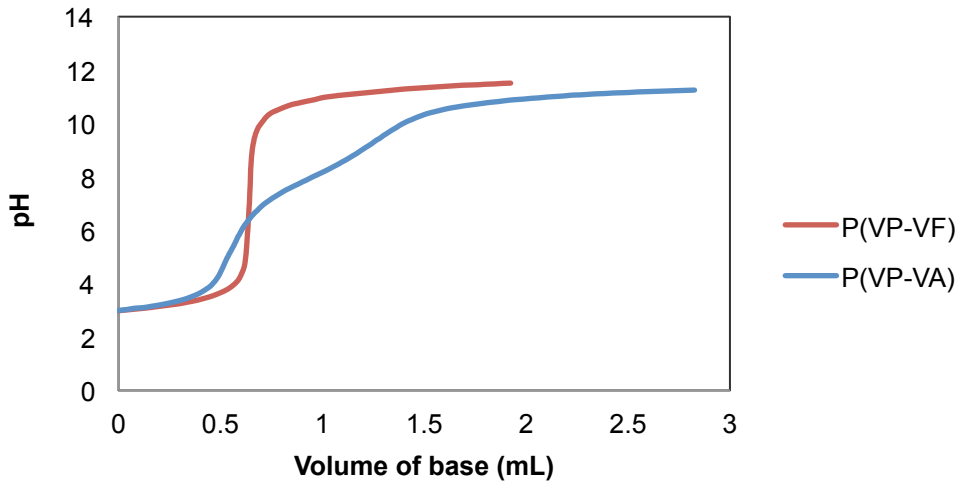


Figure 3-13 Potentiometric titration of a P(VP-VF) sample and the corresponding 85% hydrolyzed P(VP-VA)

Compiling these titration and NMR results for all polymers synthesized, the degree of hydrolysis calculated based on both ^{1}H-NMR and titration indicating the amine group concentration (and hydroxyl group byproduct concentration, based on the difference between the NMR and titration results) for a number of different P(VP-VA) samples is presented in Table 3-3.

Table 3-3 Percentage of NVF residues converted into amines and hydroxyl groups

Sample	% of amide residues converted into amine		% of amide residues converted into -OH
	^{1}H-NMR	Titration	
P(VP(90%)-VA)-4hr	66	43	23
P(VP(90%)-VA)-8hr	80	75	5
P(VP(90%)-VA)-10hr	87	78	9
P(VP(90%)-VA)-12hr	90	84	6
P(VP(90%)-VA)-24hr	100	87	3
P(VP(80%)-VA)-6 hr	77	50	15
P(VP(80%-VA)-24 hr	96	88	8

3.3 Synthesis of the Hydrophobically-Modified P(VP-VA)

Grafting short-chain alkyl halides in low concentrations to the P(VP-VA) graft platform was conducted to synthesize polymers with the capacity to form an associative physical gel with highly shear-thinning properties. Short hydrophobic grafts present in low concentrations can form hydrophobic crosslinks which can be disrupted by shear,

imparting higher zero shear viscosities and shear thinning behavior to the polymer solution.

According to Bock, more shear thinning should be expected with an increase in the hydrophobic substitution (J Bock, 1989); on the other hand, increasing the hydrophobic content decreases the solubility of the water-soluble associating polymers.(Charles L. McCormick et al., 1988). Thus, a balance must be struck in terms of the graft density to ensure optimal shear thinning while maintaining the solubility of the graft copolymer.

As suggested by François et al. in the study of hydrophobically end-capped poly(ethylene oxide), the optimal balance of graft density can be achieved by varying both the concentration and length of the hydrophobic grafts. (François et al., 1996) For example, the use of a lower concentration of C_{18} grafts may impart better shear thinning behavior than higher concentrations of C_{10} grafts. To investigate the effect of hydrophobe length on shear thinning, alkyl chains ranging from ten to eighteen carbons are evaluated. An ideal product would show maximum shear thinning and exhibit good optical clarity.

3.3.1 Experimental

Alkylated P(VP-VA) samples were prepared by reacting the P(VP-VA) grafting platform with alkyl halides (1-chlorooctane (99%), 1-chlorodecane (98%), 1-chlorododecane (≥99.5%), and 1-chlorooctadecane (96%) (Sigma Aldrich)) using a method previously reported. (Martel, Pollet, & Morcellet, 1994) (Chen, Wang, & Pelton,

2005) Briefly, 0.1 wt% P(VP-VA) was dissolved in a water/methanol (1/10 weight ratio) mixture containing 0.02wt % NaOH. The alkyl halide was slowly added at room temperature and stirred for 48 h at 65°C, after which the methanol was evaporated and the remaining solution (water + alkylated polymer) was stirred with hexanes for 4 hours to dissolve the unreacted long-chain aldehydes hydrophobically associated with the modified polymer. The mixture was then left in a separatory funnel 12 hours for the aqueous and organic phases to separate so that the lower aqueous phase containing the hydrocarbon grafted polymer and water became clear. The aqueous phase was then collected from the separatory funnel, mixed with hexanes, and again extracted; this same procedure was repeated three times to remove all residual non-reacted alkyl chains. The final solution was then dialyzed and lyophilized. Figure 3-14 shows a schematic of the alkylation reaction.

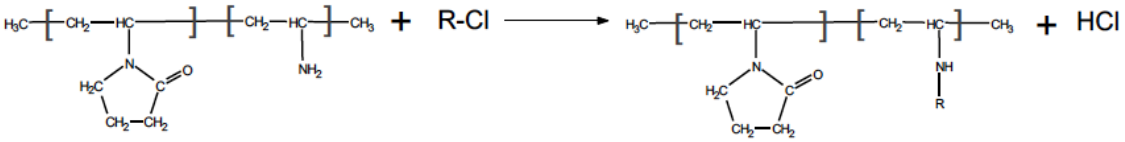


Figure 3-14 Schematic of the alkylation reaction

3.3.2 Characterization

Characterization of the degree of hydrophobic substitution is again done by both ^{1}H- NMR and conductometric-potentiometric titration. However the low degree of

hydrophobic substitution and limited sensitivity of NMR make the characterization of the hydrophobic graft density challenging.

The chemical shifts representing the CH_2 groups and the end methyl groups of the hydrocarbon grafts are completely overlapping, with the peaks associated with the protons in the polymer backbone between 0.9 -2.7ppm. Therefore, calculating the degree of substitution based on the ratio of peak areas assigned to the grafts and the polymer backbone is difficult. However calculating the increase in the whole area of the peaks ranging form 0.9 to 2.7 can achieve a quantitative result for the graft density. In order to do this, the integration of the peak area of the formamide groups in both P(VP-VA) spectra and the hydrocarbon grafted P(VP-VA) spectra is normalized to 1 and used as a reference, since the number of formamide groups in the copolymer will not change during the grafting reaction. A comparison of graft yield results from ^{1}H-NMR and potentiometric titration is shown in Table 3-4. Both titration and NMR analysis result in similar graft densities.

Table 3-4 Degree of alkylation based on ^{1}H-NMR and tirtration

Sample	**Degree of alkylation**		**Mole % of alkyl chains in polymer (Based on titration)**
	Titration	^{1}H-NMR	
C_{10}-P(VP-VA)-55 kDa	17%	14%	1.5
C_{12}-P(VP-VA)-55 kDa	19%	-	1.7
C_{18}- P(VP-VA)-55 kDa	17%	-	1.5
C_{10}-P(VP-VA)-170 kDa	17%	16%	1.5
C_{12}-P(VP-VA)-170 kDa	18%	15%	1.6
C_{18}-P(VP-VA)-170 kDa	20%	20%	1.8
C_{10}-P(VP-VA)-185 kDa	18%	19%	1.6
C_{12}-P(VP-VA)-185 kDa	21%	-	1.8
C_{18}-P(VP-VA)-185 kDa	19%	16%	1.6
C_{10}-P(VP-VA)-255 kDa	14%	16%	1.2
C_{12}-P(VP-VA)-255 kDa	14%	-	1.2
C_{18}-P(VP-VA)-255 kDa	13%	12%	1.1

Since both ^{1}H-NMR and titration give analogous results for the graft density, for the rest of the samples, the degree of substitution was calculated based on potentiometric titration alone. However, due to the lack of solubility of the reactive hydrophobic side chains and the difficulty in assessing the relationship between the total polymer mass and the number of polymer chains following grafting, a modified titration procedure was used to ensure high accuracy graft density measurements. For this purpose, 250 mg of the non-modified P(VP-VA) precursor was dissolved in 25 mL water and titrated to determine the number of available amine groups. The titrated solution was mixed with 250 mL of methanol and the grafting reaction was performed based on the procedure explained earlier in this section. Upon completion of the grafting reaction, the methanol was evaporated and the mixture was titrated to find the number of remaining non-reacted amine groups. Figure 3-15 compares the titration curves for the P(VP-VA) graft platform and P(VP-VA) grafted with different hydrocarbon chain lengths, showing a clear decrease in the number of titratable functional groups following grafting indicative of the consumption of the primary amine groups by the hydrophobic graft The degree of substitution can be calculated based on the ratio of the number of grafted amine groups to the initial number of available amine groups. (Table 3-4)

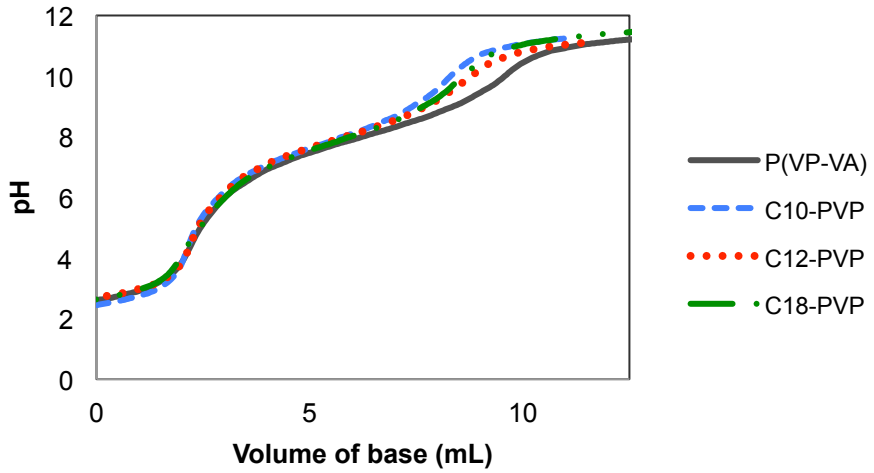


Figure 3-15 Potentiometirc titration for 250 mg of a P(VP-VA) sample before and after being alkylated

3.4 Synthesis of the Phenylboronic acid (PBA)-grafted PVP:

The PBA functional group is grafted to the P(VP-VA) graft platform through a reductive amination reaction, producing a secondary amine linkage. This secondary amine bond is essential to reduce the pK_a of the PBA functional group to form the boronate at eye pH and thus increase its ability to form bonds with the sialic acid residues in mucin.

3.4.1 Reductive Amination

Primary, secondary or tertiary amines can be formed through the reaction of aldehydes or ketones in the presence of reducing agents with ammonia, primary or secondary amines respectively. Such reaction is known as reductive amination of

carbonyl compounds (such as aldehydes and ketones) or reductive alkylation of amines. (Abdel-Magid, Carson, Harris, Maryanoff, & Shah, 1996)

The reducing agent must selectively reduce imines (or iminium ions) over aldehydes or ketones under the reaction conditions and therefore the nature of the reducing agent determines the success of the reaction.

In order to graft the PBA moiety to the P(VP-VA) graft platform with optimized substitution degree and subsequent mucoadhesion ,two different hydride reducing agents, sodium triacetoxyborohydride ($NaBH(OAc)_3$) and sodium cyanoborohydride (NaBH3CN) were used in our experiments.

3.4.1.1 Sodium triacetoxyborohydride-based Reductive Amination

Sodium triacetoxyborohydride [$NaBH(OAc)_3$] is a mild reducing agent due to the steric and the electron- withdrawing effects of the three acetoxy groups that stabilize the boron-hydrogen bond. Gribble et al. reported successful reductive alkylation of amines using this reducing agent. (Gribble, Jasinski, Pellicone, & Panetta, 1978)

Aldehydes can be reduced with sodium triacetoxyborohydride ($NaBH(OAc)_3$)(Abdel-Magid et al., 1996). Thus, the possibility exists that the reduction of the aldehyde would compete with the reductive amination process under the standard conditions. This should particularly be considered in our experiments since $NaBH(OAc)_3$ is reported to rapidly reduce aldehydes to hydroxyl groups in methanol, which is a

preferred solvent for P(VP-VA) and 4-formyl-phenylboronic acid in our experiments. Therefore, choice of an appropriate solvent to gain high substitution degrees is complicated

3.4.1.2 Sodium cyanoborohydride -based Reductive Amination

The second hydride reducing agent selected for screening was sodium cyanoborohydride ($NaBH_3CN$). The advantage of using sodium cyanoborohydride is its solubility in hydroxylic solvents such as methanol, and its different selectivities at different pH values(Borch, Bernstein, & Durst, 1971). At pH 3-4 it reduces aldehydes and ketones effectively, but this reduction becomes very slow at higher pH values. Borch et al. showed that reduction of the imminium moiety with BH_3CN^- was rapid at pH 6-7 while aldehydes and ketones were negligibly reduced in this pH range (Borch et al., 1971). Thus they proposed that aldehydes and ketones could be reductively aminated by simply reacting the carbonyl compound with amines at pH 6 in the presence of BH_3CN^-.

One major draw back of this method is that sodium cyanoborohydride is highly toxic and produces toxic byproducts such as HCN and NaCN upon workup (Abdel-Magid et al., 1996), demanding careful purification of the final product prior to end use.

3.4.2 Experimental

4-Formyl phenylboronic acid (**≥95.0%**, Sigma Aldrich) and triethylamine (**≥99%**, Sigma Aldrich) were used as received.

