INFLUENCE OF PAPER SURFACE CHEMISTRY ON ANTIBODY ACTIVITY

INFLUENCE OF PAPER SURFACE CHEMISTRY ON THE ACTIVITY OF IMMOBILIZED ANTIBODIES

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Abstract

With a long-term view to developing bioactive paper that can detect pathogens both in the laboratory and in the field, it is important to understand whether wet-strength papers are suitable supports for antibodies. This thesis describes the influence of polyamide-epichlorohydrin (PAE) and polyvinylamine (PVAm), which are typical wet-strength resins, on antibody activity. Two kinds of antibodies were employed: conventional Anti-Rabbit IgG (AR-Ab, whole molecules) and engineered anti-S. aureus single domain antibody with cellulose binding domain (CBM-Ab). The results of the activity studies surprisingly showed that the typical loadings of reactive, cationic wet-strength polymers, used to strengthen wet paper, did not interfere with the antibody assays. However, higher content of wet-strength resins impeded the function of antibody. Also, conventional AR-Ab adsorbed very well and retained its functionality on paper surface without the aid of cellulose binding domain. A preliminary study was also performed to investigate the effect of alkylketene dimer (AKD), as a sizing agent, on AR-Ab activity. The results in the early stage revealed that AR-Ab functioned better on AKD-treated papers than on original paper.

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Chapter 1 Introduction

Since 2005, there has been a tremendous research effort in Canada to develop technology platforms for bioactive paper.[1] One of the specific goals of the Canadian effort is to produce paper-supported sensors that will detect the presence of pathogens (bacteria and viruses) and produce a direct visible indication without the use of sophisticated and expensive instruments. Such type of paper is essentially a pathogen-detecting paper that is analogous to pH sensitive litmus paper. A number of strategies have been adapted from a wide range of available detection technologies and focused on this work, such as bacteriophages,[2] DNA aptamers,[3] and antibody fragments (immunoassays). Reported in this work are results from an initial assessment of the influence of paper surface chemistry on paper-supported immunoassays.

Paper filters have a long history in the biochemistry laboratory for filtration and as chromatography supports. Paper-based filters for laboratory use are made from fully bleached cotton or wood fibers that are nearly composed of pure cellulose with no chemical additives. However, these pure cellulosic papers possess low mechanical strength when they are wet. Modern laboratory cellulosic filters with enhanced wet strength are usually "acid hardened" or may contain some synthetic fibers. Outside the laboratory, printing and packaging papers present a much greater diversity in both

physical and chemical properties. For example, domestic coffee filters are often brown, indicating some lignin in the paper, and the filters are impregnated with wet-strength resins. Polyamide-epichlorohydrin resin (PAE) is one of the most common commercial wet-strength resins used in paper-related industries.[4] By contrast, printing papers often contain mineral fillers and have been treated with hydrophobizing (sizing) agents to control water and ink penetration.

The long-term goal of this work is to develop robust pathogen detection technologies that will work on a wide range of paper surfaces. Hence, the antibody performance on filter paper which had been impregnated with various levels of two wet-strength resins and sizing agent was evaluated in the early stage of the bioactive paper development. In particular, the influence of reactive cationic wet-strength resins and sizing agent on the denaturation of antibodies immobilized on paper surface was one of the concerns in the development of pathogen detecting filter paper. Surprisingly, the result shown in this work concluded that this did not turn out to be the case.

Chapter 2 Literature Review

2.1 Antibody

2.1.1 Immune system

Immune system is a collective network of cells, tissues, proteins and organs, which can identify pathogens and tumour cells, and kill them in order to protect its host against foreign agents and infectious organisms.

The immunity can be generally divided into two systems: innate (natural) immune system and adaptive (acquired) immune system. The innate immunity relies on phagocytic cells which can release inflammatory mediators and kill pathogens. [5] As the term suggested, it is naturally present and generally nonspecific. As such, it is considered as the first line of defence. If the innate immunity is not strong enough to protect against the invading pathogens, the adaptive immunity may work effectively. Unlike innate immunity, the adaptive immunity needs some time to develop and reacts specifically with the organism that provokes the response. Additionally, adaptive immunity also possesses immunological memory.[6] In general, adaptive immunity is composed of T lymphocytes and B lymphocytes. T lymphocytes are developed in the thymus and responsible for guarding against invaders while B lymphocytes are grown in the bone marrow and can

produce a variety of proteins, referred to as antibodies or immunoglobulins.[7]

2.1.2 Antibody Structure

Antibodies (Immunoglobulins or Igs) are glycoprotein molecules which are produced by plasma cells to recognize foreign objects such as bacteria and viruses in immune system.

All antibodies are, at least, composed of four subunits as their basic structure: two identical heavy chains (50-70 kDa) and two identical light chains (~23 kDa). The subunits are responsible for the globular structure and they are connected by inter-chain disulfide bonds and non-covalent interactions to form a Y-shape structure (shown in **Figure 1**). Within the subunits, each polypeptide chain also has intra-chain disulfide bonds.[7]

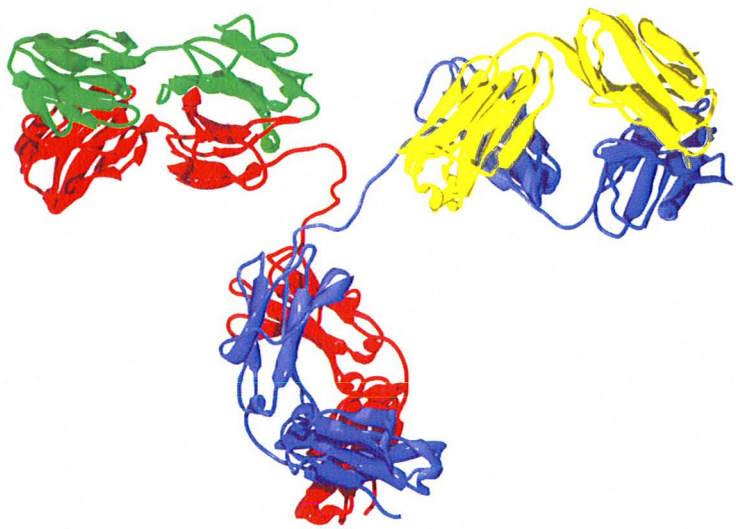


Figure 1 X-ray structure of IgG (http://en.wikipedia.org/wiki/File:Antibody_IgG2.png) [8]

Antibodies can be categorized into five classes (IgA, IgD, IgE, IgG and IgM) according to their types of heavy chains and subunit structure which are summarized in **Table 1** and **Figure 2.** The first type of antibodies is IgA which plays an important role not only in mucosal surfaces, such as the linings of the lungs, gastrointestinal and respiratory tracts, but also in the external secretions such as saliva, tears and breast milk with a fluid-type secretion. The IgA can be of monomers, dimers or secretory structure.[9] IgD, on the other hand, presents a small amount in blood serum and its function has not been well-identified. Despite its enigmatic function, IgD still remains as a topic of interest for scientists to explore.[10] IgE is a monomeric protein, present in blood in a low concentration. It detects and responses to parasitic worms and allergens.[11] IgM consists of five Y-shape molecules joined by a J (Joining) chain with a molecular mass of approximately 900 kDa and is the first antibody to appear in the immune reaction. Although IgM has 10 binding sites, it could not bind 10 antigens at the same time and diffuse well because of its large physical size.[12] IgG is the most abundant immunoglobulin of all the Ig classes. It makes up 80% of the immunoglobulins in adult plasma and is most commonly used in the application of therapeutic and biosensors. IgG is produced during the secondary immune response and can be further divided into four major subclasses: IgG1, IgG2, IgG3, and IgG4.[13]

Table 1: Properties of Different Immunoglobulins[7, 14]

Class	Heavy Chain	Light Chain	Molecular Form	Molecular Weight (kDa)
IgA	α	γ or κ	Monomer, Dimer, Secretory	360-720
IgD	δ	γ or κ	Monomer	160
IgE	ε	γ or κ	Monomer	190
IgG	γ	γ or κ	Monomer	150
IgM	μ	γ or κ	Pentamer	950

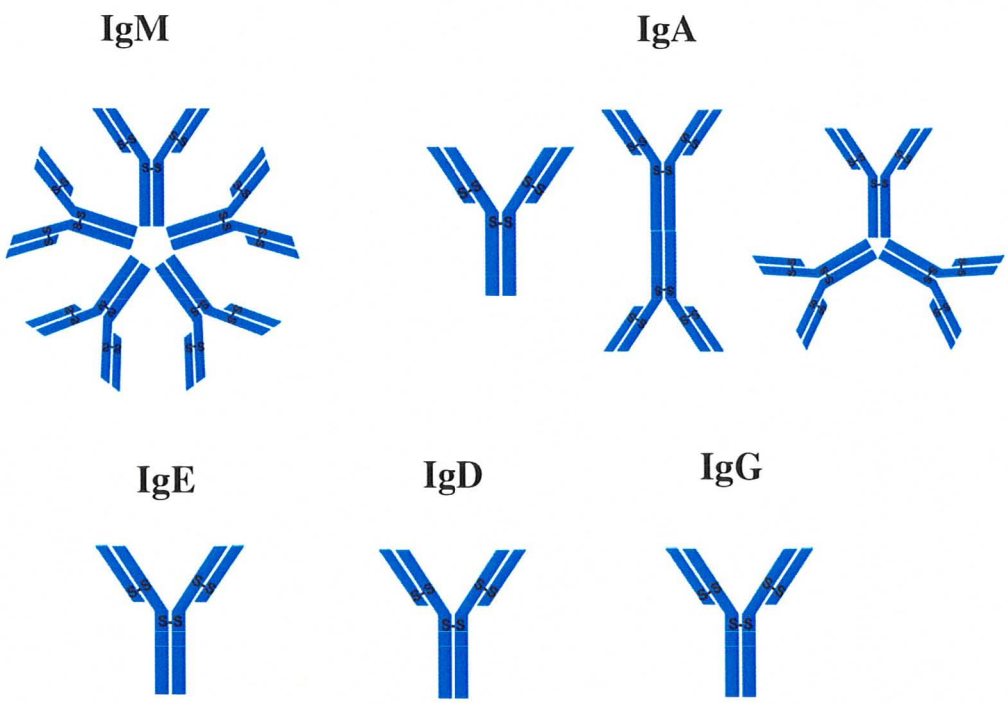


Figure 2 Structure of different immunoglobulins

Figure 3 presents a crude image of IgG structure of human. IgG has a Y-shape configuration which is defined by two broad regions. The top of Y-shape antibody is composed of two hands, generally known as Fab fragments. On each side of the hands, there are two sets of constant (C_L, C_H1) and variable domains (V_L, V_H), which compose the light and heavy chains. The tail of Y-shape antibody represents Fc fragments, which is crystalizable, and it is made up of four heavy constant domains (CH_2, CH_3).[7, 15]

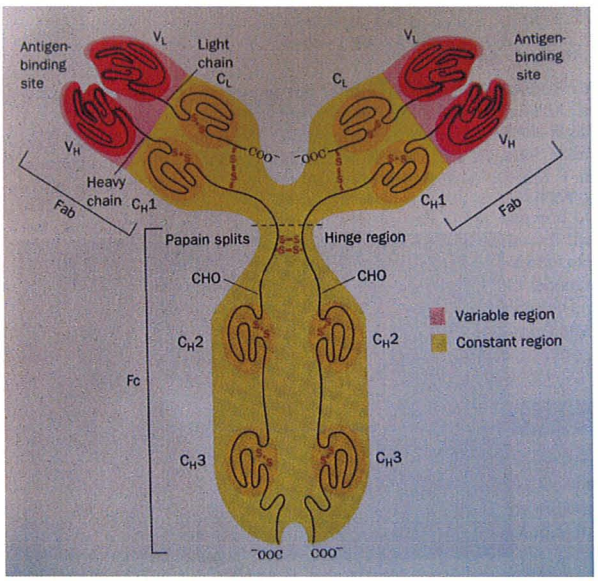


Figure 3 Typical structure of human immunoglobulin G (IgG)[7]