A method similar to that used by Winblade et al. was performed with the use of two

different reducing agents explained in the previous sections. (Winblade et al., 2000)

A 10mg/mL solution of the P(VP-VA) polymer in methanol was prepared and the mild $NaBH(OAc)_3$ reducing agent (2 eq) and 4-formyl phenylboronic(5eq) were added. Triethylamine (2eq) was also added to convert amine salts in the P(VP-VA) copolymer to free amine groups. The solution was then purged with nitrogen and stirred for 24 hours at room temperature. Upon completion of the reaction after 24 hours, the PBA grafted polymer (P(VP-PBA)) was purified by neutralizing the reduction via addition of 1N aqueous saturated $NaHCO_3$ solution. Following evaporation of the methanol, the solution was dialyzed against MQ water at pH 9 for 3 days and lyophilized. The same procedure was repeated with the use of the other reducing agent (Sodium cyanoborohydride). Figure 3.16 shows a schematic of the reductive amination reaction.

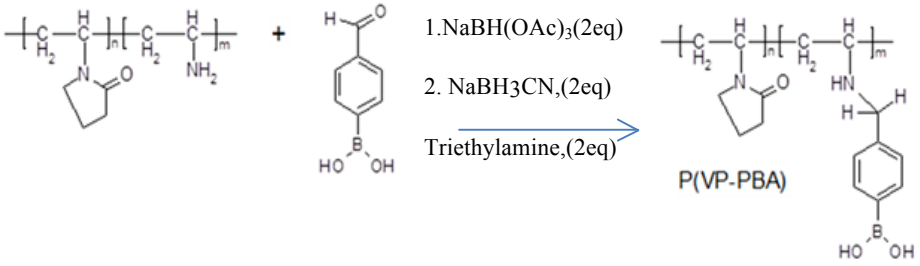


Figure 3-16 Schematic of the reductive amination reaction

3.4.3 Characterization

^{1}H-NMR was used to determine the total degree of PBA substitution by comparing the aromatic proton peak area with chemical shifts between 7-8 ppm and to the proton

peaks from the polymer backbone with chemical shifts between 1.5-4.5ppm.

High amounts of PBA substitution may increase the residence time of the artificial tear on the ocular surface too significantly and/or reduce the solubility and water binding capacity of the polymer. Consequently, the total number of amine residues on the graft polymer (and thus the resultant PBA content) was targeted to be no more than 10mol% of the total residues in the polymer backbone. A 10% PBA polymer will be the initial synthetic target, with subsequent targets adjusted based on the mucoadhesion results obtained.

Figure 3.17 shows the ^{1}HNMR spectra of the P(VP-VA) graft platform and the PBA grafted P(VP-VA). For reductions performed with sodium triacetoxyborohydride, NMR indicated that ~55% of the total available amine groups were grafted with PBA, corresponding to ~4.5 mol% of total monomer units in the polymer backbone. In comparison, the substitution degree in the case of sodium cyanoborohydride was ~80% of available amine groups, corresponding to up to 7 mol% of the total monomer units in the polymer backbone. Thus, sodium cyanoborohydride is a significantly more effective reduction reagent in this protocol and was selected for all future experiments.

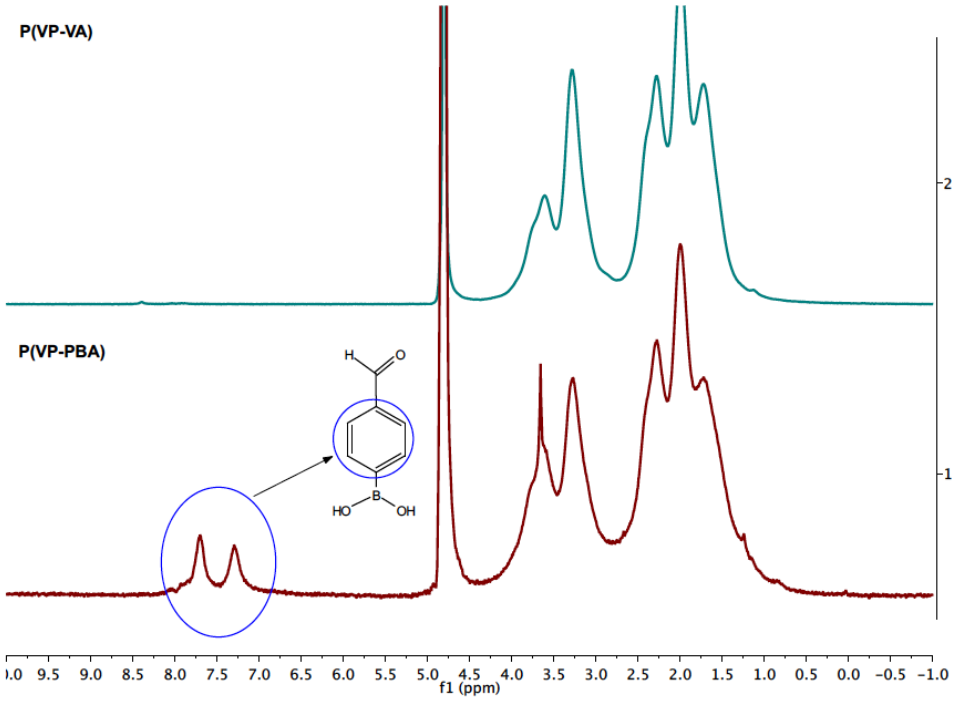


Figure 3-17 ^{1}H-NMR spectra of P(VP(90%)-VA) and P(VP(90%)-PBA)

Figure 3.18 compares the degree of functional group titration as a function of pH for P(VP-VA) and the corresponding P(VP-PBA). Higher degrees of titration are observed for the PBA grafted polymer compared to the P(VP-VA) precursor at any given pH, with ~50% of groups titrated at pH <6.5 and ~78% of groups titrated at physiological pH. Titration signal may arise from either titration of the secondary amine resulting from the reductive amination reaction or the conversion of boronic acid to boronate. Considering that the resulting PBA grafted product has a structure analogous to benzylethyl (pK_a=9.68) and the remaining non-grafted amine groups (primary amines) have a structure analogous to ethylamine (pK_a=10.63), the lower overall pK_a of the PBA-

grafted product cannot exclusively be attributed the inductive lowering the pK_a of PBA groups so that they are primarily ionized at pH 7.4. However, since the grafted product shows 100% conversion at pH ~8.1, it is clear that the reductive amination has successfully lowered the pK_a of the phenylboronic acid groups (pK_a ~8.4 in the absence of an inductive substituent)

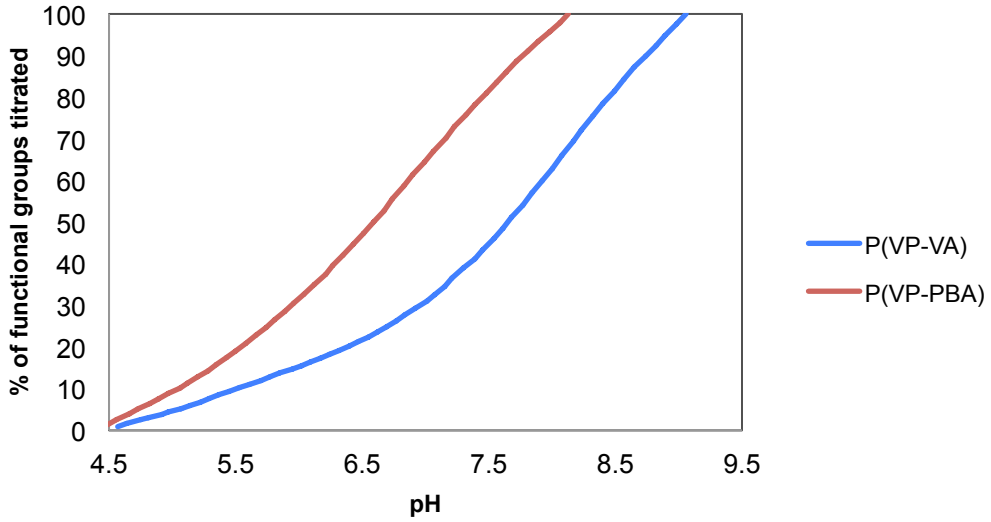


Figure 3-18 Degree of functional group titration for P(VP(90%)-VA)- 55 kDa and the corresponding P(VP-PBA)

3.5 Combining Mucoadhesive and Shear-Thinning Grafts in a Single Polymeric Vehicle

Hydrophobes and phenylboronic acid groups can be sequentially grafted to the same polymer backbone to optimize both shear thinning and mucoadhesive properties of the PVP-based polymer. The hydrophobic grafts are stable under the NVF hydrolysis conditions. Therefore, as outlined in section 3.2.1, the NVF residues can be partially hydrolyzed to add the hydrophobic grafts and then fully hydrolyzed to free more amine groups and conjugate the PBA groups.

^{1}H-NMR and conductometric-potentiometric titration were conducted to confirm the sequential conjugation of the PBA to the hydrophobic grafted polymers. Figure 3-19 shows the ^{1}H-NMR spectra for the C_{12}-PVP and the C_{12}-P(VP-PBA) polymers. Signals for both the hydrophobic grafts as well as the aromatic peak associated with phenylboronic acid groups are clearly present, indicating a successful conjugation. Table 3-5 indicates that the percentage of the PBA moieties in the polymer chains for the various samples is consistent at ~6 mol% total monomer units irrespective of the molecular weight of the graft polymer or the length of the hydrophobic grafts, suggesting the reductive amination reaction is highly robust and adaptable to a range of different graft chemistries.

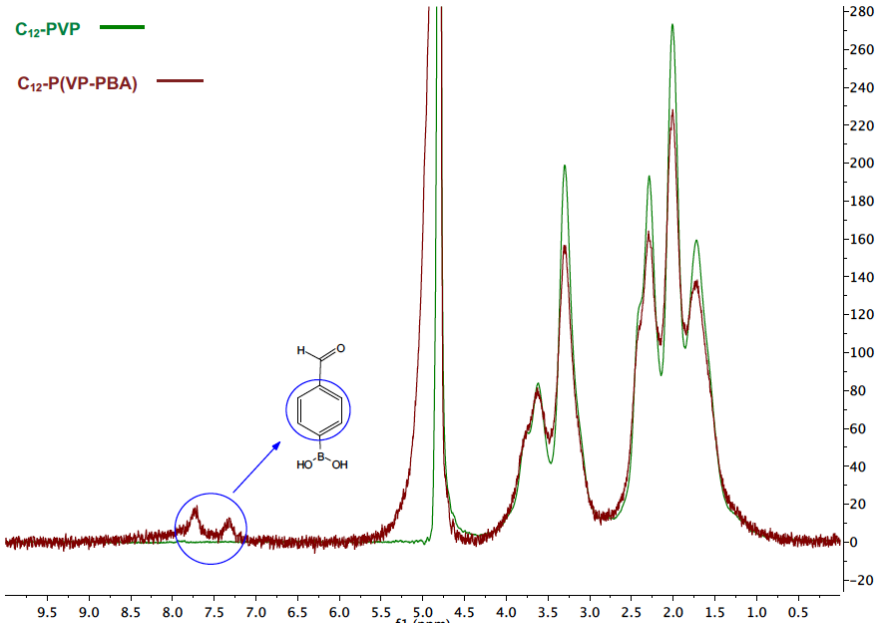


Figure 3-19 ^{1}H-NMR spectra of P(VP(90%)-VA)- 170 kDa grafted with C_{12} and PBA

Table 3-5 PBA content of dual grafted polymers

Sample	% of PBA (of total monomer residues)
C18-P(VP-PBA)-55 kDa	6
C12-P(VP-PBA)- 170 kDa	5.5
C12-P(VP-PBA)-255 kDa	6.5
C18-P(VP-PBA)-255 kDa	6

3.6 Cell Viability Testing

Cell viability was tested *in vitro* with the use of human corneal epithelial cells (HCEC). These cells were selected to be the most relevant cells for modeling the cytotoxicity of the polymers to the corneal surface, given the ultimate application of the polymers as components of eye drop formulations.

"Complete" KSFM (Gibco #17005-042) was used as the proliferation media and was prepared by adding the following supplements to the bottle:

- Contents of the bovine pituitary extract & EGF supplement vials that come with purchased KSFM
- 1x Penicillin/streptomycin (Invitrogen #15140122) in a 1:100 dilution

A detailed procedure for cell splitting and culturing is given in Appendix B. Tests were conducted in polystyrene 24 well plates ($2cm^2$) containing HCEC cells at a concentration of **100,000** cells and 1 mL of the media per well. Cells were allowed to adhere for 24 hours.

Solutions of the alkylated P(VP-VA) samples with different MWs and different graft chain lengths were made at 2mg/mL concentration in MQ water and sterile filtered using a 0.2μm syringe filter. Cells were exposed to 1mL per well of the sterilized

polymer solutions diluted with the media at concentrations ranging from 100 µg/mL to 10,000 µg/mL for 24 hours. A blank well containing no cells and wells containing cells but no polymer solutions were used as controls. Each experiment was replicated 4, with the error bars representing the standard deviation of the measurements.

3.6.1 MTT Assay

Cell viability was assessed via the thiazolyl blue tetrazolium bromide (MTT) assay, modified from manufacturer's protocols. Thiazolyl blue tetrazolium bromide (MTT) assay is a metabolic process in which the concentration of the purple metabolite of the MTT dye can be correlated with the overall level of cell metabolism and thus the total number of viable cells. The MTT stock solution (3mL) was reconstituted in 10 mM PBS at a concentration of 40mg/mL and sterile filtered. When applied to cells, the solution was diluted to 0.4mg/mL with the “complete” KSFM medium. Following a 24 hour exposure of the cells to the polymer solutions, 150 µL of the diluted MTT solution was added and allowed to incubate for 24 hours. The resulting insoluble formazan precipitate was then dissolved via addition of 250 µL DMSO to each well. Plates were shaken for 10-20 minutes or until formazan was fully dissolved. 200 µL of the resulting solution was transferred to a 96 well plate and read in a microplate reader (Biorad, Model 550) at 570nm. Viability was measured as a function of formazan absorption at 570nm relative to a blank cell-only control, in which no polymer was added to the cells. Absorbance due to the plate and medium itself was used as a control and was subtracted out. Cell viability was calculated based on the following equation:

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

3.6.2 Cell Cytotoxicity

Figure 3-19 shows the cell viability of HCEC cells in the presence of the low molecular weight (60kDa) polymer grafted with hydrophobic groups of different chain lengths (C_{10} & C_{18}). No significant cytotoxicity was observed even at concentrations up to 1mg/mL, typically considered high for an *in vitro* cytotoxicity measurement; indeed, the C_{18} polymer slightly increased the observed metabolic activity of the cells relative to the cell-only control, potentially attributable to the cell-polymer adhesion interactions. Figure 3-20 indicates that the higher molecular weight (135kDa) polymers grafted with C_{10}, C_{12} and C_{18} exhibit no significant cytotoxicity at low concentrations (<0.5mg/mL) but minor cytotoxicity at higher concentrations (1mg/mL), particularly for polymers grafted with longer hydrophobic chains. Thus, the lower molecular weight graft platforms induce less cytotoxicity than higher molecular weight graft platforms and will be the focus of application studies moving forward.

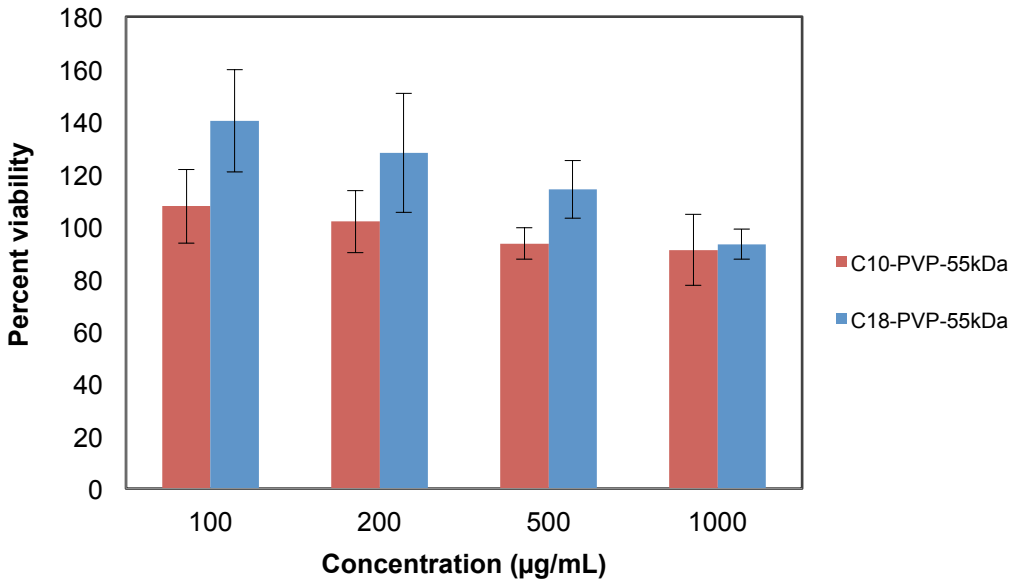


Figure 3-20 Percent cell viability of HCEC cells exposed to a 55kDa polymer grafted with C_{10} and C_{18}

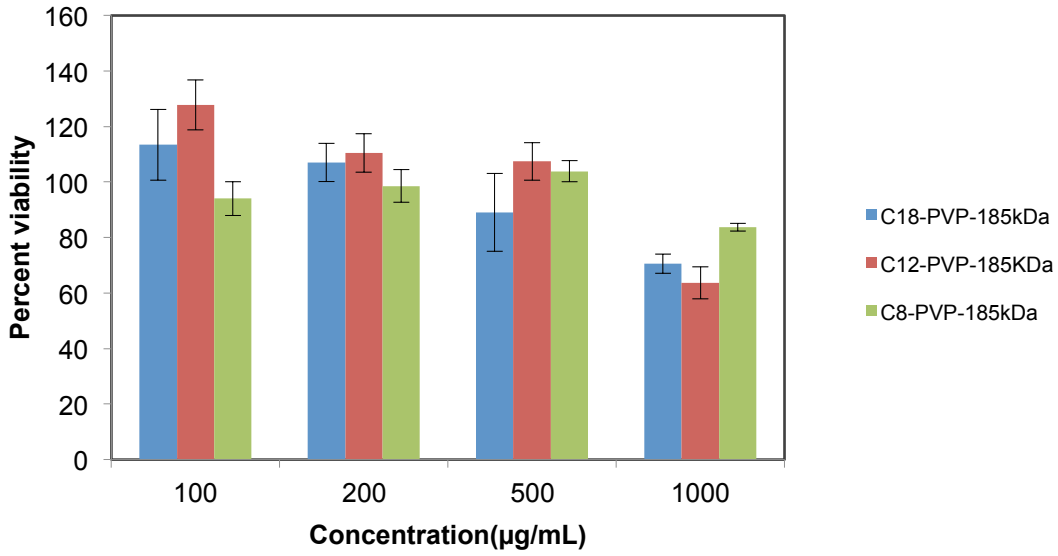


Figure 3-21 Percent cell viability of HCEC cells exposed to 135kDa polymer grafted with hydrophobic groups of different chain lengths

3.7 Transmittance By UV-Vis Spectroscopy Measurement

In order to be useful as an ophthalmic material, the solutions of the graft copolymers must be optically transparent. To screen the transparency of these materials, the % transmittance of the samples was measured over the full UV and visible spectrum using a DU 800 UV/Visible Spectrophotometer (Beckman Coulter). P(VP-VA) samples of molecular weights 255 kDa and 170 kDa grafted with C_{18},C_{12} and C_{10} as well as samples grafted with both hydrophobic chains and PBA were dissolved in 1 mL of Milli Q water at a concentration of 10wt%. Samples were then placed in PS cuvettes and a wavelength scan was performed from 300nm to 700 nm.

The % transmittance as a function of wavelength is shown in Figure 3-22 while the single wavelength (600nm) % transmittances are shown in Table 3-2. C_{18}-grafted polymers exhibit significantly lower transmittance profiles than the shorter grafts over all wavelengths, perhaps indicative of the formation of nanodomains of hydrophobic aggregates when longer hydrophobes are incorporated. However, above 450nm, all other samples showed transmittance values of >85%, with transmittances consistently >93% at the single wavelength measurement at 600nm. Note that this measurement is performed on a 1cm path length cell, while the tear film has a significantly smaller thickness (~10 microns) in which transparencies would be universally high. Thus, these formulations

are appropriate for tear film use. In addition, as shown in Figrue 3-22, the %transmittance of the samples drops significantly at wavelengths < 450nm and in particular < 400nm. Therefore these materials can provide UV protection, which can be useful when used in ophthalmic applications.

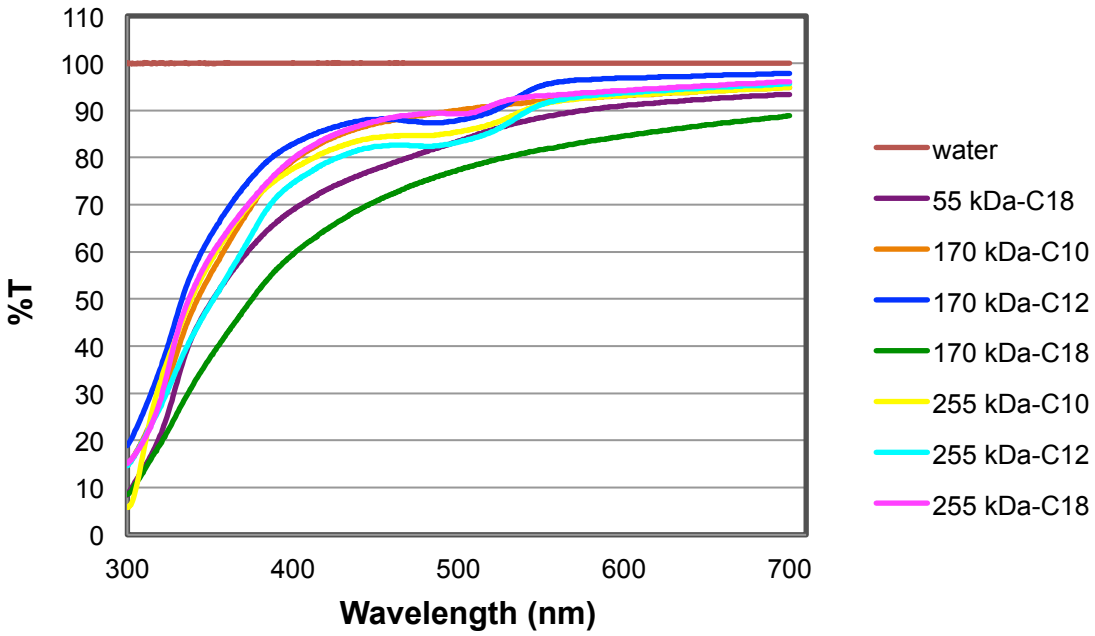


Figure 3-22% Transmittance of different hydrophobically-modified polymers at solution concentrations of 10 wt% as a function of wavelength

Table 3-6 % Transmittance of hydrophobically-modified polymers at 600 nm

Sample	% Transmittance
C_{18}-PVP-55 kDa	91
C_{10}-PVP-170 kDa	93.1
C_{12}-PVP-170 kDa	96.8
C_{18}-PVP-170 kDa	84.5
C_{10}-PVP-255 kDa	93.1
C_{12}-PVP-255 kDa	93.7
C_{18}-PVP-255 kDa	94.2

Despite the many favourable properties noted herein for the use of these graft polymers in the eye, it is essential that the rheological properties of the graft copolymers be optimized for application in the eye, as will be discussed in the next chapter.

4. Rheology of Dual Hydrophobic/Boronic Acid Grafted Copolymers

4.1 Shear Thinning

High molecular weight liquids, which include polymer melts and solutions of polymers, usually display non-Newtonian rheological properties in that the viscosity is a function of the shear rate applied to the sample. In the case of shear-thinning fluids, viscosity decreases with increasing shear rate, the typical behaviour of a polymer solution. A typical viscosity versus shear rate plot for a shear-thinning fluid is shown in Figure 4-1.

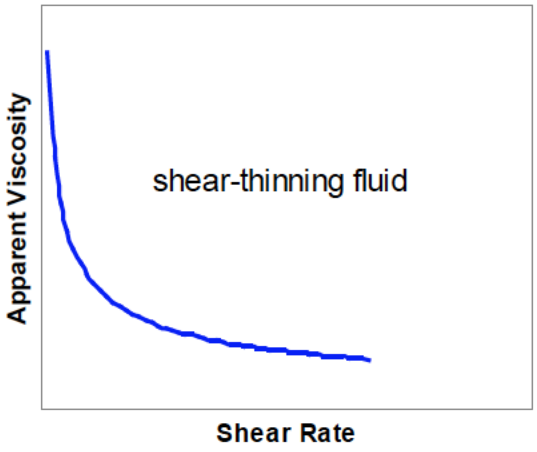


Figure 4-1 Viscosity versus shear rate for a shear thinning polymer

Hydrophobically-modified polymers represent a well-known class of highly shear thinning materials (Wang, Iliopoulos, & Audebert, 1988)(Taylor & Nasr-El-Din, 1998). Several factors including the average polymer molecular weight, type and degree of substitution of hydrophobic groups as well as the distribution of hydrophobes determine the rheological properties of the hydrophobic modified polymers(Taylor & Nasr-El-Din, 1998). For effective use as an eye drop formulation, the rheology of the polymer solution

should be tuned such that can be easily administered to the eye via an eye dropper but then form a weak gel on the corneal surface to prolong the residency time of the polymer in the eye. Stated in another way, the higher the viscosity gradient between low and high shear (i.e. the magnitude of shear thinning), the more effective the formulation for ophthalmic application. In this light, the purpose of this chapter is to investigate the effect of hydrophobic functionalization on rheology of the aqueous solutions of PVP-based polymers. The physical properties of PVP-based polymer solution with and without the presence of the hydrophobic and PBA grafts were examined using a controlled stress rheometer.

4.2 Experimental

Rheological measurements on solutions of the P(VP-VA) graft platform with different molecular weights grafted with hydrophobic groups of various chain lengths were carried out with a controlled stress rheometer (ATS, Rheologica Instrument, USA)) using the cone/plate configuration with cone diameter of 4 cm and cone angle of 2^{o}. Solutions at concentrations of 5 wt%, 10 wt% and 15 wt% of the polymers were prepared in Milli Q water and allowed to stay in the vials for 48 hours prior to testing. All measurements were done at room temperature and solutions were handled carefully during sample loading to avoid the introduction of any bubbles. After loading the sample solution onto the rheometer, sufficient time was allowed for for any residual stresses in the material as a result of transfer to the rheometer to dissipate. Shear dependent viscosity was measured by applying stresses from 0.01 to 100 Pa and measuring the resulting strain. The range of the stress was divided by 35 intervals and an integration time of 60

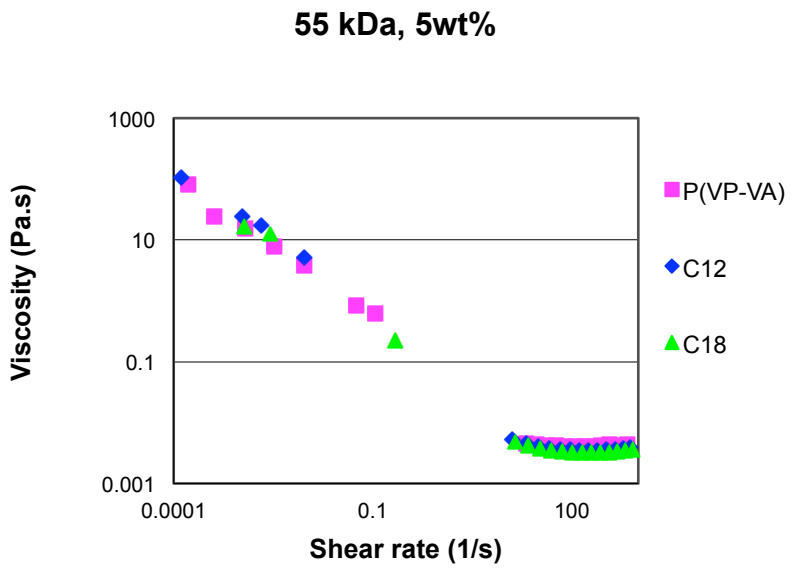
seconds was applied at each shear stress.