2.1.3 Antibody-Antigen Binding

The entire mechanism of antigen-antibody interaction consists of two stages, namely site recognition and non-covalent binding. The variable domain, found in antibody, is formed by the linkage of a three- and a four-stranded anti-parallel βsheets via disulfide bond. The sheets are interconnected by three hypervariable loops which are

responsible for the recognition of complementary site (epitope) of antigen. Since these loops carry unique amino acid sequences, the site recognition of antibody and antigen is analogous to a key and a lock concept.[7]

Once the recognition is achieved, the antibody captures the antigen via a group of non-covalent interaction forces, such as van der Waals force, hydrogen bonding, electrostatic interaction, and hydrophobic bonding. The resulting antibody-antigen binding strength is extremely strong due to their high dissociation constants (10^{-4} to 10^{-5} M) as well as the synergistic effect of the interaction forces.[7] In some circumstances, this interaction is so strong that the binding may lead to a dramatic change in conformation. Nevertheless, the binding of antibody and antigen is reversible because their interaction is through non-covalent bonds.[16]

2.1.4 Recombinant Antibody

With the significant advancement in genetic engineering and research, the conventional antibody can be re-engineered using the recombinant DNA technology. Such recombinant is commonly performed by expressing the genes of the antibody of interest in bacterial, yeast, plant, immune tissue, insert cell or hybridoma cell lines to fuse the genes, allowing the encoding of the variable region (V_L and V_H) with the constant region of immunoglobulin. The recombinant antibody is also known as "chimeric

antibody".[17-19] Single chain antibody (scAb) and single domain antibody (sdAb) are the two typical kinds of recombinant antibodies, which just contain function fragments.

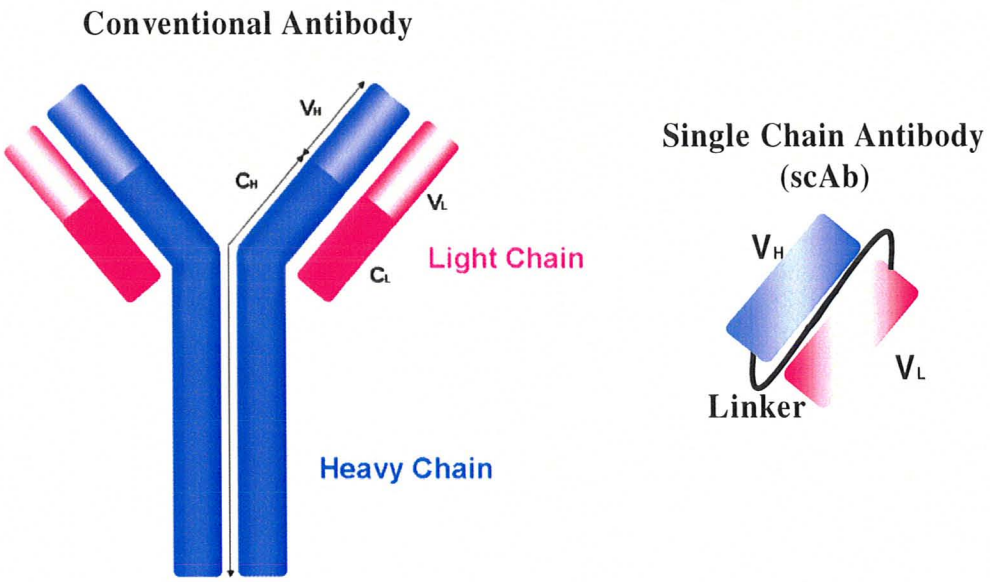


Figure 4 Typical structure of conventional antibody and single chain antibody (scAb)

Single Chain Antibody (scAb), as shown in **Figure 4,** is made of variable domains of heavy and light chains (V_H and V_L) with a molecular mass, ranging from 27 to 30 kDa. It is also known as single chain variable fragment (scFv). Two variable domains are linked by a polypeptide with 10 to 25 amino acid residues through the carboxyl terminus of one variable domain to the amino group of the other. The non-specific interaction between polypeptide linker and variable domains and the instability of polypeptide linker can lead to deficiency of scAb.[20] The first scAb was engineered by Huston et al.[21] using protein engineering principles and recombinant DNA technology.

They successfully yielded genetically fused variable domains of the 26-10 anti-digoxin monoclonal antibody merely by expressing them in E. coli, followed by isolating them as a sigle chain and refolding them to become a functional antibody combining site. These small antibody fragments are very suitable for use in imaging or therapy application. They can penetrate tissue boundaries more easily and be cleared from the serum faster because of their tiny size.[22] However, the disadvantages of using scAb include the susceptibility of degradation of hydrophilic polypeptide linker by proteolysis and the tendency of the linker to induce scAb aggregation.[23]

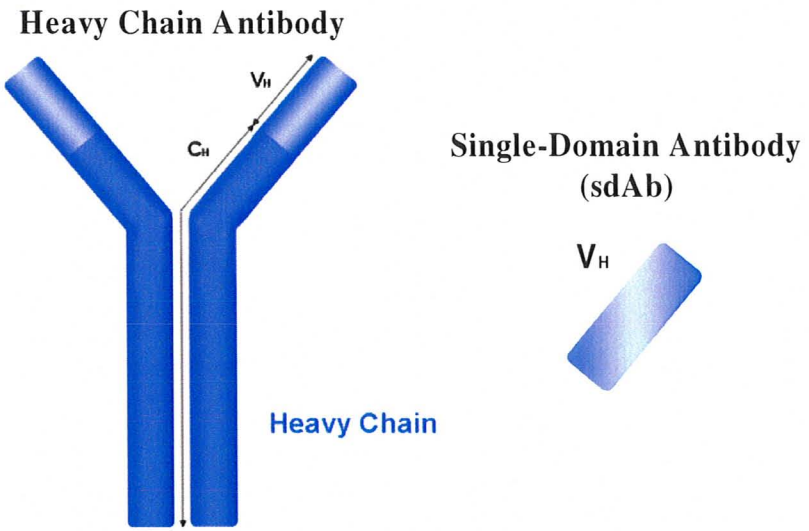


Figure 5 Typical structures of heavy chain antibody and single domain antibody (sdAb)

Single Domain Antibody (sdAb), which is just composed of variable domain from heavy chain (V_H), was first described by Ward et al.[24] They expressed and secreted

V_H domains, obtained from mice, in E. coli. The isolated V_H domains show strong affinity to the corresponding antigens because the domains retain the antigen-specificity of the parent antibody. However, they also found that the isolated V_H domains are quite sticky because of their hydrophobic surface which causes them to become insoluble and easily aggregated.[25] It was reported that the serums, obtained from camelidae (camels and llamas) and cartilaginous fish (wobbegong and nurse sharks), have a peculiar type of antibodies which have no light chains and is lower in molecular weight, as illustrated in **Figure 5**. As such, the solubility of sdAb can be improved by using these types of antibodies.[26] Hamers-Casterman et al.[27] and Ghahroundi et al.[28] reported that the sdAb from heavy chain antibody showed excellent stability, high solubility and strong affinity towards antigen. The other attractive features of sdAb include incredibly small size of natural single domain, high yields in expression and low cost.[29]

In general, intact and recombinant antibodies are quite different. The intact antibodies are polyvalent molecules which contain two binding sites or more. IgM is an example of intact antibody which has ten Fab arms. Compared to polyvalent antibody, the scAb and sdAb are both monovalent, which contain one binding site from the corresponding intact antibody. Although the intact antibodies have higher avidity than scAb and sdAb, they are still not very efficacious in some applications. For instance, the intact antibody was reported to be too large to penetrate into tumour effectively in a

cancer targeting study, leading to high retention in organ, such as liver, because of the slow clearance.[30] On the contrary, the monovalent Fab and Fv had high off-rates because of its single binding site and its small size, which resulted in rapid clearance from the blood.[31] Hence, multimerization of small functional fragments using chemical or genetic engineering, which can offer more binding sites and appropriate molecule size, is a strategy to solve the inefficiency problem with intact antibodies.[30, 32] Le Gall et al.[33] had successfully developed dimers, trimers and tetramers of scAb linked by different linkers, and compared their binding characteristics to the corresponding antigen. They concluded that the increase in avidity and size of scAb could be beneficial for imaging and immunotherapy of B-cell disease. Zhang et al.[34] constructed the pentamer of sdAb from phage libraries which has improved functional affinity for immobilized antigen, in addition to excellent thermostability and protease resistance. The sdAb was fused to the B-subunit of verotoxin and formed a homopentamer by self-assembly of verotoxin.

2.2 Paper Fundamentals

Paper is one of the greatest inventions in the history which plays an important role in our modern life. Nowadays, people cannot live without paper products as paper has been widely used for wrapping, packaging, cleaning, writing, printing and so on. More than 300 million metric tons of paper, as well as paper board, are produced every year in the world. Papermaking was first developed by Chinese in 100 AD using bamboo or

mulberry fibers.[35]

2.2.1 Chemical Properties

The major constituent of paper is wood fibers which bind to one another and form a porous paper web. The wood fibers are mainly composed of cellulose (40%-50%), hemicelluloses (15%-35%) and lignin (15%-35%).[36] Cellulose is a polysaccharide with a chemical formula of $(C_6H_{10}O_5)_n$, where n is the degree of polymerization, typically ranging in between 600 and 1500. The structure of cellulose is show in **Figure 6**. Each repeating unit contains two consecutive glucose anhydride units, called cellobiose, and six free hydroxyl groups which account for the high mechanical strength of cellulose via hydrogen bonding.[35]

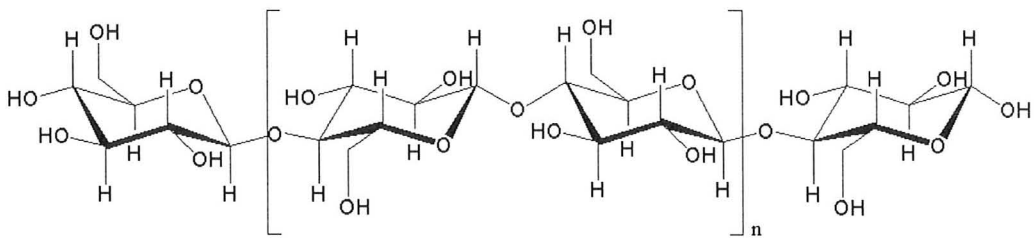


Figure 6 Structure of cellulose

Hemicelluloses are a blend of polymers containing glucose, mannose, galactose, xylose and arabinose.[35] Compared to cellulose, hemicelluloses have lower molecular weight with repeating units in between the range of 100 to 400. The structure of

hemicelluloses is random and predominantly amorphous. As such, it is less chemically stable but more readily soluble than the cellulose.[36] Nevertheless, hemicelluloses facilitate fiber-fiber bonding by amending the ability of fibers to take up water.[37]

Lignin is an aromatic polymer with complex and amorphous structure. It mainly consists of groups of methoxyl, phenolic hydroxyl and benzyl alcohol groups.[38] Due to the nature of mechanical pulping process, lignin cannot be completely removed from wood fibers and therefore papers are inherently brown and brittle. However, bleaching process can remove the remaining lignin to make the papers white.[35]

Besides the major components, there are also a number of organic extractive and inorganic materials such as resin acids, fatty acids, alcohols and calcium. These chemicals are usually water or neutral organic solvent soluble and typically make up less than 10 % of the native paper fibers. [35, 36]

Cellulose fibers in paper are ribbon-like hollow tubes which are approximately 1.5 mm in length, 20 μm in width and 2 μm in wall thickness.[39] Native cellulose fibers are negatively charged majorly due to the carboxyl groups which were formed during bleaching and alkaline oxidation processes.[38]

2.2.2 Structural Properties

Typical structural properties which are used to characterize the arrangement of the components in papers include but not limited to grammage, thickness, formation, smoothness and porosity. Grammage defines the weight per unit area of the paper in g/m^2. This property can be measured, especially during the papermaking process, using either β-ray emission or ultrasonic wave propagation. However, it is affected by the additives, moisture content of paper and so on, which can introduce errors while taking the measurement. Thickness indicates the distance between the two principal surfaces of the paper, as measured by micrometer, usually under the pressure of 50 ± 2 kPa, according to TAPPI Test Method T 411. Similar to grammage, there are plenty of factors, such as the amount of pressing, the sheet moisture and the additives, which can influence the thickness measurement. The formation refers to the uniformity of fibers distribution and other solid components on the paper. There are two ways to measure the formation. The most direct and practical method is visual examination and comparison by eyes. This method, however, is less accurate. On the other hand, the use of sophisticated instrumentation is much more accurate as it detects the variation in paper surface by measuring the transmitted light when it scans across the paper samples. The formation may also be affected by paper machine, furnished variability and so on. Smoothness, which is quite important for writing and printing, describes the outer structure of paper.