4.2.1 Viscometric Measurements Of The Hydrophobically Modified Polymers

Figure 4-2 compares the viscosity profile of a 55 kDa non-modified P(VP-VA) polymer with the viscosity profiles of the same P(VP-VA) polymer grafted with hydrocarbon chains of different lengths at solution concentrations of 5wt% and 10wt%.

In each result, grafting a hydrophobic chain to the graft platform slightly decreases the viscosity at high shear rates while enabling measurement of accurate viscosity values at significantly lower shear rates relative to the P(VP-VA) graft platform alone, indicative of the presence of intermolecular interaction between the hydrophobic grafts. The effect is also a function of hydrophobic chain length; longer hydrophobes lower the high shear viscosity to a greater extent than shorter hydrophobe

a



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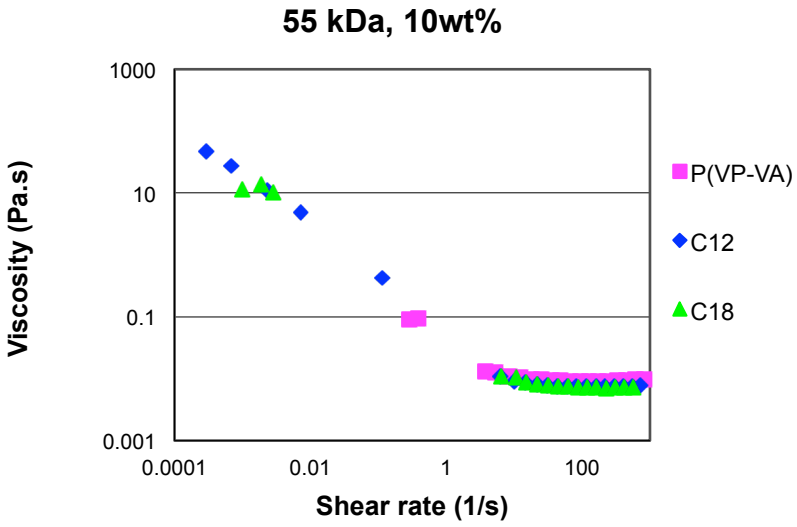
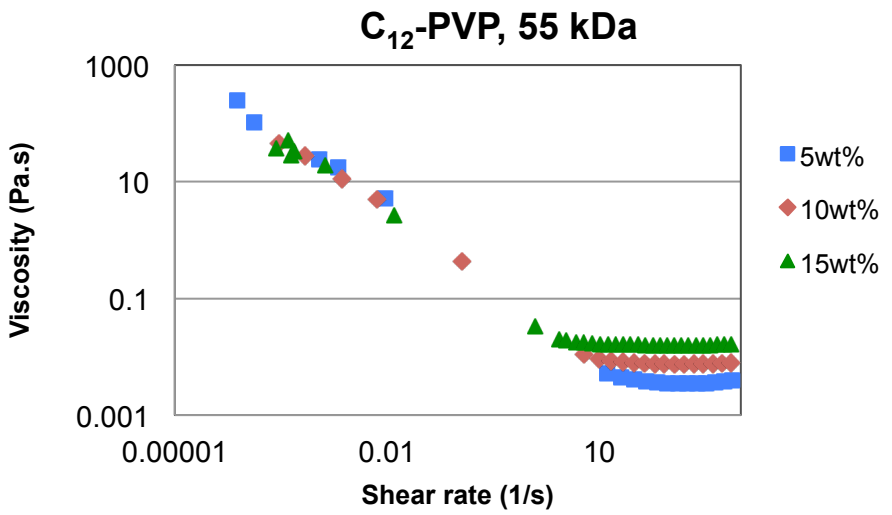


Figure 4-2 Viscosity profiles of P(VP-VA)-55 kDa, C_{18}-PVP-55 kDa and C_{12}-PVP-55 kDa at solution concentrations of a) 5wt% b) 10wt%

The concentration of the polymer solution has a significant impact on the rheology. Figure 4-3 shows that for the 55 kDa graft platform, the viscosity of the grafted polymers at the concentration of 5 wt% is significantly lower than the viscosity of 10 wt% and 15 wt% solutions for all graft lengths at high shear rates. However, at low shear, there appears to be no significant difference in the viscosity as a function of concentration, with lower concentration solutions actually exhibiting higher viscosities at extremely low shear rates than more concentrated solutions. This result indicates that in more dilute solutions, self-diffusion of the hydrophobic grafts occurs more efficiently, leading to higher degrees of physical cross-linking, higher low shear viscosities, and higher shear thinning for each hydrophobic graft length. Furthermore, the viscosity of the non-modified polymers and the hydrophobic grafted polymers at high shear rates are similar, which indicates that the viscosity at high shear rates is more controlled by the polymer backbone concentration and properties.

Shear-dependent viscosities of the solutions of non-modified polymer and hydrophobic grafted polymers at a concentration of 15wt% are shown in Figure 4-4. Higher viscosities at low shear rates and lower viscosities at high shear rates are observed for longer hydrophobic graft chain lengths. Consequently, significantly higher magnitudes of shear thinning are observed for polymers grafted with longer alkyl chains.

a



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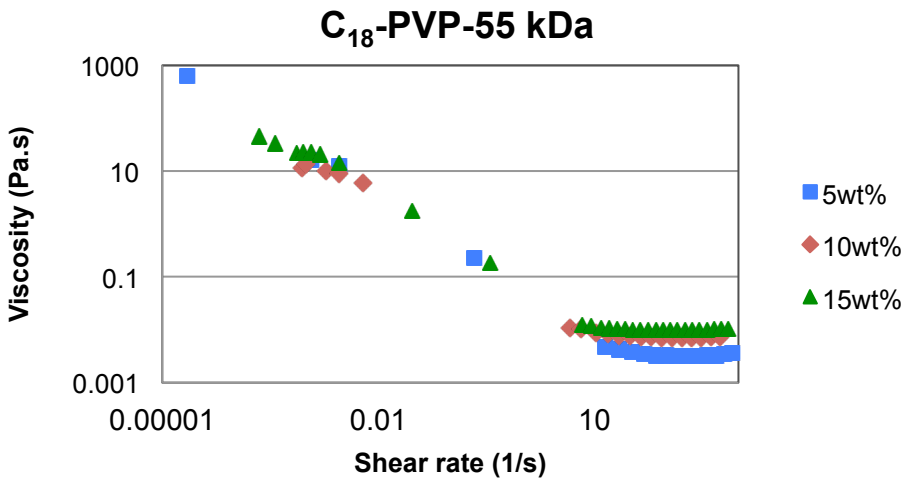


Figure 4-3 Viscosity profiles of a 55 kDa P(VP-VA) grafted with a)C_{12} b)C_{18} at different solution concentrations

Combining information from these results together, conclusions can be drawn regarding the effect of polymer concentration and graft length on the solution rheology. From Figure 4-2, it can be concluded that the viscosity of solutions at concentrations less than 15 wt % of the 55 kDa grafted P(VP-VA) at high shear rates is controlled primarily by polymer concentration, with only a minimal impact of the graft length on the viscosity. In comparison, Figure 4-4 indicates that at a solution concentration of 15wt% grafts of any length have a more significant impact on the viscosity (higher at low shear, lower at high shear) and the viscosities have a significantly higher dependence on the graft chain length, particularly at high shear rates.

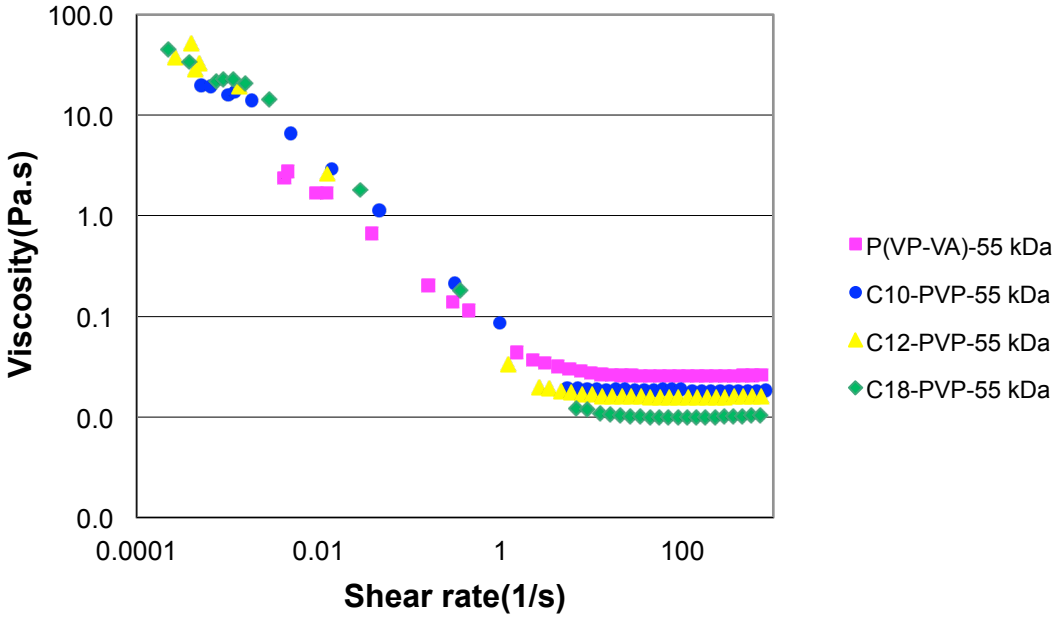


Figure 4-4 Effect of hydrophobic graft length on shear thinning; 55 kDa polymer graft platform, 15 wt% polymer concentration

By increasing the molecular weight of the graft platform, the rheological properties can again be further tuned. Figure 4-5 shows the viscosity profile of a 170 kDa P(VP-VA) polymer versus the viscosity profiles of the same polymer grated with hydrocarbon chains of varying lengths at the solution concentration of 15wt%. In this case, the graft length has virtually no effect on the measured viscosity at any tested shear rate. Comparing Figure 4-5 with Figure 4-4, it can be concluded that increasing the molecular weight of graft platform decreases the efficacy of grafts on shear-thinning. This result is expected since the polymer itself contributes more strongly to the overall viscosity of the solution and restricts the conformational mobility of the hydrophobic grafts to form associative thickening interactions.

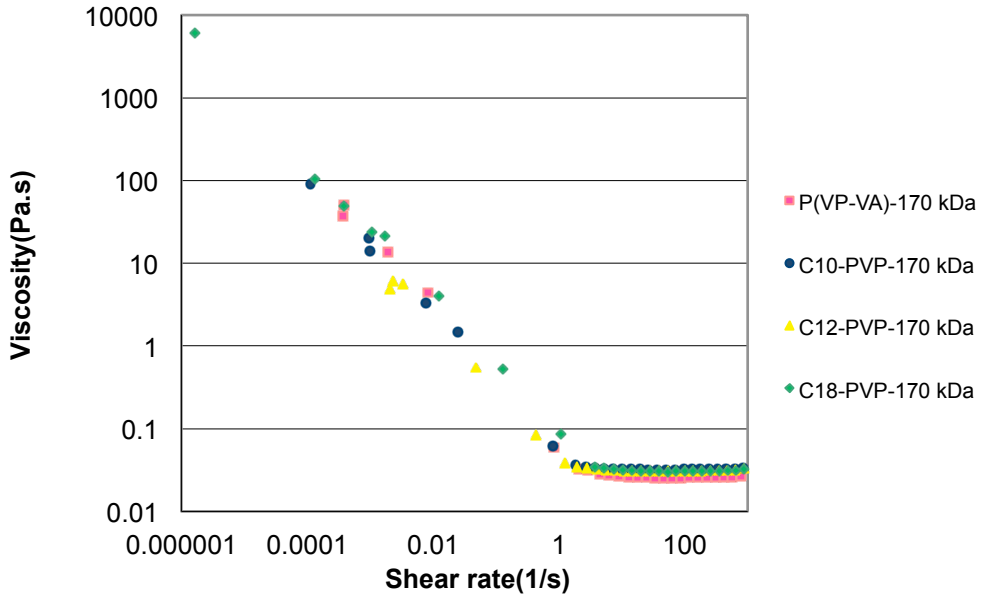


Figure 4-5 Effect of hydrophobic graft length on shear thinning;170 kDa polymer graft platform; 15wt% polymer concentration

The observation that higher molecular weight graft platforms are less influenced by hydrophobic grafting is confirmed when the molecular weight of the graft platform is further increased to 255 kDa (Figure 4-6).