TAPPI 479 is the most common method to measure the smoothness of paper. The raw materials and processing variables in papermaking can influence the smoothness of paper. Also, using shorter and finer fibers can improve smoothness. Manufacturing parameters, such as pressure and moisture, can also affect the smoothness of paper. Porosity is the ratio of all pore volume to total volume of the paper.[37]

2.2.3 Mechanical Properties

The important mechanical properties of paper are mainly tensile properties, tearing resistance, and stiffness. Tensile strength represents how strong and durable the papers are when they are subjected to direct tensile stresses. Many methods such as increasing the long fiber content, basis weight and wet pressing can improve tensile strength. Tearing resistance indicates the ability of paper to withstand any tearing strain during converting or in its end use. Stiffness can be defined as the ability of papers to resist bending and is affected by thickness, grammage and Young's modulus (elastic modulus). [37]

2.2.4 Fiber-Fiber Bond

Paper is a three-dimensional network of cellulose fibers which are held together by intermolecular forces, such as hydrogen bonds and van der Waals forces. The hydrogen bonds between fibers are predominant while the van der Waals forces are weak

and, nevertheless, also provide some contribution to keep fibers in contact. The hydrogen atom from the hydroxyl group of cellulose or hemicelluloses, and the oxygen atom from the other hydroxyl group or carbonyl group form the hydrogen bond with the bonding energy of 8-32 KJ/mol while the bonding energy of covalent bond is 150-500KJ/mol.[40] Hydrogen bonds are sensitive to water content because water molecules can interfere with the hydrogen bonding between fibers. When the paper is wet, part of the hydrogen bonds would be broken, causing the paper to lose about 90% to 97% of its original dry strength. The strength of hydrogen bonds decreases with increasing water content in paper.[41] Therefore, some chemical wet-strength resins have been developed to help paper retain some strength when wet by adding and enhancing the bonding areas.

The commercial wet-strength resins are usually cationic, water soluble and chemically-reactive polymers. They can adhere to anionic pulp spontaneously by adsorption through electrostatic interaction, crosslink among themselves and form an insoluble network. Such strong network, as shown in **Figure 7**, can enhance the contact between fibers and prevent them from falling apart. When the paper is rewetted, these wet-strength resins can restrict the movement of fibers, thereby preserving part of original dry strength. Additionally, the covalent and ionic bonds, occurring between wet-strength resin and fibers can further step up paper wet strength. Since covalent bonds cannot be

broken easily by water, a stronger and more permanent network is resulted. Also, the covalent bonds play a significant role in reinforcing the natural strength of hydrogen bonding in the dry sheet while the ionic bonding provides a weaker and more temporary form.[4, 41]

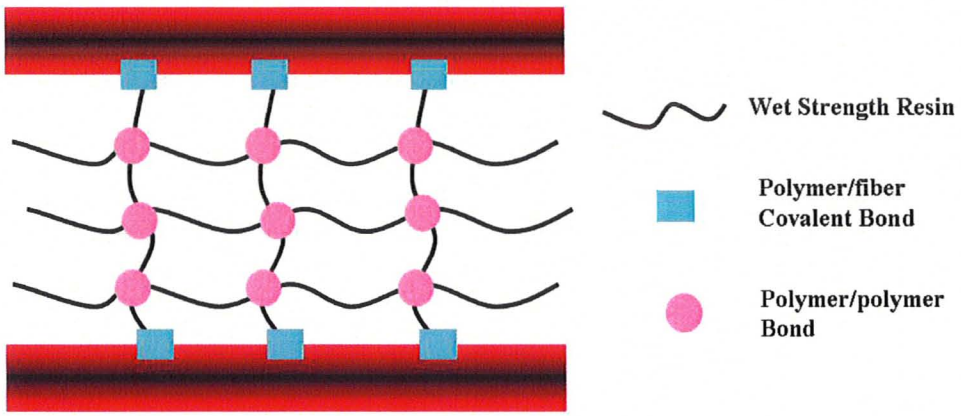


Figure 7 Structure of fiber network treated with wet-strength resin

2.2.5 Wet-Strength Resins

The common wet-strength resins used in paper include phenol formaldehyde resin, polyamide-epichlorohydrin resin (PAE) and other special additives, such as polyethylenimine, polyacrylamide-glyoxal and dialdehyde starch.[41]

PAE resin is the most common and efficient wet strength resin in papermaking industry. It is synthesized by the reaction of polyamideamine and epichlorohydrin to form polymer chains with four-membered azetidinium rings (AZR), as shown in top of Figure

8.[42, 43] Although PAE resin can be applied in alkaline, neutral or slightly acidic media, the best pH range is between 6 and 8. Since PAE resin contains tertiary and quaternary amino groups, it has net positive charge and, therefore, can absorb onto cellulose fibers via electrostatic interaction.[44] The possible reactions between PAE and cellulose are shown in the bottom of **Figure 8**. The azetidinium group of PAE can either reacts with carboxyl group of cellulose fibers or amine group of the other PAE chains to form a homo-crosslinking network.[41, 45] PAE resin can provide a long-term wet strength in paper. Hence, it is often applied in kitchen towels and coffee filters. However, other wet-strength resins, such as polyacrylamide-glyoxal, show temporary strengthening effects, which are suitable for products that are exposed to moisture in a short time (i.e. facial tissue and packaging applications).[39]

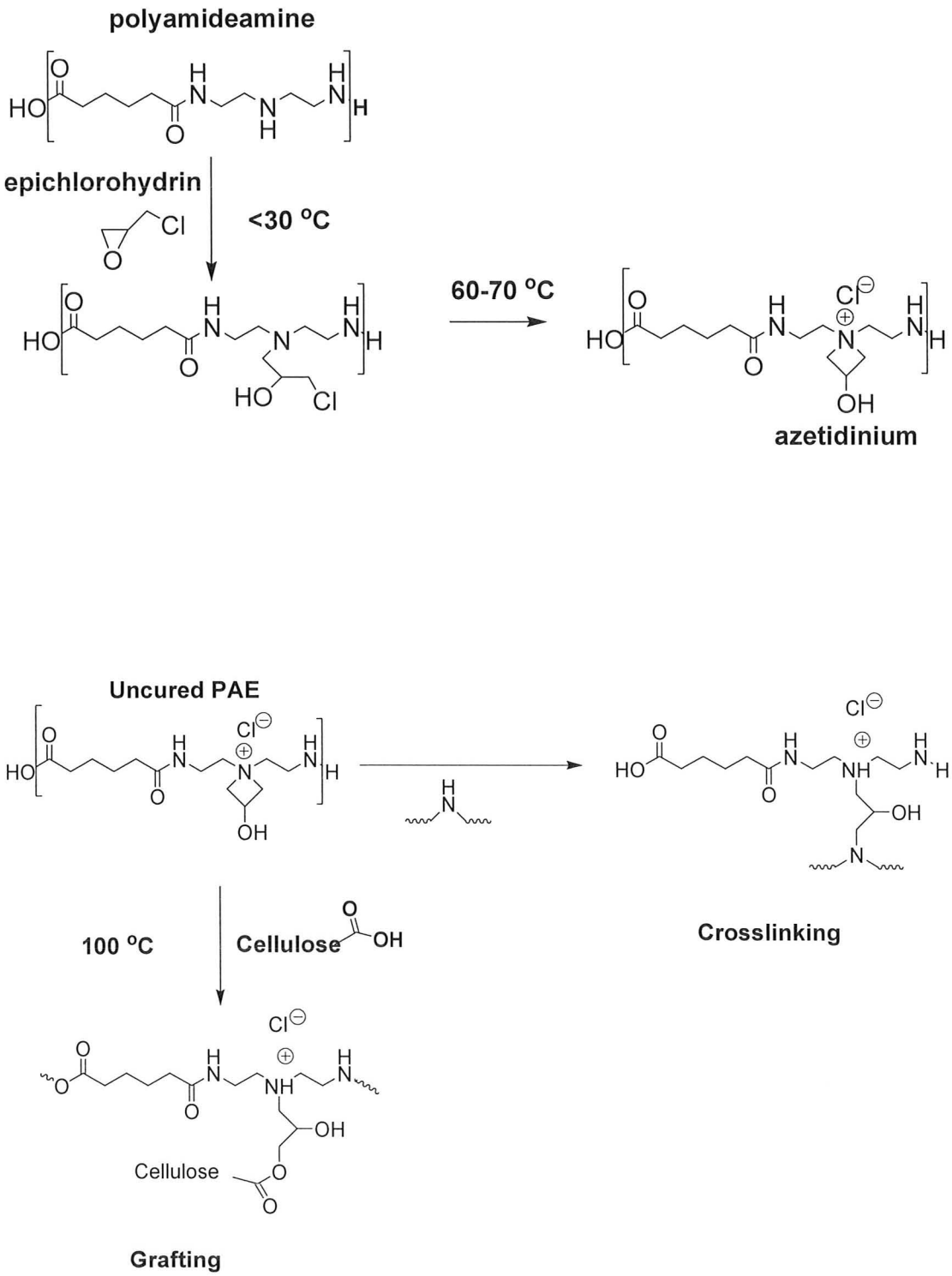


Figure 8 Preparation of PAE(top) and reaction between PAE and fibers(bottom)[41, 43, 45]

Polyvinlyamine (PVAm) resin is a relatively new papermaking chemical. It is a linear polymer with primary amines on every repeating unit, as illustrated in **Figure 9**. At neutral pH, approximately half of the amine groups in PVAm are protonated and yield a highly positively charged polymer.[46, 47] PVAm resin is the hydrolysis product of poly (N-vinylformamide) (PNVF), which is commercially available.[38] Wang et al.[48] reported that partially aminated PNVF can help increase both dry and wet strength of paper. Pelton and Hong[49] investigated the wet and dry strength properties of newsprint which was impregnated with PVAm. The studies concluded that the wet and the dry strength properties are significantly improved by PVAm and partially convert PNVF-PVAm copolymers. About half of the primary amine groups in PVAm can be protonated to ammonium groups by water, and adsorb on fibers through electrostatic attraction. The other primary amine groups would either react with aldehyde and ketone groups to form imines or carboxylate groups to form ammonium carboxylate, both of which are responsible for the improvement of paper wet-strength.[38, 49, 50]

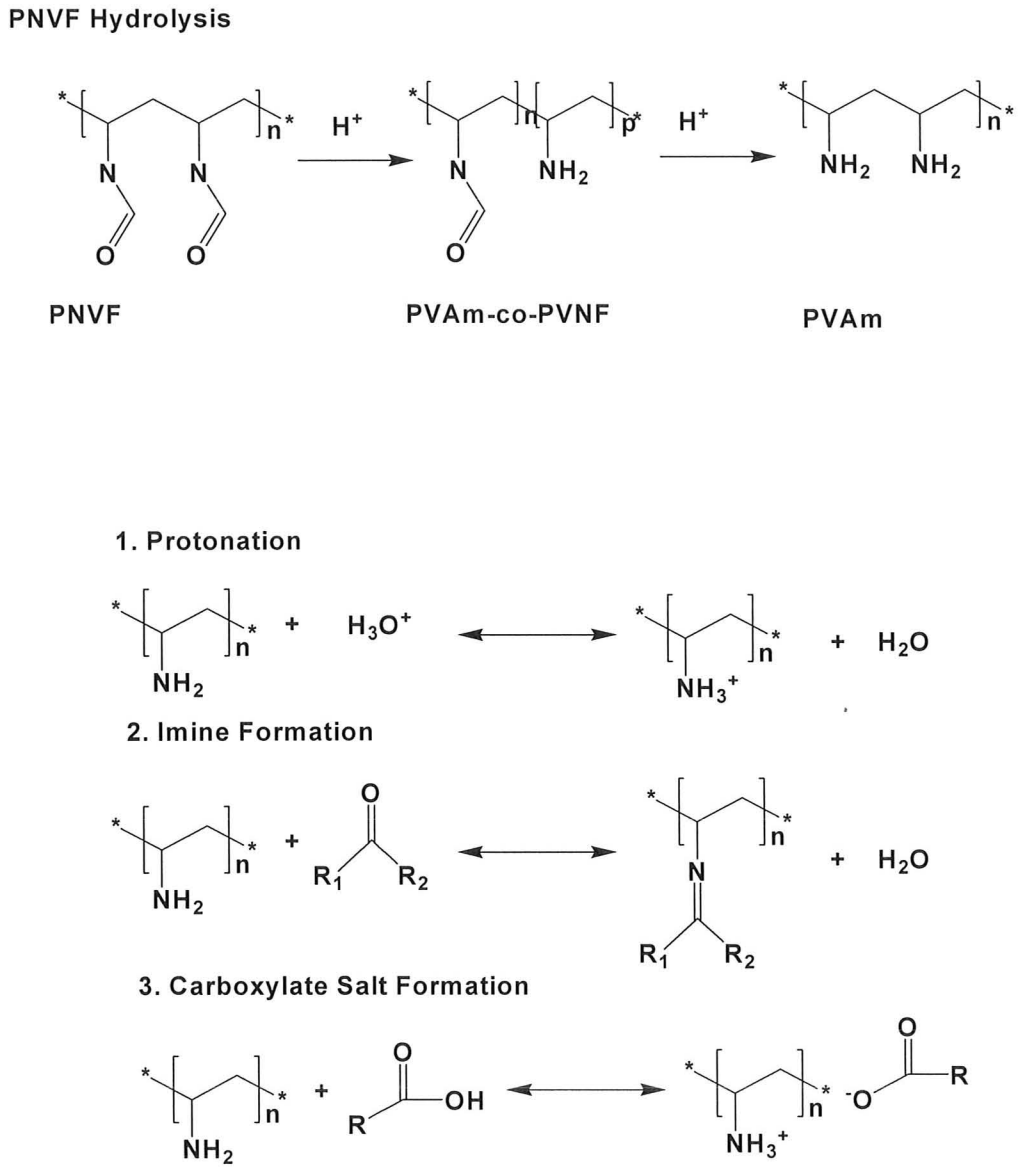


Figure 9 Preparation of PVAm(top) and reaction between PVAm and fibers(bottom)[49]

2.2.6 Sizing

Sizing is typically utilized in papermaking process to prevent the penetration of aqueous solutions, usually water, through capillaries. It can also improve the other properties of paper, such as porosity, smoothness, brightness, abrasiveness and surface strength. Sizing can either be applied at the wet-end during paper making (Internal Sizing) or coated to the paper surface (Surface Sizing). The basic idea of sizing is to create a hydrophobic, low-energy fiber surface which can impede the rate of liquid penetration during the drying stage of papermaking.[35, 51] Rosin-alum sizing was used in acidic condition with no covalent bonds formed between the sizing and the fibers. The new synthetic sizing agents, which can form covalent linkage with cellulose fibers and be applied in neutral or slightly alkaline pH, alum-free condition, were used in papermaking industry in the 1950's.[52] One of the most typical synthetic sizing agents is alkylketene dimer (AKD) which is usually prepared by long fatty acids and their acid chlorides. The lactone ring of AKD can react with water and cellulose to form the β -keto acid and β -keto ester, respectively. The β -keto acid can further decarboxylate to form ketone which is unable to react with cellulose. The formation and reaction of AKD are shown in **Figure 10**.[53]

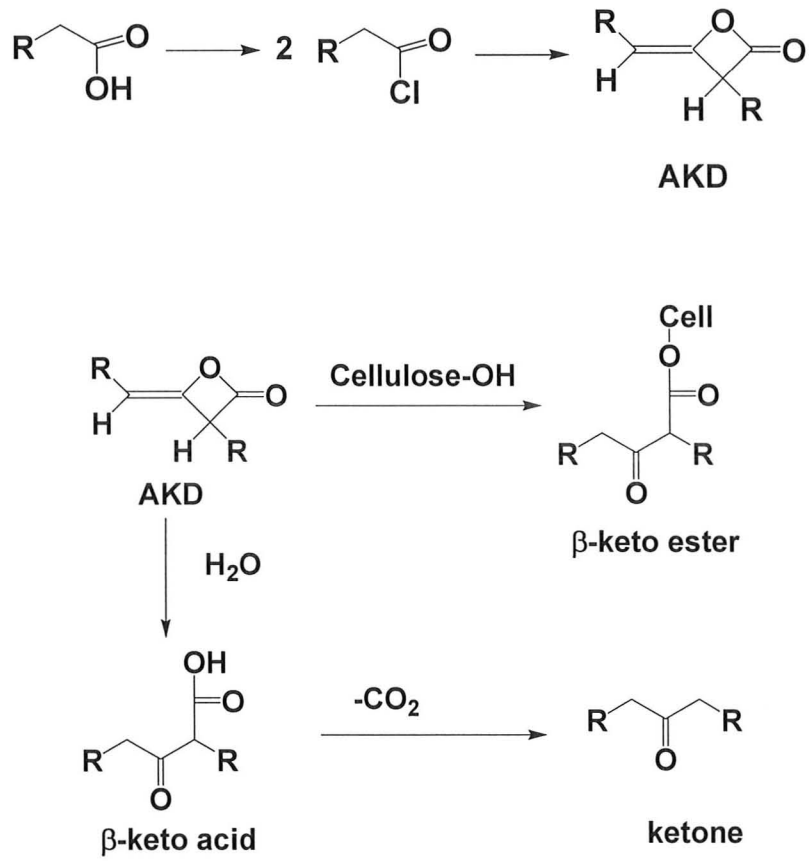


Figure 10 Preparation of AKD(top) and reaction between AKD and fibers(bottom)[53]

2.3 Paper-Based Biosensor Development

Recently, a pandemic of swine flu (H1N1) is spreading all over the world. More than 70 countries have reported cases of H1N1 infection with about 100,000 confirmed cases and 429 deaths.[54] Six years ago, the another outbreak called Severe Acute Respiratory Syndrome (SARS) also spreaded across 32 countries, which had resulted in nearly 800 deaths and more than 8000 infected cases.[55] Humans are constantly threatened

by health problems and people are vulnerable to new diseases. The world has become very concerned about public health and huge investments have been channelled to research and development field to develop technologies that can detect, capture and inactivate pathogens. Paper has widely been applied as filter media, barrier media and sterile packaging because it is low-cost, biodegradable, porous and bimolecular-friendly. Recently, a new "bioactive paper" concept has been developed which is a paper product that can give a direct indication to detect waterborne and airborne pathogens without any instrument.[56] Sentinel bioactive paper network led by Dr. Pelton in Canada and Whitesides's group in USA are the two major spearheads in bioactive-paper research.[39]

In order to develop "bioactive paper", the biorecognition agents (biosensors), such as enzymes, antibodies, bacteriophages and DNA aptamers must be immobilized on the paper first before reporting the information. The immobilization of biosensors can be broadly classified into four categories: physical immobilization, chemical immobilization, biochemical coupling and bioactive pigments.[39]

2.3.1 Biosensor Immobilization

Physical immobilization, also known as physical adsorption, is the easiest and the most direct way for biosensor binding. Such binding relies on van der Waals force and

electrostatic interaction between biosensor and adsorbent.[57] Karra-Châabouni et al.[58] have investigated the physical adsorption of Rhizopus oryzae lipase onto oxidized cellulose fibers and confirmed that the adsorption was mainly driven by electrostatic interaction between enzyme and cellulose substrate. However, the physical adsorption is not a sophisticated strategy for biosensor immobilization because this method is found to be not strong, robust and efficient. Brash et al.[59] have reported the poor adsorption of fibrinogen from plasma onto cellulose. Su et al.[60] have shown that the immobilization of ATP-binding DNA aptamer onto the cellulose through physical adsorption is rather weak and reversible.

Chemical immobilization, on the other hand, can provide a more efficient covalent coupling of biosensor to polymer substrate with little side effect. Modified cellulose substrates are used more often than the native substrate for chemical immobilization of biosensors because pure cellulose lacks the functional groups required for bioconjugation.[39] The common paper, which consists of cellulose, hemicellulose, lignin and other extractives, can offer even more reactive sites for bioconjugation. Kong et al.[61] have used epichlorohydrin activation as a method for coupling DNA to cellulose. Bora et al.[62] have developed a photo-reactive cellulose membrane for antibody coupling and successfully applied it in enzyme-linked immunosorbent assays (ELISA). The

hydroxyl group of the cellulose membrane was first activated with fluoro-group of 1-fluoro-2-nitro-4 azidobenzen (FNAB), after which azido-group was transformed into highly reactive nitrene, under UV light, to bind protein. After confirming the weak and reversible physical adsorption of DNA aptamers onto cellulose, Su et al. [60] have described a covalent coupling between DNA aptamers and oxidized cellulose through the aldehyde groups of oxidized cellulose and amine groups of DNA aptamers. In some cases, the modification via covalent binding may lead to decrease of biosensor activity and, possibly, introduce some toxic components.[63]

One example of **biochemistry coupling** for paper-based biosensor is using cellulose binding modules (CBMs), which were first found in Trichoderma reesei and cellulomonas fimi.[64, 65] CBMs can naturally bind to cellulose with exceptionally high affinity. Additionally, this binding has low desorption rate and irreversibility.[63] Shoseyov's group[66, 67] has developed a bio-conjugated method which can detect pathogenic microbes in food samples. The CBM-conjugated antibody was immobilized onto cellulose matrix, such as cotton gauze, and used to filter large volumes of liquid and capture the bacteria on the matrix. Cao et al.[68] have reported the immobilization of antibodies on magnetic cellulose microspheres via CBMs-Protein A linkage. Protein A is a cell-wall protein which can bind specifically onto the Fc region of IgG while CBMs

have high affinity with cellulose microspheres. Compared to chemical immobilization, this method gives much higher efficiency. Recently, Mackenzie's group[29] has engineered a bispecific pentermer sdAb which can bind on the cellulose substrate through CBMs and recognize human pathogen Staphylococcus aureus (S. aureus) through human sdAbs. CBM is fused to N-terminus of VTB and the sdAb is fused to the C-terminus of VTB. After expression of single polypeptide chains of sdAb-VTB-CBM in E. coli, VTB assists polypeptides in self-assembly and forms a bispecific pentamer.