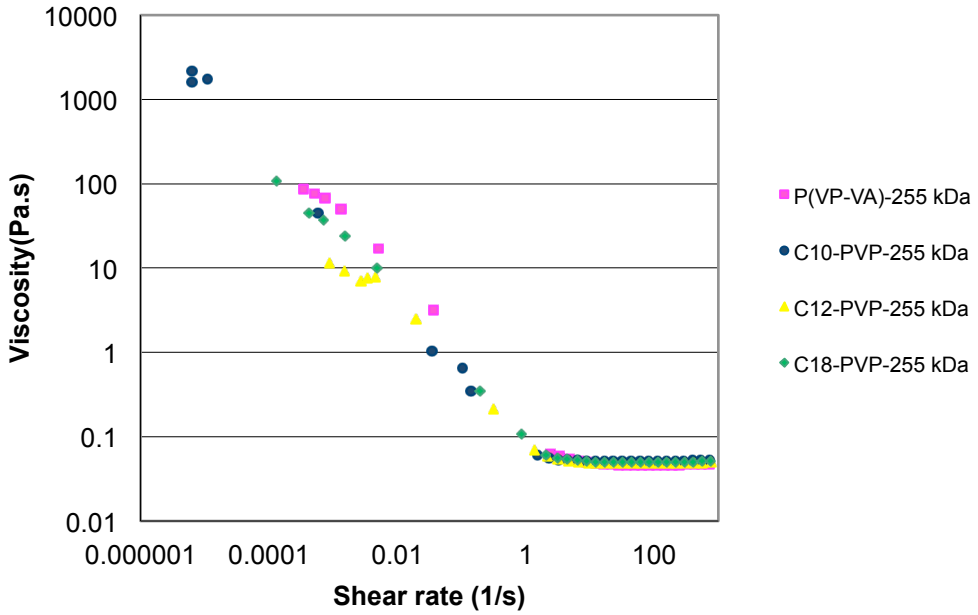


Figure 4-6 Effect of hydrophobic graft length on shear thinning of a 255 kDa polymer graft platform; 15wt% polymer solution

As the molecular weight of the polymer backbone increases, hydrophobic grafts exhibit less effect on the magnitude of shear thinning. However, as the concentration of the polymer is reduced such that the polymer contributes less to the overall viscosity of the solution, the presence of hydrophobic grafts begins to again affect the solution rheology, as shown in Figure 4.7. Figs. 4.7 **a** and **b** indicate that for hydrophobic graft lengths of C_{10} and C_{12}, solutions at 10 wt% concentration show the most shear thinning, whereas Figure 4.7 **c** indicates that for C_{18} grafted polymers, 5wt% solutions exhibit more shear thinning than the 10 wt% and 15 wt% solutions. Thus, as the graft length increases

and the graft platform molecular weight increases, more shear thinning is observed at lower overall polymer concentrations.

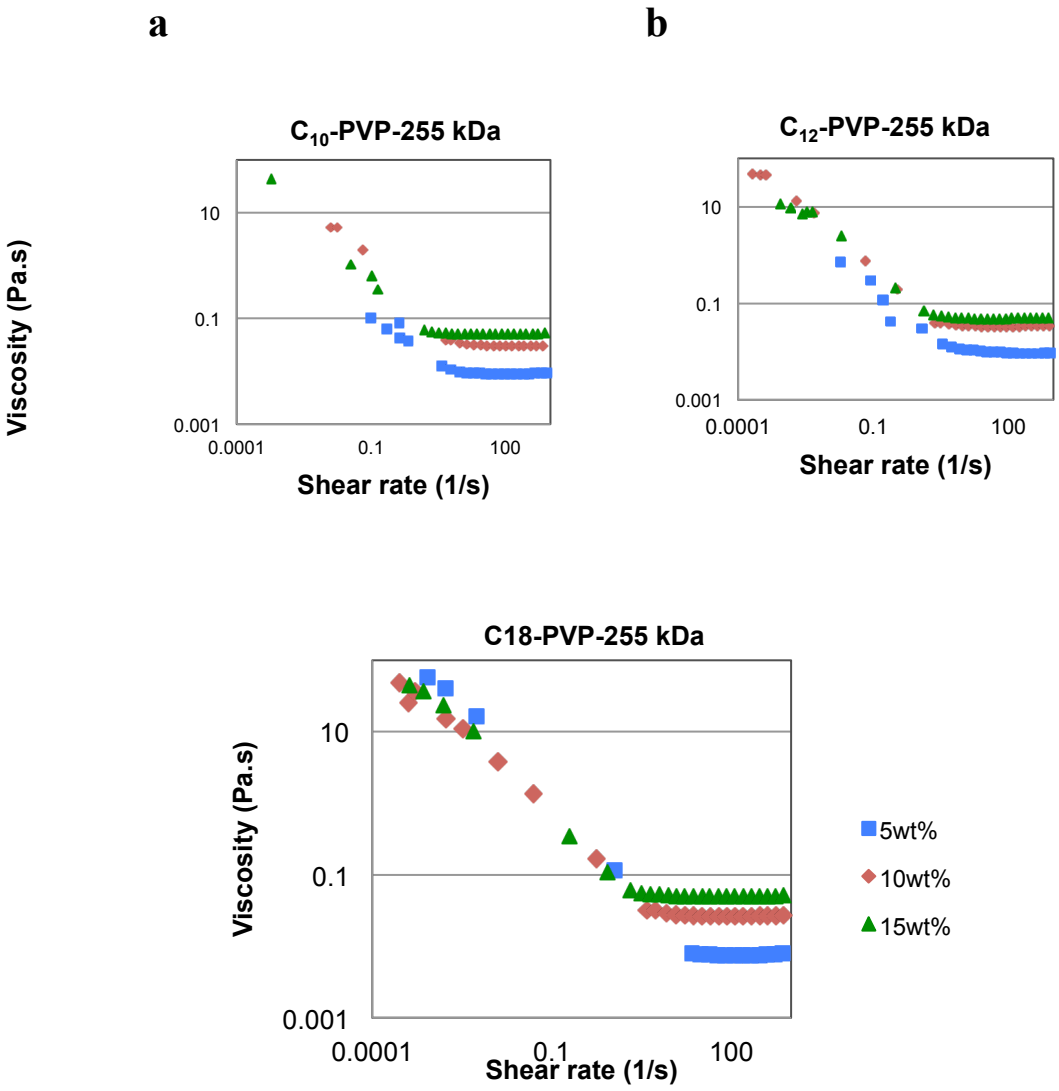
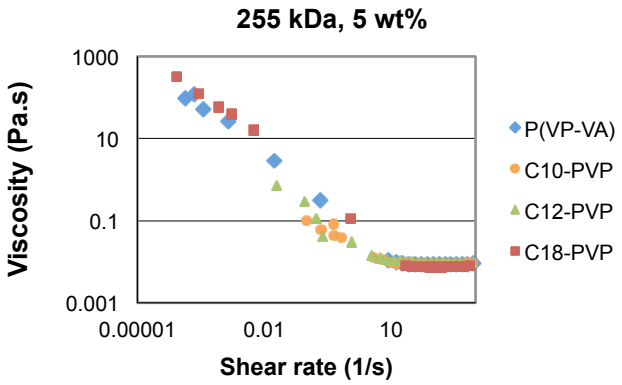


Figure 4-7 Viscosity profiles of a 255 kDa P(VP-VA) grafted with a) C_{10}, b)C_{12}, C) C_{18} at different solution concentrations

As the molecular weight of the graft platform is further increased, the presence of hydrophobic grafts (and thus the reduction in the solubility and hydrodynamic volume of the graft platform polymer) can have a negative effect on the viscosity of the grafted product, particularly at lower polymer concentrations at which intermolecular associations become less probable. Figure 4-8 shows that the viscosity of the non modified 255 kDa P(VP-VA) graft platform and the C_{18} grafted P(VP-VA) are both higher than the C_{10} and C_{12} grafted polymers in the more dilute 5wt% solutions. In this case, the high viscosity of the backbone polymer limits the mobility of the hydrophobic grafts such that intermolecular hydrophobic interactions become less likely, while the large number of hydrophobes per chain on the high molecular weight graft platform increase the likelihood of intramolecular associations. The longer C_{18} grafts can engage in more intermolecular associations and thus allow for some of this viscosity to be recovered. At higher concentrations of 10wt% and 15wt%, intermolecular interactions become more probable and the C_{10} and C_{12} grafted polymers exhibit similar viscosity values to the unmodified and C_{18} graft polymers.

a



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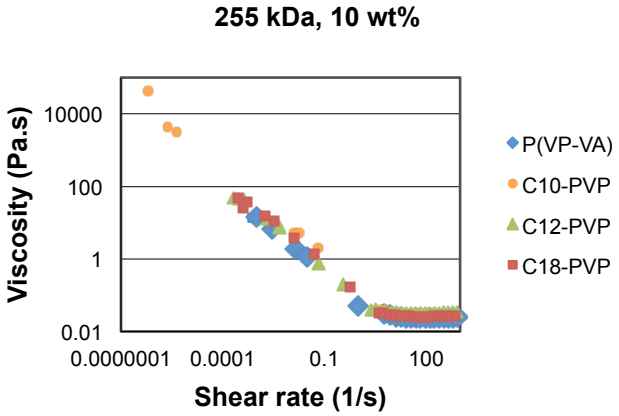

c

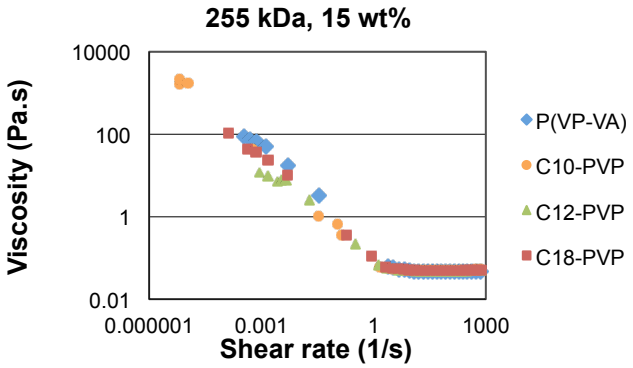

Figure 4-8 Effect of solution concentration and hydrophobic graft chain length on viscosity profiles of a 255 kDa graft platform a)5 wt% b) 10 wt% c) 15 wt%

The effect of the graft platform molecular weight on the rheology of the grafted polymers at a solution concentration of 15 wt% is shown in Figure 4-9, which compares the viscosity profile of three P(VP-VA) graft platform samples with different molecular weights grafted with C_{10}, C_{12} and C_{18}. For each graft length, as the molecular weight of the graft platform is reduced, more shear thinning is observed, attributable to the lower inherent viscosity of the backbone polymer and the higher mobility of the hydrophobes to form associations in the lower molecular weight graft platform. The viscosity of the grafted material at low shear rates is more influenced by the hydrophobic association of the grafts than the graft platform viscosity itself; as a result, since the graft length is the same in all cases, the viscosity profiles are the same at low shear rates. In comparison, the viscosity of graft material at high shear rates (at which the hydrophobic associations are broken) is more dependent on the polymer backbone molecular weight. Thus the viscosity of the C_{10} grafted polymer with the backbone molecular weight of 255 kDa at high shear rates is higher than that of the 170 kDa and 55 kDa polymers. Of particular note, according to Figure 4-9, shear thinning over 4-5 orders of magnitude can be achieved with these polymers, a technically useful range of shear thinning potential for use in eyedrops.

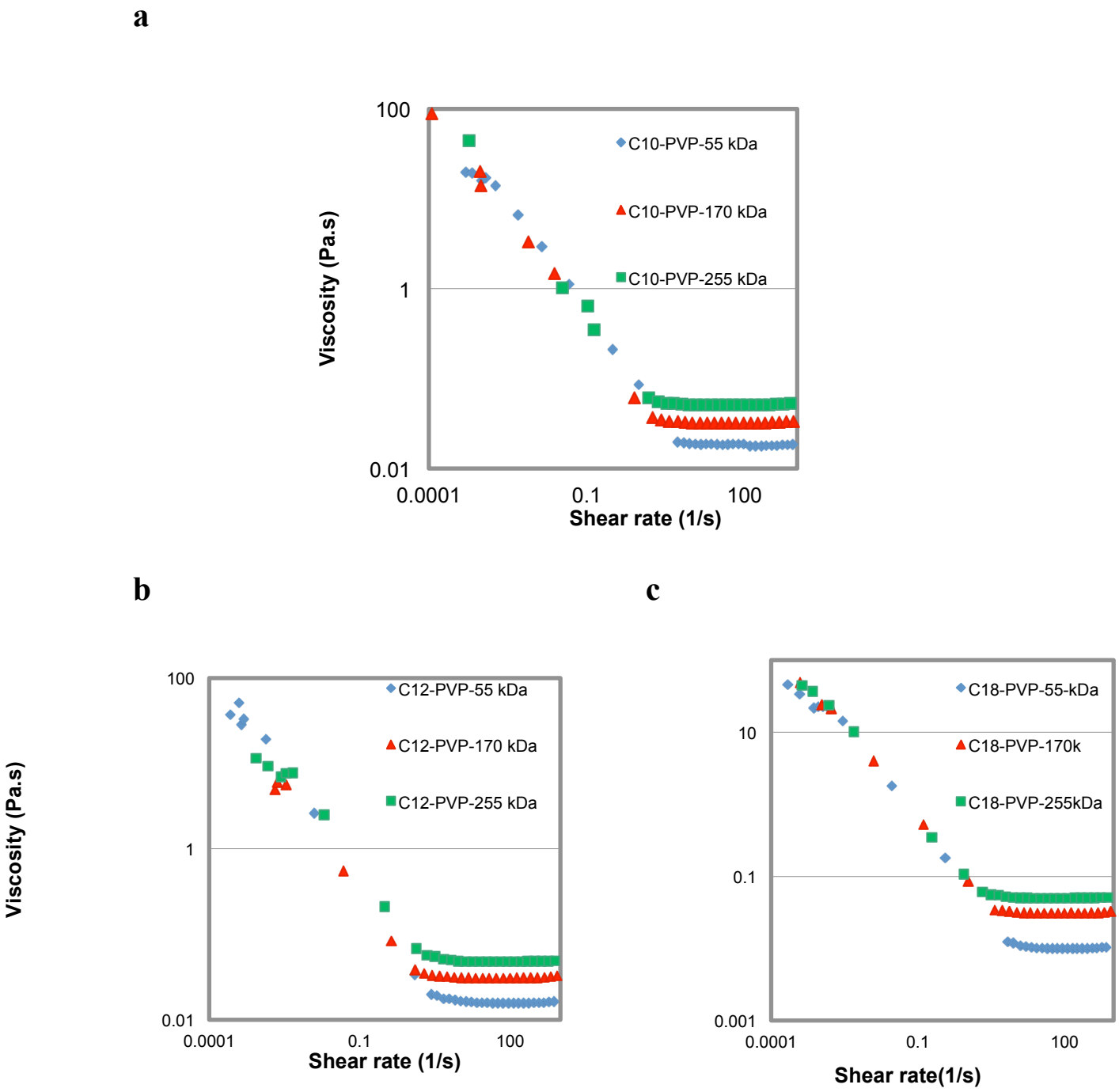

Figure 4-9 Effect of graft platform molecular weight on shear thinning of hydrophobic grafted polymers at 15wt% concentration. a) C_{10} b) C_{12} c) C_{18}