Bioactive pigments refer to the covalent coupling of the biosensors to colloid particles. The carrier particles can be printed, coated or even added during the process of papermaking.[39] Caldwell's group[69, 70] has reported the synthesis of polystyrene latex coupled DNA using streptavidin and biotin as the linker between latex and DNA. Su et al.[71] have used poly (N-isopropylacrylamide) (PNIPAM) microgels, coupled with an antibody and a DNA aptamer to make biosensor paper. They found that the bio-conjugated microgels worked very well when printed on paper. Sol-gel also can be coupled with bio-molecules and applied for screen printing. Wang et al.[72] have developed sol-gel-derived enzyme-containing carbon inks for screen-printing. Recently, Brennan's group[73] has invented the sol-gel bioinks with the ability to print on paper and detect toxins. The paper was first coated with a PVAm layer and then printed with the

acetylcholinesterase (ACHE) within two layer of sol-gel. This sol-gel printed biosensor paper was successfully applied for quantitative assessment of toxin substance using color change as the indicator.

2.3.2 Biosensor Reporting

The other critical step after immobilization of biomolecules on the papers is reporting. There are three common ways for reporting: 1) color generation of enzyme; 2) fluorescence observation; and 3) colour change of gold nanoparticles.

The reaction between enzyme and corresponding substrate can result in color generation. Whitesides' group[74-76] has performed a series of experiments on development of paper-based microfluidic device for bioassays. They used hydrophobic polymers on the hydrophilic paper to create multiple channels which allows running multi-assay on one strip of paper to detect glucose and protein. The presence of glucose can be indicated by a color change from clear to brown because of the enzymatic oxidation of iodide to iodine while the presence of protein is observed by a color change from yellow to blue using tetrabromophenol blue (TBPB). The concentration of analytes can also be quantified by color intensity based on calibration curves. The most well-known system which uses color generation extensively is Enzyme-linked Immunosorbent Assay (ELISA). The basic steps to perform ELISA are as follows: 1) Immobilization of antibody onto the plastic

surface; 2) Capture of analyte from the test sample; 3) Addition of secondary antibody conjugated with enzyme for the specific binding of the analyte; 4) Color generation of enzyme after reacting with the substrate.[77]

The fluorescence method is similar to the color generation but without using substrate to generate color. It requires to the use of light source or even more expensive instrument, such as confocal microscopy, for detection. Su et al.[60, 71] have used a fluorescent conjugated aptamer to detect the ATP bound on the paper via DNA aptamer. This method was originally reported by Nutui and Li[78], which was based on the structural switch from DNA/DNA duplex to DNA/target complex. The fluorescence-labelled DNA (FDNA) was first paired with a quenching oligonucleotide (QDNA) to form a duplex without a fluorescence signal. When the target, which could be recognized by the FDNA, was introduced, the FNDA left the QDNA and bound to the target thereby inducing a fluorescence signal.

Gold nanoparticles (AuNP) can also be used as the reporters. The color of AuNP solution depends on the interparticle distance of AuNP particles because of its surface plasmon resonance (SPR). A well dispersed AuNP solution shows red color while an aggregated AuNP solution exhibits pink or blue colour. Elghanian et al.[79] first reported a

highly sensitive, selective and colorimetric polynucleotide detection method by gold nanoparticle (AuNP) probes. The AuNPs were loaded with two different DNA strands as the probes which formed dispersed solution and showed red color. After adding the targets which were complementary DNA strands for both probes, the AuNPs aggregated and the color of the solution changed to blue. Zhao et al.[3] have developed a paper-based biosensor using the method discussed above which is convenient, rapid and inexpensive. The aggregated AuNP solution, cross-linked by DNA, was spotted as a blue-dot on the paper. After the target solution was added to cleave the DNA strands, the AuNPs became dispersed and displayed a red-dot on paper. Zhao et al.[80] also have developed a biosensor based on DNA and AuNPs by non-crosslinking mechanism. During the biosensor synthesis, AuPNs were first modified with an oligonucleotide, following by the hybridization of complementary DNA aptamer to oligonucleotide. The APT-OD-AuNPs were quite stable and well-dispersed at certain concentration. Addition of target induced the binding between target and DNA aptamer, and the aggregation of unhydridized AuPNs, which initiated a colour change.

Based on the significant progress achieved in bioactive paper development, the objective of this work is to study the versatility of biosensors on different paper surfaces. If biosensors could work on a wide variety of paper surfaces, it would become more

practical to be implemented. Since the biosensors are mostly liquid-based, the bioactive paper must have sufficient wet strength to maintain its integrity. Also, some ink-jet printing papers which contain alkaline sizing agents can be useful substrates for biosensor immobilization. Therefore, the versatility of biosensors is examined by studying the activity of different antibodies immobilized on wet-strength and hydrophobic papers, respectively.

Chapter 3 Experimental Methods

3.1 Introduction

This chapter first described the preparation and the characterization of wet-strength papers. Then, the influence of wet-strength resins on biological activity of two different types of model antibodies was examined. In the end of the chapter, a preliminary study on antibody activity under the influence of sizing agent was presented.

3.2 Materials

All papers were provided by Ahlstrom Corporation and used as support matrices on which polymers or proteins were grafted. All chemicals were used as received unless otherwise specified. Polyamide-epichlorohydrin (PAE) resin was generously provided by Hercules Inc. (Kymene 624). Polyvinylamine (PVAm) resin was supplied by BASF (ZD1989/106). Skim milk powder was purchased from BD (Becton, Dickinson and Company). Alkylketene Dimer (AKD) was provided by Kemira Chemicals Canada Inc. (Hydrores 266MB). Tween 20 was purchased from Fisher Scientific Inc. TMB, (3,3',5,5'-tetramethylbenzidine), Microwell Peroxidase Substrate System was purchased from KPL (Kirkegaard & Perry Laboratories, Inc.). Anti-Rabbit IgG (AR-Ab) produced in goat and Anti-Mouse IgG (produced in rabbit)-peroxidase antibody (AM-Ab-HRP) were purchased from Sigma-Aldrich. S. aureus Protein A conjugated with horseradish

peroxidase (Protein A-HRP) was purchased from Invitrogen Corporation. Cellulose-binding module single domain anti-S. aureus pentavalent antibody (CBM-sdAb) was engineered by Dr. Mackenzie from Institute for Biology Sciences of National Research Council of Canada, and then expressed and grown in E. coli in Dr. Hall's lab at University of Guelph. Texas Red-X dye labeled Protein A was prepared using the labeling kit supplied by Invitrogen Canada Inc. Anionic fluorescently labeled polymethacrylic acid was kindly provided by Dr. Stöver's group from McMaster University. The salts required to prepare phosphate buffered saline buffer, such as sodium chloride, potassium chloride, sodium phosphate dibasic and potassium phosphate monobasic, were purchased from Sigma-Aldrich. All buffers and protein solutions were prepared using Millipore-Q grade water and micro-filtered using a membrane filtration unit prior to use.

3.3 Paper Treatment with Wet-strength Resins

PAE resin: Ahlstrom A-55 filter papers were cut into appropriate sizes and conditioned at 23 ± 0.5 °C and 50 ± 1.0 % RH in a constant temperature and humidity (CTH) room to obtain the original weight of papers. Different concentrations of PAE (1 wt%, 10 wt%, 20wt%) solutions, adjusted to pH 7.5, were prepared and poured onto the paper samples to allow soaking to take place at room temperature for 30 min. Following liquid adsorption, the excess polymer solutions were discarded and the wet paper samples were weighed. The weighed paper samples were then subjected to further heating on a

speed dryer (SD36D, Labtech Instruments Inc.) at 120 °C for 10 min to initiate a chemical reaction between PAE and paper fibers. When the polymerization was completed, the treated papers were then conditioned at the same temperature and relative humidity as described earlier to determine the mass increase of the treated papers.

PVAm resin: PVAm solution (Degree of Hydrolysis: 89.5%; Molecular Weight: 950 KDa), received from the supplier, was freeze-dried and then dissolved in Millipore-Q water to prepare PVAm solutions at different concentrations (1 wt%, 2.5 wt%, 5 wt%). The PVAm treated papers were prepared using the same method as that of PAE treated paper.

3.4 Electrophoretic Mobility of Paper Samples

Electrophoretic mobility was used to validate the surface treatment of filter papers by measuring the electrostatic charge of treated papers. The mobility of original paper was also measured for comparative purpose. Paper samples were cut into small pieces and stirred using a magnetic stirrer in 10^{-3} M NaCl solution at pH 7.5 for 6 hr. The samples were then left to sediment before measuring the electrophoretic mobility of the supernatant using a Zeta Plus Analyzer (Brookhaven Instruments Corp.) operated in phase analysis light scattering mode (PLAS). Each sample was subjected to 10 runs with 15 cycles in each run.

3.5 Anionic Fluorescence Experiment

The surface charge of original and treated papers was also qualitatively determined by using anionic fluorescently labeled polymethacrylic acid. Untreated paper samples and PAE-treated papers were cut into discs and immersed in solutions containing 5 mg/ml of anionic fluorescent at room temperature for 1 hr to allow polymer adsorption to take place. The paper samples were then washed with Millipore-Q water for 5 times until the water was clear. After washing, the papers were evaluated for the presence of fluorescent on the papers.

3.6 SEM Characterizations

Original and treated papers were tested using **Scanning Electron Microscope (SEM，Philips 515)** to examine the morphological difference between the papers. Paper samples were cut into small pieces (2 x 2 mm) and splutter coated with a layer of gold before imaging.

3.7 Electrophoretic Mobility of Antibody

The electrophoretic mobility of the studied antibodies was examined by electrophoresis. The antibodies were diluted in phosphate buffered saline buffer (PBS, 10mM, pH 7.5) and subjected to mobility measurement by using Zeta Plus Analyzer

(Brookhaven Instruments Corp.) operated in phase analysis light scattering mode (PLAS). Each sample was subjected to 10 runs with 15 cycles in each run.

3.8 Antibody Activity Measurement

Case A: CBM-Ab

A schematic diagram of the enzyme-linked immunosorbent assay (ELISA) protocol used in the experiments is shown in **Figure 11** and is briefly described below. CBM-Ab was used as the model antibody to investigate the effect of wet strength resins on binding and activity of antibody. Paper samples were punched into small discs (6 mm diameter), soaked in water and placed onto individual wells of a 96-well Bio-Dot microfiltration apparatus (Bio-Rad Laboratories Canada Ltd.). The paper samples were used as adsorptive media for antibody binding. 10 μl of CBM-Ab (~0.212 mg/mL) was applied to paper in each well and incubated for 30 min. The remaining unoccupied wells were covered by Parafilm to facilitate efficient vacuum pumping. A vacuum was applied to filtrate the excess solution, followed by washing the paper filters with 500 μl of PBST (10 mM phosphate buffered saline containing 0.5 vol% Tween 20, pH 7.5) for ten times, with vacuum applied in between each wash. Then, 300 μl of 3 wt% skim milk solution was added to each well to block the rest of binding sites on paper and prevent non-specific binding. After 30 min of milk blocking, a vacuum was applied to remove the excess milk, followed by ten washes of filter papers with PBST. 10 μl of Protein A-HRP

conjugate (1/3000 dilution) was added, as antigen, to each well and incubated for 30 min to allow protein A-HRP to bind to active antibody on filter paper. The same washing step was carried out after the Protein A-HRP binding, followed by an additional 500 μl of PBS washing for three times.

After the antibody-antigen reaction was complete, the paper discs were removed from the Bio-Dot apparatus and placed a 96-well microtitre plate. To each well, 150 μl of TMB substrate was added to react with the bound Protein A-HRP and develop a color change. The samples were incubated in the dark for 20 min and the solution in each well was mixed gently by pipetting every 5 min. The solutions were then transferred into a second microtitre plate and the reaction between HRP and TMB substrate was terminated by adding 150 μl of 10% H_2SO_4 to each sample. Finally, the plate was placed in a microplate reader (Bio-Rad Laboratories Canada Ltd., Model 680 XR Microplate Reader) for absorbance measurement at 450 nm wavelength. Control experiments, in which no antibody was applied, were also conducted in a similar fashion to investigate if the passive binding of Protein A-HRP on blocked paper would give false signal.

Case B: AR-Ab

A procedure similar to the one described previously was also carried out to

investigate the effect of wet-strength resins on binding and activity of AR-Ab. In this study, 0.12 mg/ml of AR-Ab and 1:10,000 dilution of AM-Ab-HRP were used. AM-Ab-HRP was selected as the antigen to provide a direct detection of the activity of AR-Ab. Compared to CBM-Ab experiments, the blocking of binding sites in AR-Ab experiments was extended to 3 hr. The extension of milk blocking was necessary to minimize the non-specific binding of antigen (AM-Ab-HRP) on paper samples. Similarly, the control experiments were also performed to check for any passive binding of AM-Ab-HRP on blocked paper.

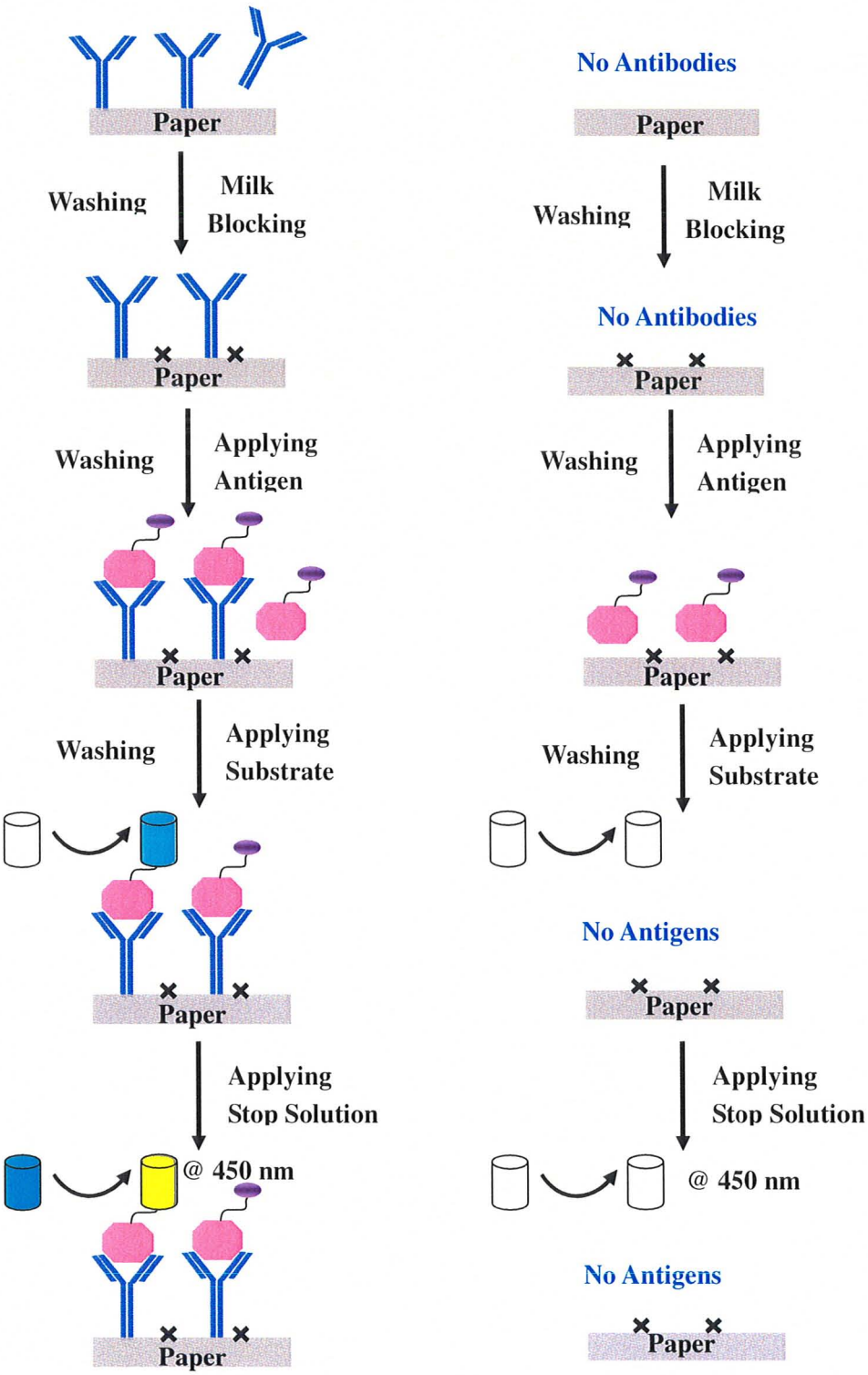


Figure 11 A schematic diagram of ELISA protocol to measure antibody activity

3.9 Confocal Laser Scanning Microscopy

In addition to ELISA technique, the qualitative analysis of antibody activities on untreated and PAE treated paper was also performed using confocal laser scanning microscopy. The method for preparing paper samples for confocal laser scanning microscopy was similar to that of ELISA experiments described previously. However, after milk blocking and washing steps, Texas Red-X dye labeled Protein A (2.5mg/ml, 1:400 dilution) was used in lieu of Protein A-HRP and incubated for 30 mins. The paper discs were then washed 10 times with 500 μl of PBST and 3 times with 500 μl of PBS, after which the paper discs were dried by vacuum before conducting the confocal microscopy. Zeiss LSM 510 laser scanning confocal microscope was used to view the bounded dye labelled protein A on paper samples at 543nm wavelength. Brightness (gain) and contrast settings were maintained in all image captures.

3.10 Antibody Binding Experiment

The binding of AR-Ab to paper samples was quantified using a membrane chromatography system, as shown in **Figure 12**.

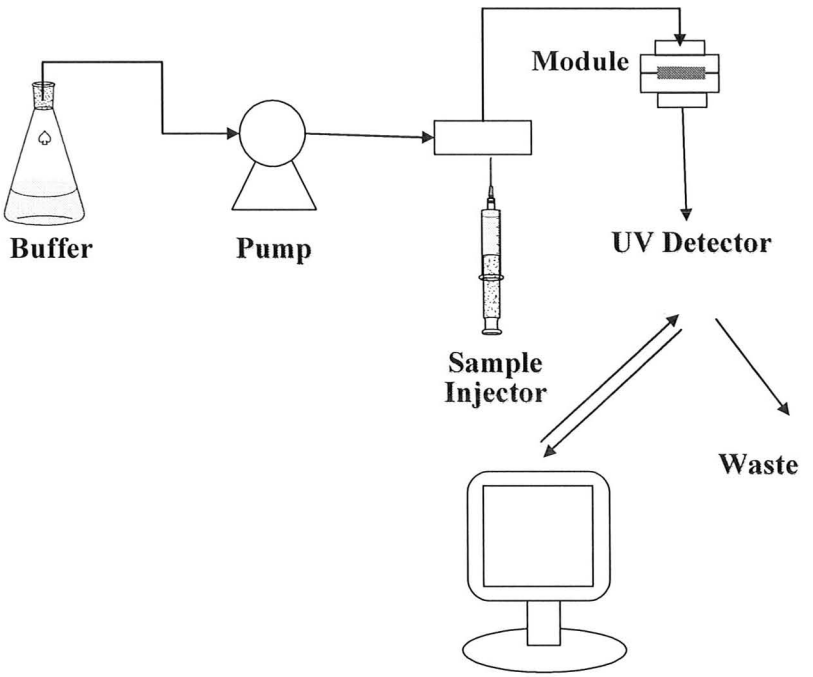


Figure 12 The schematic diagram of AKTA system

Paper samples were cut into three 8-mm diameter discs and housed within a custom-designed membrane module.[81] The module was connected to the AKTA Prime Liquid Chromatography System (GE Healthcare Biosciences), in between the pump and the UV detector. A buffer reservoir was supplied to the integrated system through a peristaltic pump. The UV absorbance (280 nm), pH, and conductivity of the effluent stream leaving the membrane module were continuously measured and logged into a computer using the Prime View software (GE Healthcare Biosciences). The binding buffer used for the experiments was phosphate buffered saline buffer (PBS, 10mM, pH 7.5). All binding experiments were carried out at ambient temperature of the laboratory

(i.e. 24 ^{o}C) and 1 ml/min flow rate. The feed samples were prepared by diluting the original antibody stock with binding buffer to 1:4 v/v.

The binding experiments were started by equilibrating the chromatography system with binding buffer, until UV absorbance at 280 nm and pressure in the system were constant. When the system became stable, the antibody was injected into the membrane module using a 100 μl sample loop, after which the binding buffer was continuously fed into the module to wash away any unbound antibody. The experiment was complete when the UV absorbance returned to the baseline. Control experiments, without paper loading in membrane module, were also conducted for comparative study.

Numerical integration of area under the curve of UV intensity (280 nm) against effluent volume was determined using Prime View. The percentage of CBM-Ab or AR-Ab bound to the paper disks was determined by calculating the difference in the area under the UV-volume curves for both control and binding experiments.

3.11 Preliminary Study: Contact Angle Measurement and Antibody Adsorption on AKD-treated Paper

Ahlstrom A-55 filter papers were treated with different concentrations of AKD solution (0.5 wt%, 1.5 wt%, 5 wt%, 12.5 wt%) using the same method as described

previously for wet-strength resins.

The contact angle of Millipore-Q grade water on untreated and AKD-treated paper was measured by sessile drop method using Krüss Drop Shape Analysis System (DSA100). Paper samples were cut to 50×50 mm and placed on the sample stage. Pictures showing the water drops on paper samples were captured by high resolution camera and the contact angles on both sides of the drop were analyzed by software. The recorded values were obtained from the average of 10 measurements.

The influence of hydrophobic sizing agent on biological activity of antibody (AR-Ab) immobilized on untreated and AKD-treated paper surface was examined. The experimental procedures used for preparing antibody on paper and determining its activity was similar to that of wet-strength resins.

Chapter 4 Results and Discussion

4.1 Treatment of paper surfaces with wet-strength resins

4.1.1 Characterization of polymer content in paper samples

The Ahlstrom A-55 filter papers were impregnated with different concentrations of PAE or PVAm solutions prior to curing at 120 °C. Both PAE and PVAm solutions were adjusted independently to pH 7.5 in order to facilitate an effective cross-linking reaction between polymers and paper fibres.[44] While preparing wet-strength papers, the paper samples were soaked in polymer solutions for exactly 30 min, as suggested in the literature, to maximize the polymer adsorption without compromising the wet tensile strength of filter paper. [38]

In this study, the treated paper samples were first characterized by their polymer content in filter papers. Two approaches were utilized to determine the amount of polymer loaded on filter paper. In the first approach, a dry weight basis was used to determine the polymer content. The percentage of polymer content in filter paper was calculated using **Equation 1**.

$$\text{PAE content } \% = \frac{\text{Treated paper dry weight - Untreated paper dry weight}}{\text{Treated paper dry weight}} \times 100\%$$

Equation 1

In the second approach, the weight of wet paper after immersing in polymer solution was measured instead. To determine the mass of polymer adsorbed on the paper, the weight difference between dry and soaked paper was multiplied by the polymer concentration in solution, as indicated by the numerator term in **Equation 2**.

$$\text{PAE content } \% = \frac{(\text{Treated paper wet weight - Untreated paper dry weight}) \times \text{PAE concentration}}{\text{Treated paper dry weight}} \times 100\%$$

Equation 2

Table 1 and **Table 2** report two different methods used in calculating the percentage of polymer content in filter papers which were prepared in solutions containing different polymer concentrations.