Since the self-diffusion of hydrophobes drives the buildup of viscosity via hydrophobic associations, a significant kinetic effect is anticipated on the viscosity profiles (note that all experiments reported to this point were performed by dissolving the polymers and waiting 48 hours). Time dependent rheology of the hydrophobically modified polymers was studied by conducting measurements as a function of resting time between consecutive shearing events, scanning the same shear range (shear stress of 0.01-100 Pa divided by 35 intervals and integration time of 60 seconds) at each time point (5, 10, 20, 40, 60 and 90 minutes of delay between each measurement). Figure 4-10 shows the resulting viscosity curves for a185 kDa P(VP-VA) polymer grafted with C_{18}. The hydrophobic grafted polymer is able to relatively quickly recover its structure to exhibit viscosities as high as 1 (Pa s) and 1.85 (Pa s) at shear rates of about $0.035s^{-1}$ after 60 and 90 minutes respectively. The ageing effect has rarely been investigated previously. Feng et.al. investigated the effect of ageing on rheological behaviours of associative polyacrylamide and observed an increase in the viscosity of solutions with time.(Feng, Grassl, Billon, Khoukh, & Francois, 2002)

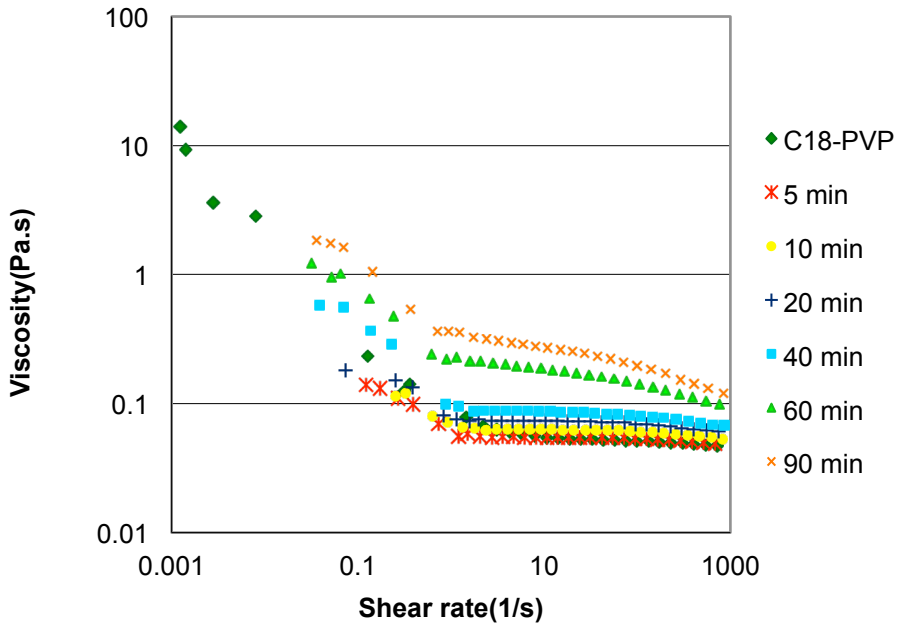

Figure 4-10 Time-dependent rheology of hydrophobically-modified polymers. (185 kDa P(VP-VA) grafted with C_{18}

The viscosity buildup of materials as a function of time can be tuned based on how the copolymer properties facilitate the self-assembly of hydrophobic grafts. Graft polymers with grafts that form stronger associations (i.e. C_{18}) or with graft platform polymers of lower molecular weights that facilitate higher graft diffusion would be anticipated to recover structure faster than shorter grafts or polymers with higher molecular weights. Figure 4-11 compares the viscosity profiles of 15 wt% solutions of the 185 kDa P(VP-VA) grafted with C_{18} and C_{12} after 48 hours and 3 weeks. As anticipated, the longer graft lengths build viscosity significantly faster than shorter graft lengths. Indeed, viscosities as high as 2.5×10^6 Pa s (analogous to an extremely stiff hydrogel) can be achieved after 3 weeks by grafting C_{18} with grafting density of only 1.5

mol% of the total residues. In the application of these materials in the eye, grafts that quickly build viscosity to maximum value (i.e. low molecular weight graft platforms) would be preferred.

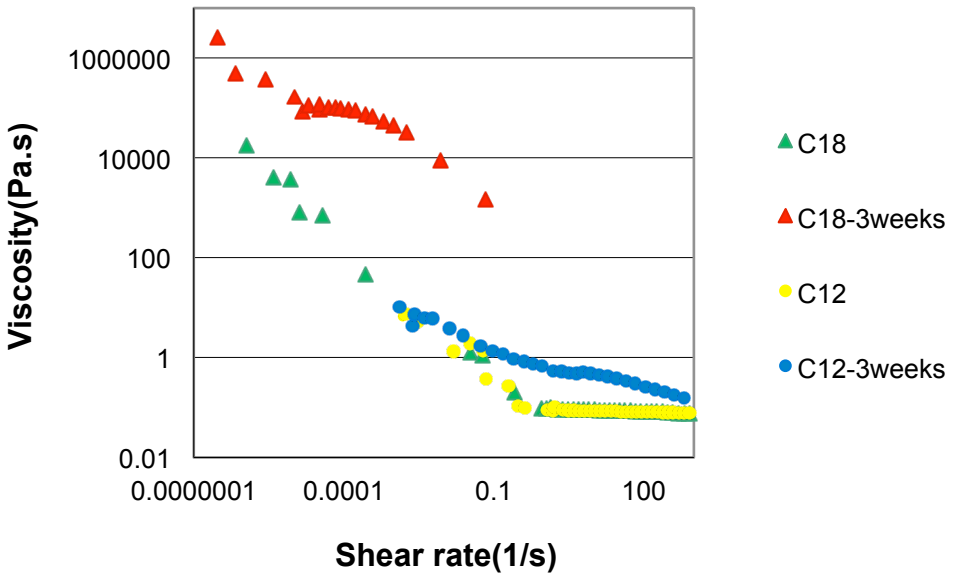

Figure 4-11 Time dependent rheology of C_{12} and C_{18} grafted polymers measured 48 hours and 3 weeks after preparing the samples

4.2.2 Rheological Measurements Of The Dual Grafted Polymers

Rheological measurements were performed on dual grafted polymers (i.e. polymers grafted with both hydrophobic chains and PBA) to study the effect of the boronic acid groups on the viscosity and shear thinning of the material. Viscosity profiles of polymer solutions of selected hydrophobic modified P(VP-VA) at concentrations of 5 wt% and 10 wt% were compared with the viscosity profiles of the same polymers subsequently grafted with PBA. C_{18}- PVP with molecular weights of 55 kDa exhibits the maximum shear thinning among all combinations tested and is therefore one of the top formulations chosen to make the dual grafted polymers. C18-PVP-255 kDa was grafted with PBA and compared to C18-P(VP-PBA)- 55 kDa to study the effect of polymer backbone molecular weight on the rheological properties of the dual grafted polymers. C_{12}-PVP-170 kDa and C12-PVP-255 kDa were also grafted with PBA to prepare a library of dual grafted material with different molecular weights and hydrophobe graft lengths. Figures 4-12 to 4-15 compare the viscosity profiles of the polymers grafted only with hydrophobic groups with the same polymers with both hydrophobic and PBA grafts. Molar compositions of PBA moieties and hydrophobic chains in the dual grafted polymers are presented in Table 4-1

Table 4-1 PBA and hydrophobic graft contents of the dual grafted polymers. Mole percentages are expressed in terms of percentage of monomer residues bearing each functional group

Sample	% alkyl chains of total monomer residues	% PBA of total monomer residues
C18-P(VP-PBA)-55 kDa	1.5	6
C12-P(VP-PBA)- 170 kDa	1.6	5.5
C12-P(VP-PBA)-255 kDa	1.2	6.5
C18-P(Vp-PBA)-255 kDa	1.1	6

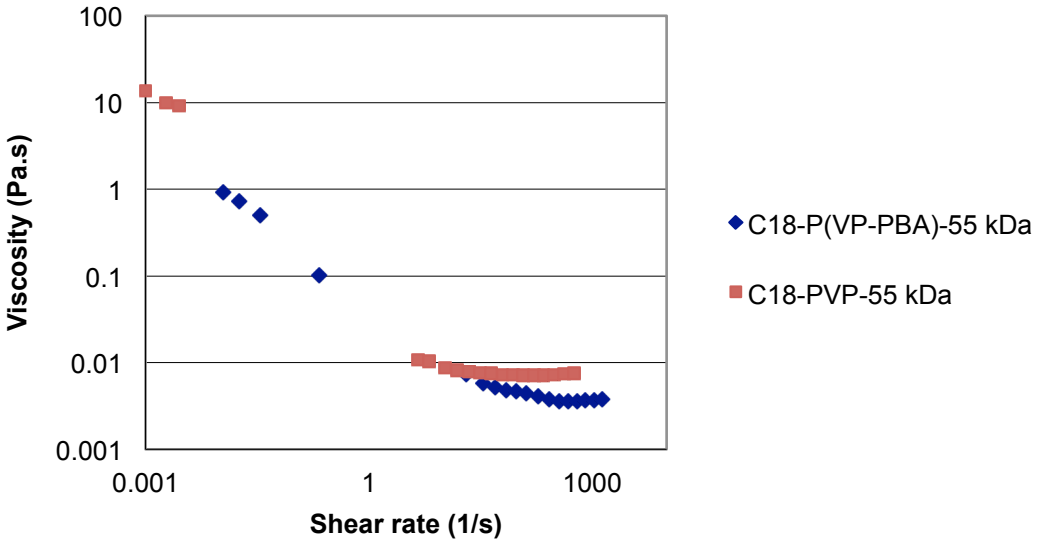

Figure 4-12 Effect of the addition of PBA grafts on the viscosity profile of a 55 kDa C_{18}-PVP

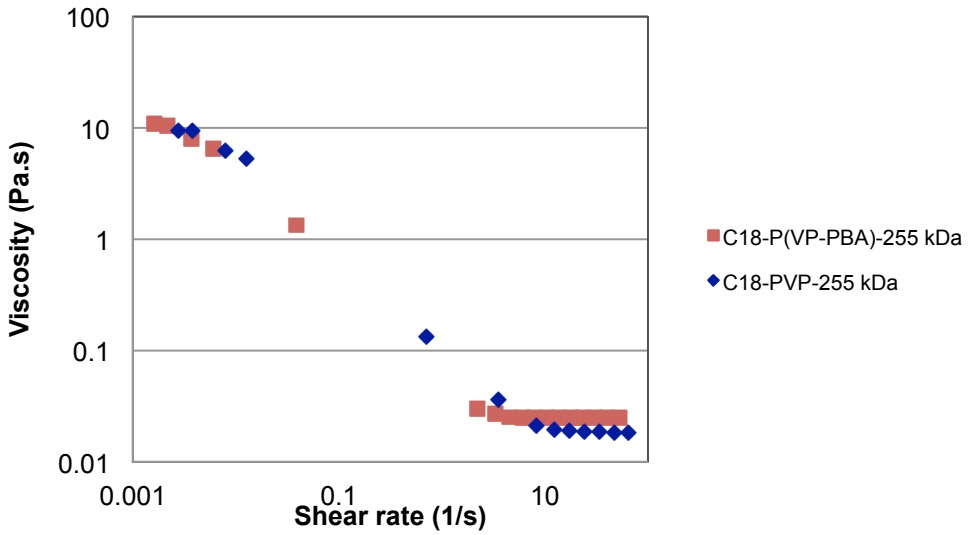

Figure 4-13 Effect of the addition of PBA grafts on the viscosity profile C_{18}-PVP-255 kDa

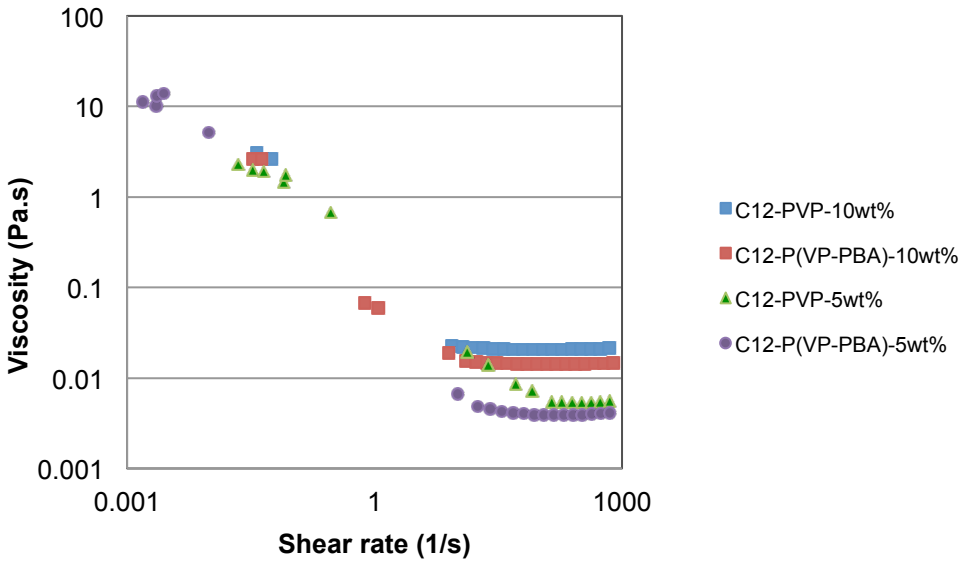

Figure 4-14 Effect of the addition of PBA grafts on the viscosity profile of C_{12}-PVP-170 kDa at solution concentrations of 5 and 10 wt%