Table 1 PAE content on filter paper (According to Equation 1)

PAE Concentration	Untreated Paper Dry Weight (mg)	Treated Paper Dry Weight (mg)	Mass of PAE on Paper (mg)	% PAE Content
1%	290.1	293.6	3.5	1.2
10%	287.2	355.8	68.6	19.3
20%	291.6	506.3	214.7	42.4

Table 2 PAE content on filter paper (According to Equation 2)

PAE Concentration	Untreated Paper Dry Weight (mg)	Treated Paper Wet Weight (mg)	Mass of PAE on Paper (mg)	% PAE Content
1%	298.0	775.6	4.8	1.6
10%	296.5	986.8	69.0	18.9
20%	295.2	1268.6	194.7	39.7

As shown in the tables, the results of both calculated methods were found to be in good agreement with one another. The percentage of PAE content in filter papers was strongly dependent on the PAE concentration in polymer solution. The relationship between percentage of PAE content and its concentration in polymer solution appeared to be linear, such that the polymer content on filter paper increased with the concentration of PAE in polymer solution.

Similarly, the percentage of PVAm content in filter paper was found to increase linearly with PVAm concentration in polymer solution. Percentages of PVAm contents of 2.3%, 4.2% and 10.9% in treated papers were obtained when untreated filter papers were modified with 1%, 2.5% and 5% PVAm solution, respectively.

4.1.2 Paper characterization

Charge Characterization

Electrophoresis is a longstanding analytical tool that measures the electrophoretic mobility of charged colloidal particles. This technique relies on the migration of charged colloidal particles or molecules under the influence of an electric field to determine the mobility of colloidal particles. This method has been widely applied in electrokinetic study of cellulosic materials. Therefore, it is useful in characterizing the surface charge of treated and untreated filter paper.[82-84] The electrophoretic mobility measurements of both

untreated and PAE-treated papers were conducted in 1mM NaCl solution (pH 7.5) and the results of the experiments are summarized in **Table 3**.

Table 3 Electrophoretic mobility of PAE-treated Papers

Paper Type	Mobility ($10^{-8}m^2/Vs$)	Std. Err. ($10^{-8}m^2/Vs$)
Untreated Paper	-1.65	0.01
1.2% PAE Paper	0.79	0.03
19% PAE Paper	1.49	0.06
42% PAE Paper	1.81	0.05

As expected, the original filter paper was found to be negatively charged while the PAE-treated papers were found to be carrying excess positive charges. Since PAE is a cationic polymer, the mobility of the PAE treated papers was expected to increase with the percentage of PAE content in treated paper. Similarly, PVAm-treated papers were also found to be positively charged and the mobility of the PVAm treated paper increased the PVAm content in filter paper. These results were consistent with those reported by Liu[82] and Sandberg et al.[85].

Figure 13 shows the results of anionic fluorescent adsorption on untreated and different PAE-treated paper samples. This figure clearly shows that PAE-treated papers are, in fact, positively charged. Such positively charged treated papers could adsorb anionic fluorescent through electrostatic interaction and illuminate green color while

original filter paper did not produce any color. Also, the colour intensity was found to increase with the PAE content in on filter paper. This observation suggested that filter paper became more positively charged when more PAE was loaded on filter paper. Based on the anionic fluorescent adsorption experiments, the result was consistent with that of electrophoretic mobility study.

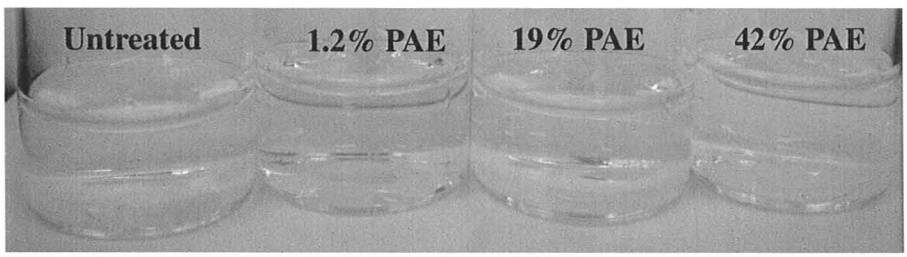


Figure 13 Anionic fluorescent adsorption on untreated and different PAE-treated paper samples

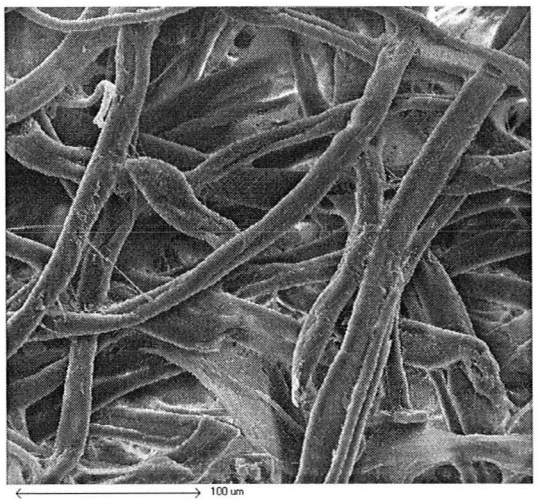
Porosity Characterization

Paper porosity, which can also be defined as the total pore volume of paper, is a critical parameter in paper treatment. It can be easily determined using **Equation 3** when the weight of dry and wet papers, and the density of cellulose and water are known. [39] The total pore volume of filter paper used in this study was found to be approximately 70% when the density of solid component of wood fibre of 1540 kg/m^3 was used. [39]

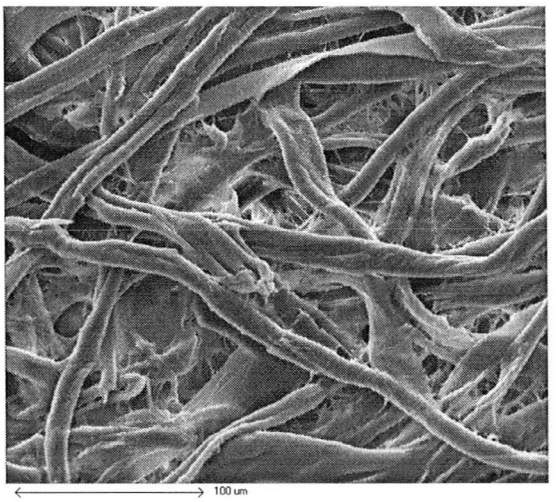
$$\text{Total Pore Volume} = \frac{(\text{Wet weight} - \text{Dry Weight})/\text{Water denstiy}}{\text{Dry weight}/\text{Wood fiber dentsity} + (\text{Wet weight} - \text{Dry Weight})/\text{Water denstiy}}$$

Equation 3

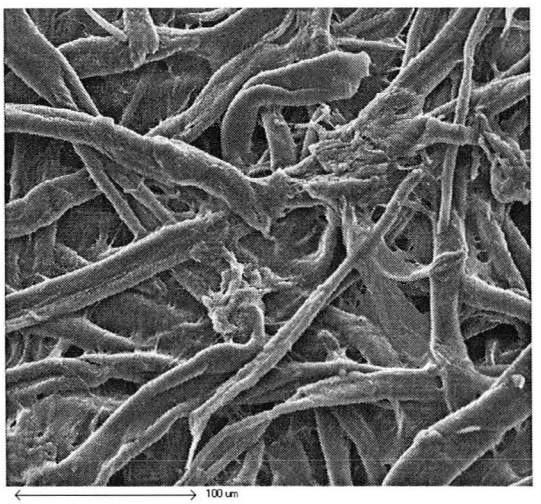
When wet-strength polymers were adsorbed on filter paper, they occupied the pore space and changed the porosity of paper. In particular, since the adsorbed polymers could not diffuse into cellulose fibres, they could only reside on the pore surface after the paper had been dried. Hence, it is reasonable to assume that, from the way the filter paper is treated, the total pore volume of paper is decreased when the polymer are loaded on paper.[86, 87] For example, when the highest concentration of PAE solution (20 wt%) was used to soak filter paper, it is believed that roughly 20% of the pore volume would be occupied by the polymers.



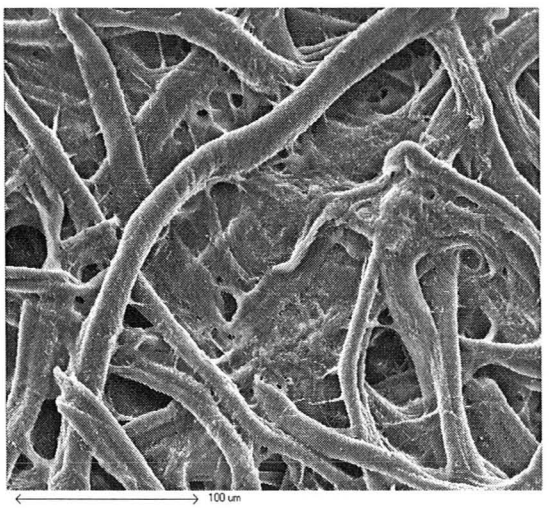
Untreated Paper



1.2 % PAE-treated Paper



19 % PAE-treated Paper



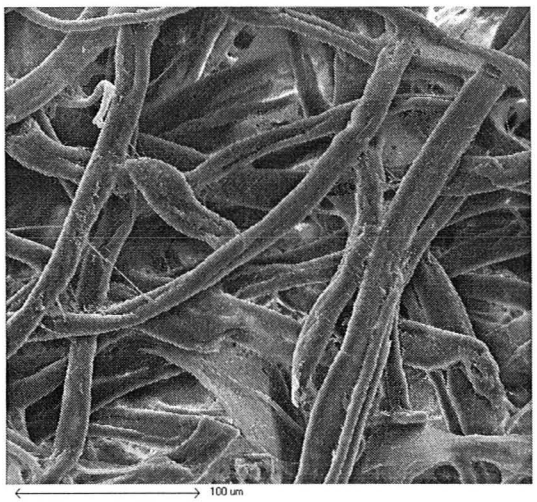
42 % PAE-treated Paper

Figure 14 SEM images between untreated paper and PAE-treated papers

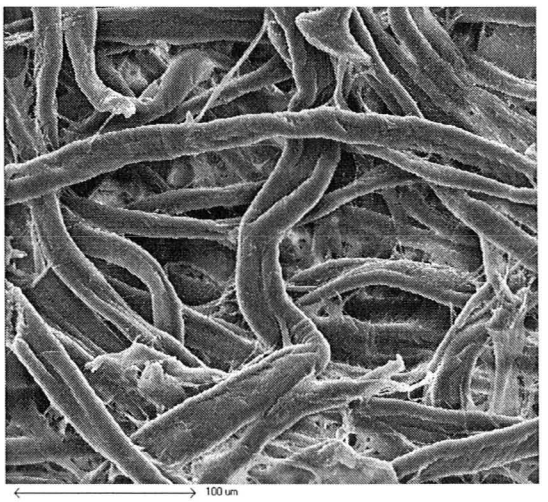
Figure 14 presents the comparison of the morphology differences between untreated and PAE-treated papers at different coating levels. Based on the morphological images, the surface structures of untreated and 1.2% PAE-treated paper were not so different. However, the morphological difference became evident when 42% PAE-treated

paper was compared against nascent filter paper. The untreated paper had clear and big pores with smooth surface on individual fibres. When PAE was added onto the filter paper, the surface of fibres was partially covered by a layer of polymer. Such effects were manifested in high PAE loading paper.

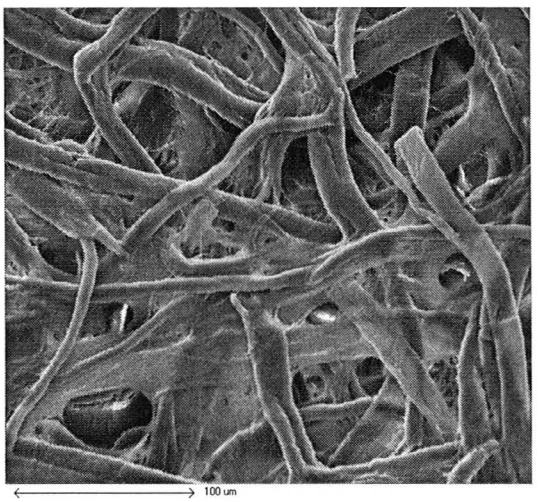
Due to these effects, the pore size of filter paper was reduced and some pores were occluded by the polymers as they occupied the pore space in filter paper. As such, the observations in the morphology of PAE-treated paper were consistent with the reducing porosity assumption discussed in previous paragraph. The morphological displays of PAE-treated paper clearly demonstrate the function of wet-strength resins in improving paper wet strength as the fibres were seemed to hold together by the polymer, as a result of the polymer cross-linking.