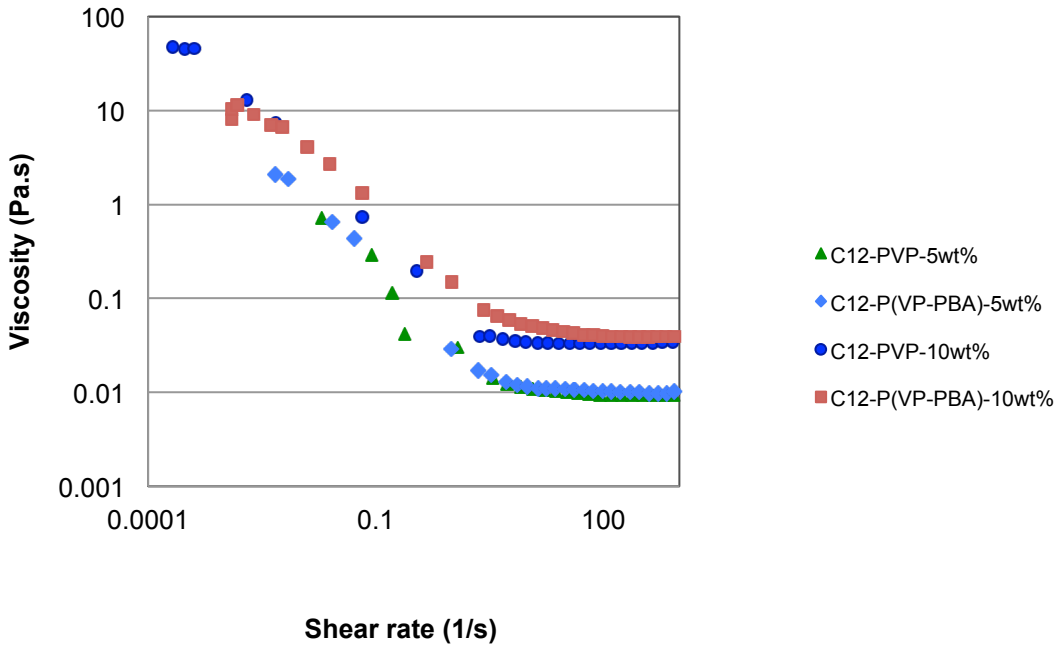

Figure 4-15 Effect of the addition of PBA grafts on the viscosity profile of C_{12}-PVP-255 kDa at solution concentrations of 5 and 10 wt%

Dual hydrophobe-PBA grafted polymers shear thin to the high shear plateau at slightly lower shear rates than the hydrophobe-only grafted polymers at all graft platform molecular weights and hydrophobe chain lengths tested. However, over the full range of polymer molecular weights, graft lengths, and polymer concentrations tested, the viscosity and shear thinning of the polymers are not significantly affected by the addition of the PBA grafts to the polymer. This result again suggests that a significant percentage of the PBA groups are in the anionic, boronate state under the test conditions, as un-ionized PBA is relatively hydrophobic and would be expected to reduce the viscosity (via polymer condensation) if uncharged. Thus, the rheological properties and the

mucoadhesive properties of the graft polymers can largely be independently tuned to optimize both the viscosity of the tear film and the mucin-polymer interactions.

5. Conclusions and Recommendations

5.1 Conclusions

- Copolymers of NVP and NVF with tailored compositions of both monomers were synthesized. Copolymers with the targeted NVF content (10%) and molecular weights ranging from 55 kDa-400 kDa were made for subsequent grafting.
- The hydrolysis kinetics of NVF were studied, allowing for precise control over the number of amine groups produced as a function of time and thus the degree of grafting achieved.
- Polymers with hydrophobic graft contents ranging from 1-1.8% using C_{10}, C_{12} and C_{18} grafts and PBA contents of ~6% were synthesized.
- Polymer solutions exhibit optical transparency (>90% transmission at 600 nm wavelength) even at high concentrations (15 wt%), facilitating their application in artificial tear formulations.
- Cytotoxicity studies using HCEC exposed to concentrations of hydrophobic grafted material suggested these materials exhibit no significant cytotoxicity.
- All formulations deviated from Newtonian behaviour, resulting in shear thinning flow over 4-5 orders of magnitude.
- The rheological properties of the hydrophobe-grafted polymer solutions were evaluated to determine the dependence of shear thinning on the length of the graft chains, concentration and molecular weight of the polymer backbone.

- Improved gelation behaviour was observed for hydrophobic grafted polymers compared to the non grafted P(VP-VA).
- Longer hydrophobic grafts lower the high shear viscosity to a greater extent than shorter hydrophobic grafts.
- As the graft length increases and the graft platform molecular weight increases, more shear thinning is observed at lower overall polymer concentrations.
- For each graft length, as the molecular weight of the graft platform is reduced, more shear thinning is observed.
- The viscosity of the grafted material at low shear rates is more influenced by the hydrophobic association of the grafts than the graft platform viscosity itself.
- The viscosity of graft material at high shear rates is more dependent on the polymer backbone molecular weight than the length or concentration of the hydrophobic grafts.
- The rheological behaviour of the graft copolymers is strongly dependent on time, with significant increases in viscosity observed as the formulation is kept at low shear.
- The longer graft lengths build viscosity significantly faster than shorter graft lengths.

• The rheological properties of the dual grafted polymer solutions were evaluated to determine the effect of the phenylboronic acid groups on the viscosity and shear thinning of the hydrophobically modified material

- The viscosity and shear thinning of the polymers were not significantly affected by the addition of the PBA grafts to the polymer.
- The rheological properties and the mucoadhesive properties of the graft polymers can largely be independently tuned to optimize both the viscosity of the tear film and the mucin-polymer interactions.

Overall, the dual hydrophobe-PBA grafted copolymers show the appropriate transparency, cytotoxicity, and rheological properties for potential application in the eye.

5.2 Recommendations

The results of this thesis work have demonstrated that the graft copolymers developed have appropriate optical, rheological, and biological properties for potential use *in vivo* as eye drop formulations or vitreous substitutes. To further assess the applicability of these materials for the desired ophthalmic applications, the following experiments should be conducted to move this work forward:

- *Dilution assays:* Given the highly diluting nature of the tear film, the response of these hydrophobic-associated materials to a dilution challenge should be assessed to ensure that the optimized polymer solution formulations have the potential for maintaining a gel over the course of at least six hours and ideally up to one day at normal tear turnover volumes. A flow cell can be used to pass simulated tear fluid past a hydrogel sample at the same volumetric flow rate as observed in the eye to perform this assay.
- *Optimization of boronic acid/hydrophobic graft ratio:* Gel properties should be optimized as a function of both hydrophobic graft and boronic acid group densities. This is important to ensure adequate gelation together with mucoadhesion and lipid layer stabilization. The observation that PBA grafting does not significantly influence the viscosity of the polymers (section 4.1.2) will facilitate this study, as both the rheology and the mucoadhesion can be independently tuned.

- *Testing of mucoadhesion:* Mucoadhesion of hydrogels of various formulations to a model mucin-coated surface simulating the cornea should be tested. While strong adhesion is not necessary or even desirable (to ensure eventual clearance of the material from the eye), affinity for mucin is required to increase the residence time of the hydrogel at the corneal surface for long term drug delivery.
- *Use of native materials in the tear as gelation aids:* Both PBA and the hydrophobic grafts have been documented to interact with species naturally present in the tear film, offering the potential to further modulate the rheological properties *in situ* following administration to the eye. For the PBA groups, the influence of a small amount of glucose in the polymer formulation on the gel properties will be investigated. Glucose, naturally present in tears at a concentration 5-10 fold lower than the blood glucose concentration,(Badugu, Lakowicz, & Geddes, 2003) has two cis-diol functionalities and thus can crosslink boronic acid-containing polymers; however, glucose undergoes lower affinity binding to boronic acid groups relative to mucin and thus should be replaced by mucin at the corneal surface only. Conversely, native proteins in the tear (e.g. albumin, present in the tear at a concentration of (~0.05 mg/mL) (Luensmann & Jones, 2008)have been reported to interact with hydrophobic grafts and increase the solution viscosity.(Borrega, Tribet, & Audebert, 1999; Tribet, Porcar, Bonnefont, & Audebert, 1998) Screening the impact of both albumin and glucose at tear-relevant concentrations will assess how the native tear influences both the rheology and the transparency of the grafted polymers. This strategy may be useful for further enhancing the gel strength via a labile bonding strategy without using any additional chemistry.

- *Drug delivery:* Drug release kinetics from these hydrogels for a drug appropriate for front-of-the-eye drug delivery should be measured when drug is (1) directly loaded into the gel or (2) loaded into nanoparticles that can be immobilized at the corneal surface using the gel. The latter experiment may be conducted with Frank Gu's laboratory within the 20/20 network, as they have prepared a range of nanoparticles with ophthalmic drugs.
- *Ocular compatibility:* Tolerability of the optimized formulations should be tested in the eye. Fluorescently labeling the polymer (easy to do using the sequential modification chemistry established) would allow for real-time probing of the residence time of the polymers in the eye and assessment of the duration over which the gel is maintained in the tear film.

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Appendices

A.Copolymer Composition by Nuclear Magnetic Resonance Spectroscopy

The chemical composition of each P(VP-VF) copolymer and corresponding hydrolyzed P(VP-VA) graft platform was confirmed using ^{1}H-NMR. Figure A-1.a shows the ^{1}H-NMR spectra of a P(VP(90%)-VF(10%)) copolymer sample conducted on a 600 MHz Bruker spectrometer. Spectra were analyzed by finding characteristic proton peaks corresponding to the theoretical polymer structure and integrating the areas (comparing areas on 1:1: hydrogen basis) to calculate the mol% of NVF residues relative to NVP residues, as detailed in section 3.1.3.1. Peaks assigned to the CH_2 group in the pyrrolidone ring are located between 2.9 ppm to 3.4 ppm and peaks assigned to the proton in the formamide group are located between 7.4 ppm to 8.1 ppm, divided into two multiplets at chemical shifts between 7.4–7.7 ppm and 7.75–8 ppm (Figure A.1.b). The two multiplets are derived from formamide protons accommodated in chains with diversified tacticity. The more intense signals at the higher chemical shifts corresponds to the *cis* conformation of the formamide proton and the signals at the lower chemical shifts correspond to the *trans* conformation. (Witek et al., 2007) Higher intensity of the peaks assigned to the polar *cis* conformation is a result of polymerization in a polar medium (isopropanol as solvent) (Kirsh, 1998). The molar composition of the NVF residues in the copolymer chains calculated form equation 3.1 is equal to 11%, close to the molar composition of the NVF in the monomer feed (10%) as expected for an azeotropic copolymerization.

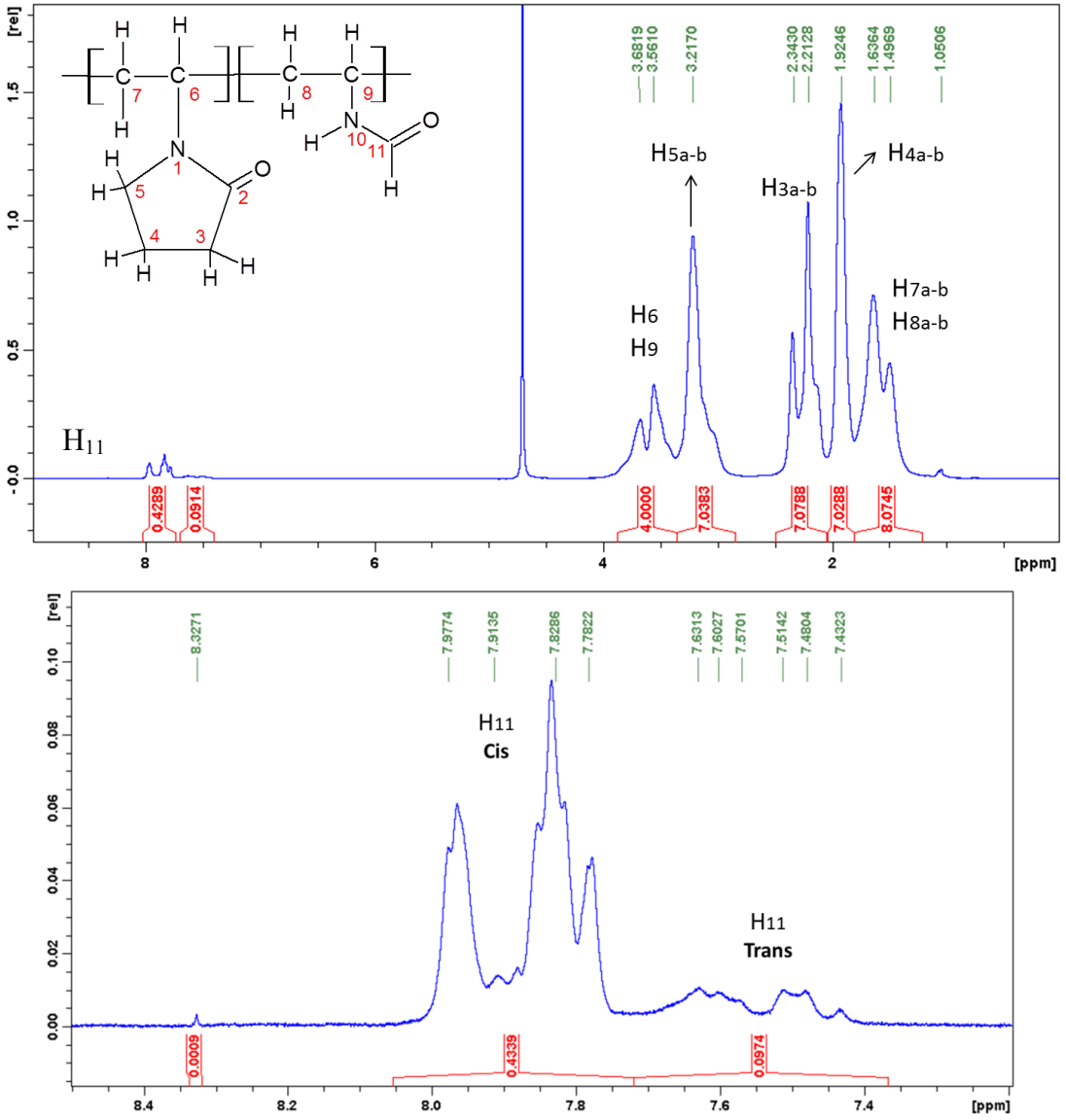

Figure A-1 ^{1}H-NMR spectra of a P(VP(90%)-VF(10%)) copolymer. a: Full NMR spectrum, b: Peaks assigned to the *cis* and *trans* conformations of the formamide groups

Figure A-2 shows the ^{13}C-NMR spectra conducted on a 600 MHz Bruker spectrometer of the same polymer. The peak assigned to the carbon in the pyrrolidone ring of the PVP is located at 177 ppm and the peak assigned to the carbonyl group of formamide is located at 163 ppm. The ^{1}H-NMR spectra of the P(VP-VA) polymers is explained in detail in section 3.2.3.1. Figure A-3 compares the ^{13}C-NMR spectra of a P(VP-VF) sample and the corresponding P(VP-VA), showing the reduction in intenstiy of the C=O peak as the amides convert into amine groups upon hydrolysis.

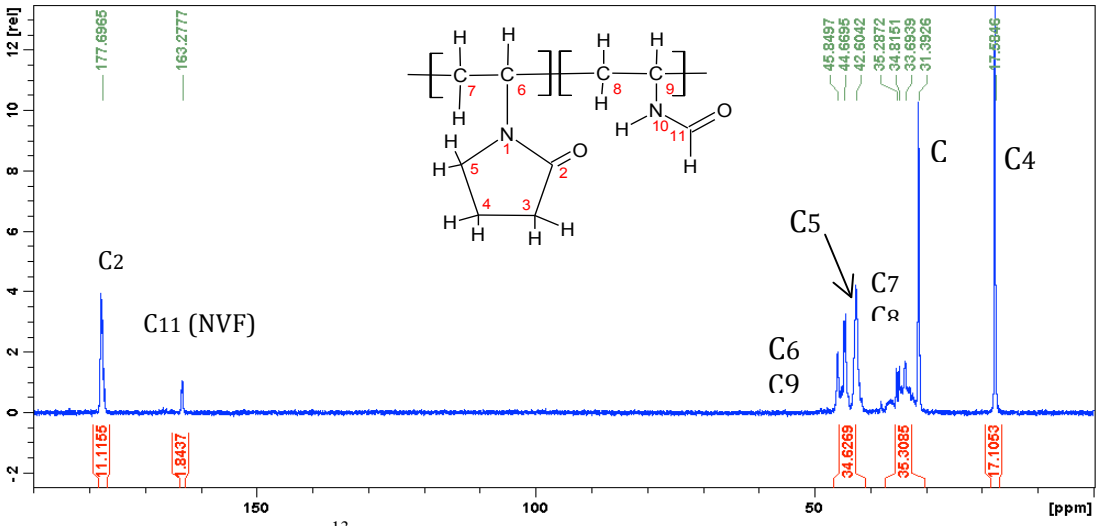

Figure A-2 ^{13}C-NMR of a P(VP-90%-VF(10%) copolymer

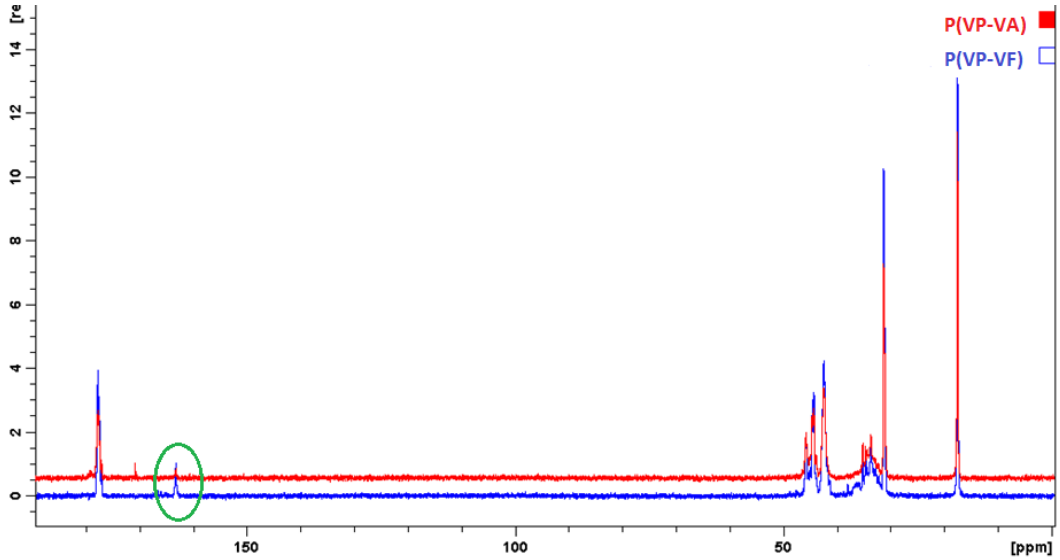

Figure A-3 ^{13}C-NMR of a P(VP(90%)-VF(10%)) copolymer and the corresponding P(VP-VA).

B. Titration Curve example

Figure B.1 shows a sample potentiometric-conductometric titration curve for 50 mg of a P(VP(90%)-VA) sample. The volume of base added (and thus the number of titratable functional groups in the polymer) was found based on two different methods which gave the same results. In the first method, the end point of the titration corresponds to the intersection of the extrapolated linear portions of the conductometric titration curve (green points in Fig. B-1). In the second method, the first derivative (d pH/d volume) of the potentiometric titration data is plotted versus the volume of base used. The spikes in the curve point correspond to equivalence points (red points in Fig. B-1)

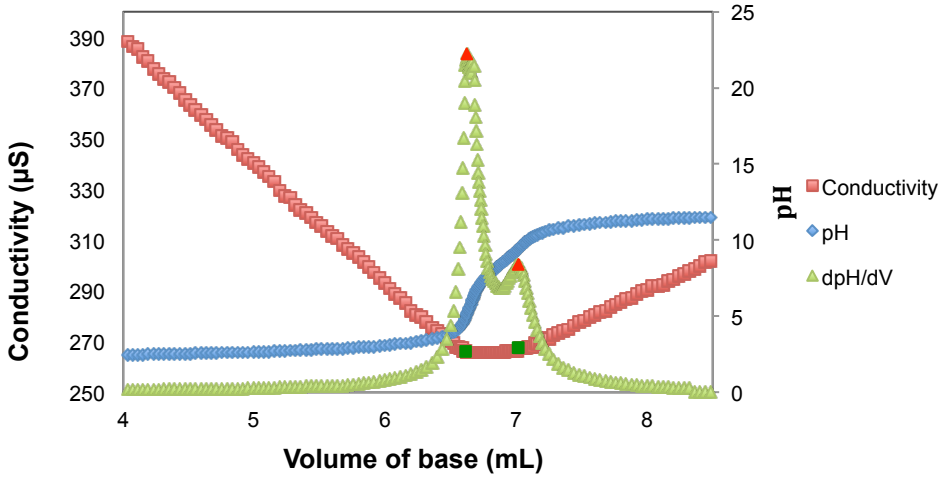

Figure B-1 Potentiometric-conductometric titration curves for 50 mg of a P(VP-VA) sample

Point	Volume of base used (mL)
1	6.6234
2	7.0156

Delta volume (mL)	0.3922
Concentration of NaOH used	0.1M
Moles of amine	3.92×10^{-5}
Molar amine content	8.5%

C. Cell Culture Procedures: Human Corneal Epithelial Cells

Medium Preparation

"Complete" KSFM (Gibco #17005-042) should be prepared by adding the following supplements to the bottle:

• Bovine pituitary extract & EGF supplement (add contents of vial that comes with purchased KSFM)

• 1x Penicillin/streptomycin (Invitrogen #15140122). Add to KSFM in a 1:100 dilution (for 500mL M199, add 5 mL).

Cell Thawing

1. Prepare complete KSFM, pre-heat to 37^{o}C in a water bath.
2. Disinfect the biological hood with UV light and by wiping the work surfaces with 70% ethanol. Prep and wipe down supplies with 70% ethanol (disposable pipettes, culture dish, etc.).

3. Remove cryovial from dry ice storage. Holding the cryovial, dip the bottom ¾ of the cryovial in a 37°C water bath and swirl gently until the contents are thawed.

4. In the biological hood, transfer cryovial contents to 15 mL Falcon tube.

5. To the Falcon tube, add slowly and drop wise 8 mL of KFSM.

6. If the cryovial contained DMSO, centrifuge (1000 rpm for 5 min) and replace supernatant with fresh KSFM.

7. Transfer cell suspension to a 10 cm^2 culture dish.

8. Incubate at 37°C with 5% CO_2.

9. Change medium every 2-3 days.

10. Split culture dish when 90% confluent.

Cell Splitting

1. Preheat TrypLE Express (1X) (GIBCO #12605, Invitrogen 12605-028) and KSFM to 37°C in a water bath. Disinfect surfaces with 70% ethanol.

2. Aspirate medium from culture dish.

3. Rinse cells with 2 mL sterile PBS (Ca^{2+} and Mg^{2+} free) (Dulbecco #14190-144) for 2 min, then aspirate off.

4. Add 2 mL TrypLE Express. Rock the dish to ensuring all cells are coated, and incubate for 10-30 min at 37°C, or until cells have detached from surface. Tapping the dish may help detach cells.

5. Transfer the cell suspension to a 13 mL Falcon tube.

6. Rinse the dish with 2 mL of fresh KSFM or PBS to collect any remaining cells. Add to Falcon tube.

7. Centrifuge cells (1000 rpm for 5 min). Carefully remove supernatant.

8. Re-suspend cells in KSFM and split as required (1:4 is recommended).

9. Incubate at 37^{o}C and with 5% $CO_{2(g)}$.

10. Replace medium every 2-3 days.

Counting Cells:

1. Aspirate off old media.

2. Add 5mL trypsin-EDTA into flask and gently tilt back and forth to detach cells.(5-10 minutes)

3. Add 6mL of the complete KSFM media into flask and move contents to 15mL centrifuge tube.

4. Centrifuge at 1000 rpm for 5 minutes.

5. Aspirate off media and add 15mL freshly warmed KSFM containing media.

6. Remove 100 µL of mixture and place in hemocytometer.

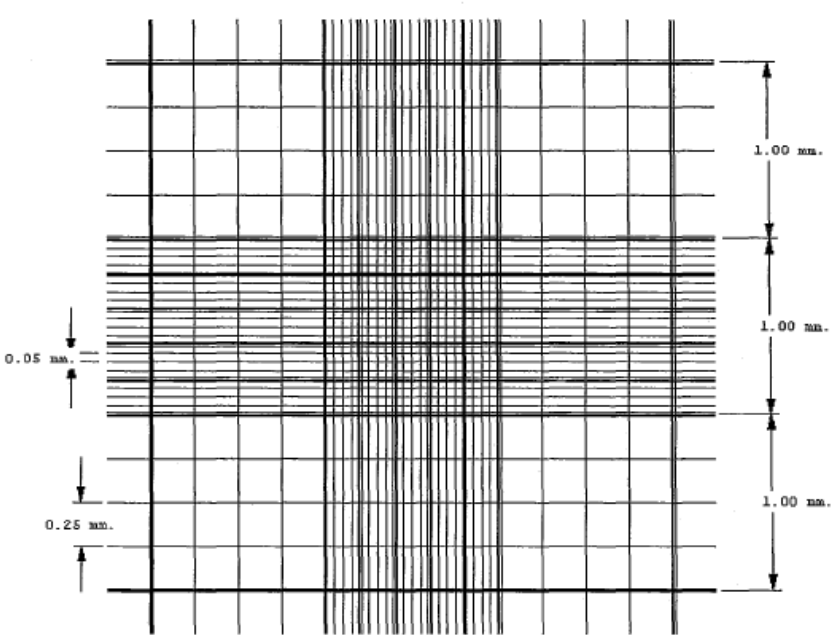

Figure C-1 Hemocytometer Grid

7. Count number of cells in outer quadrants (single quadrant area = 0.1mm^3) and calculate cells per quadrant. (See Figure C.1):

$$Number\ of\ cells = Cell\ Count\ (cells/0.1mm^3) \times 10000(0.1mm^3/mL) \times 15mL$$

Cell Freezing

1. Prepare an appropriate amount of KSFM by adding 10% dimethyl sulfoxide (DMSO).Mix well.
2. Perform steps 1 - 7 from the Cell Splitting instructions.
3. Re-suspend cells in freezing medium.
4. Distribute into cryovials.

5. Freeze the cryovial in –80°C freezer at least overnight (no longer than 3 months), and then transfer frozen cryovial into liquid nitrogen for long-term storage.

MTT Assay

Day 1:

1. Remove cells from flasks and plate in 24 well plates at a concentration of **100,000** cells per well.
2. Add 1 mL of media per well.
3. Allow cells to adhere for 24 hours.

Day 2:

1. Cells are exposed to polymer concentrations in solution using a volume of 1mL per well.
2. Perform replicates of 3-4 wells per sample.
3. Expose polymers to cells for 24 hours.

Day 3:

1. Reconstitute the MTT stock solution (about 3mL) in PBS and sterile filter. The concentration should be 40mg/mL with 10mM PBS.
2. Dilute to 0.4mg/mL with appropriate cell serum containing medium.
3. Expose cells, in a 24-well plate, to 150μm of MTT solution for 24hrs.

4. Add 250μL DMSO to dilute the formazan precipitate.

5. Shake plates for 10-20 minutes or until formazan is dissolved.

6. Transfer 200μL of solution to a 96 well plate and read in UV/vis plate reader at 570nm (baseline).