Untreated Paper



2.3 % PVAm-treated Paper



4.2 % PVAm-treated Paper

10.9 % PVAm-treated Paper

Figure 15 SEM images between untreated paper and PVAm-treated papers

Figure 15 shows the SEM images of untreated and PVAm-treated paper with different polymer content. The results were similar to those obtained in PAE-treated paper. The adsorption of PVAm resins on paper fibres was observed and the polymer network was formed as the content of PVAm in filter paper increased. Again, the images showed

that the fibres were held together by the polymer, thereby reinforcing the mechanical strength of filter paper.

4.2 Antibody Mobility Measurements

In the present work, two different sets of model antibodies were studied. The first set was composed of a classic anti-rabbit IgG (AR-Ab), as antibody, and anti-mouse IgG (produced in rabbit)-peroxidase IgG (AM-Ab-HRP), as antigen. The other pair involved a complex CBM-Ab, which consisted of five identical single-domain anti-S. aureus antibodies, and protein A–peroxidase produced from S. aureus as antigen. Five cellulose binding modules were coupled to the anti-S. aureus antibodies to facilitate immobilization of the antibodies on filter paper through verotoxin B.

Table 4 lists the electrostatic charge of different model antibodies in PBS buffer solution (10 mM, pH 7.5). The electrophoretic mobility of antibodies was examined to determine the electrostatic charge of the antibodies. This was important to investigate the effect of wet strength resins on biological activity of antibodies. From **Table 4**, the mobilities of both antibodies were reported in negative values which indicated that both antibodies were negatively charged. Since the isoelectric points of VTB-anti-protein A and CBM are 6.65 and 4.84, respectively, CBM-Ab carried a slightly negative charge under the experimental condition (pH 7.5). Similarly, AR-Ab also exhibited slightly

negative value because its isoelectric point was below pH 7.5 as indicated by the negative sign of the mobility measurement. These results could infer that the negatively charged antibodies might interact with the positively charged wet strength resins on filter paper. As a result, it was possible that the activity of the two antibodies could be interrupted and, consequently, deactivated by the wet-strength resins via electrostatic interaction.

Table 4 Electrophoretic mobility of antibodies

	Mobility ($10^{-8}m^2/Vs$)	Std. Err. ($10^{-8}m^2/Vs$)
CBM-Ab	-0.370	0.110
AR-Ab	-0.217	0.069

4.3 Antibody Activity Measurements

Since the goal of bio-active paper study was to investigate the effect of wet-strength resins present in filter paper on antibody activity, ELISA technique was used to measure the activity of antibodies immobilized on filter papers treated with various wet-strength resins. In this technique, filter paper was used, in place of micro-well plate, as the working substrate for antibody binding. However, there were several potential problems associated with the use of paper as substrate in the modified ELISA technique.

First, the bound antibodies might fail to capture the antigens and produce an active signal, if the bound antibodies were denatured or poorly oriented. Secondly, the

antigens could bind directly on the paper surface via non-specific interaction which might lead to false positive result. Hence, to eliminate the non-specific binding of antigen on filter paper, the bare surface of filter paper must be blocked using large and neutral particles, such as bovine serum albumin (BSA) and skimmed milk protein, before applying the antigen on the antibody-immobilized papers.[88] In this work, skimmed milk protein was used to block the remaining binding sites, which were unoccupied by the antibodies, on paper surface. Also, sufficient washing steps were applied to ensure loosely bound antibodies or antigens were completely washed out.

In general, the modified ELISA method was consisted of four major steps. In the first step, antibodies were immobilized on filter paper, via cellulose binding domain (CBM-Ab) or non-specific adsorption (AR-Ab). Milk blocking was applied in the subsequent step to block the remaining binding sites after which the antigens, conjugated with HRP enzymes, were added to filter paper for antigen-antibody recognition. Finally, the activity of bound antibodies was detected by reacting HRP enzymes with TMB substrates, which converted to a colored product.

Figure 16 shows the colour formation of different paper samples, both untreated and PAE-treated ones, treated with TMB substrate solution. The colour development was

attributed to the enzymatic reaction between TMB substrate and HRP. The paper samples, which had already been treated with antibody and antigen loading, were placed in the multiplates containing substrate solutions. Each vertical group was consisted of four replicated samples and the PAE content of paper was indicated at the top of each column. The blue color designated the presence of active paper-immobilized antibodies which were recognized by the enzyme-labelled antigens. As can be seen from the figure, all wells exhibit blue color with the highest color intensity found in untreated and 1.2% PAE-treated paper. Beyond 1.2% PAE content, the colour intensity decreased as the PAE content increased.

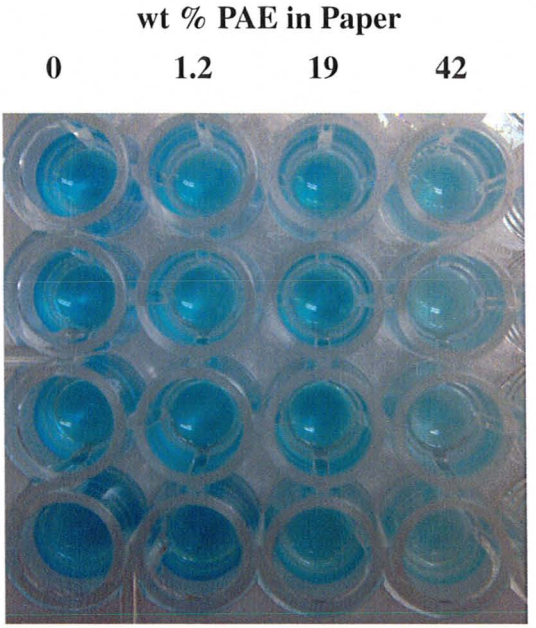


Figure 16 Immunoassay results of Anti-Rabbit IgG (AR-Ab) immobilized on untreated and PAE-treated filter papers. AM-Ab-HRP, added in a second step, binds to the AR-Ab and catalyzes color formation when the substrate (TMB) to HRP is added.

Figure 17 shows the control experiment, in which no AR-Ab was added on the paper samples. The absence of blue color confirmed that there was no non-specific adsorption of secondary antibody, AM-Ab-HRP, on papers which had been previously treated with milk. **Figure 16** and **17** clearly demonstrate high signal to noise ratio, indicating that milk blocking technique was effective in eliminating the non-specific binding of antigen on filter paper.

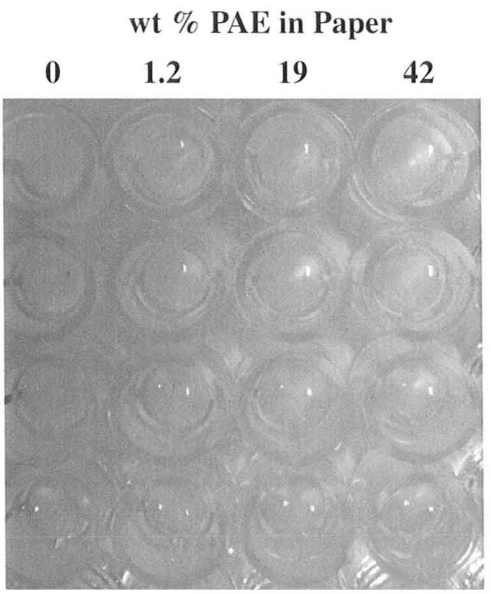


Figure 17 Control experiments of AM-Ab-HRP added to untreated and PAE-treated filter papers without immobilized AR-Ab

The color intensity generated by the paper-supported antibody assay was accurately quantified by UV-Visible spectrophotometer and the values corresponding to the samples in **Figure 16** and **Figure 17** are summarized in **Figure 18**. When compared against the untreated paper, 1.2% PAE-treated paper was found to improve the assay.

Above 1.2% PAE content, the assay sensitivity decreased when the PAE content increased. The corresponding results for the CBM-Ab pentamer are also shown in **Figure 18**. The concentration of active CBM-Abs bound on paper was determined using protein-A horseradish peroxidase conjugates (Protein A-HRP) which specifically recognizes CBM-Ab. The activity of CBM-Abs was also found to exhibit similar profile as that of AR-Ab when the CBM-Abs were immobilized on PAE treated paper.

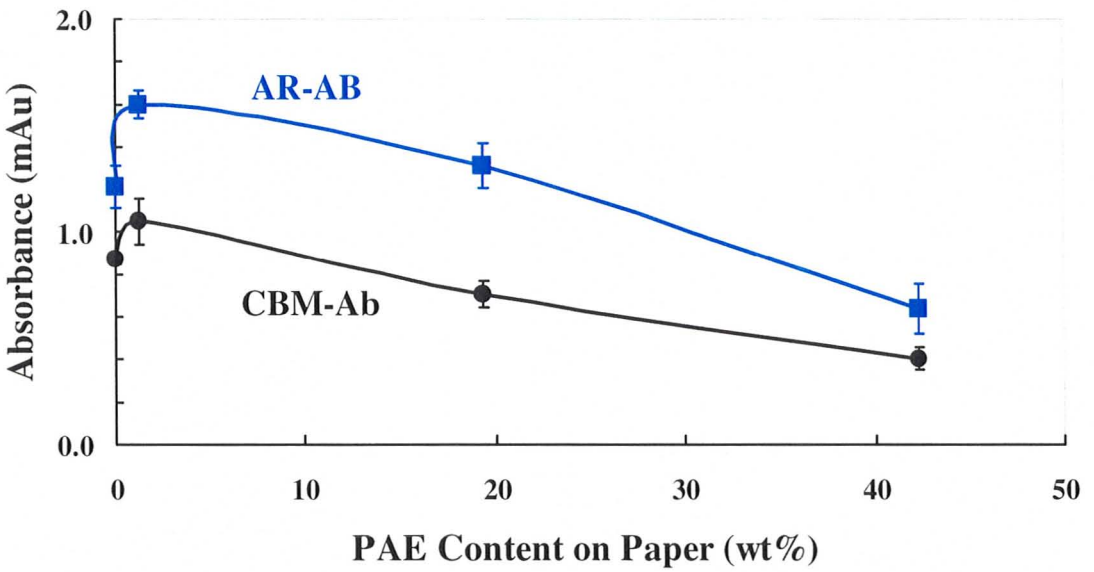


Figure 18 The influence of PAE on color generation by paper on which AR-Ab or CBM-Ab was immobilized

Alternatively, the activity of bound CBM-Abs could also be detected from confocal laser scanning microscopy technique by using protein A conjugated with a dye, as antigen. **Figure 19** shows the confocal images of different paper samples treated with CBM-Ab and Texas Red-labelled protein A, along with the corresponding control

experiments. As expected, the confocal images give clear evidence that CBD-Ab retained its functionality on both original and PAE treated papers. The red color indicated the presence of dye labelled protein As which were captured by CBD-Abs on paper samples. The control papers showed no red spots, confirming that no non-specific binding of antigen was observed after treated with milk. Hence, the red colors displayed on the paper samples treated with CBM-Ab were due to the affinity interaction of antigen and antibody. Similar to the results obtained in UV-Visible spectrophotometer studies, the highest intensity of red colour could be observed in original paper and 1.2% PAE treated paper, whereas PAE-treated papers with higher PAE content had lower red color intensity, showing the reduction of assay sensitivity.

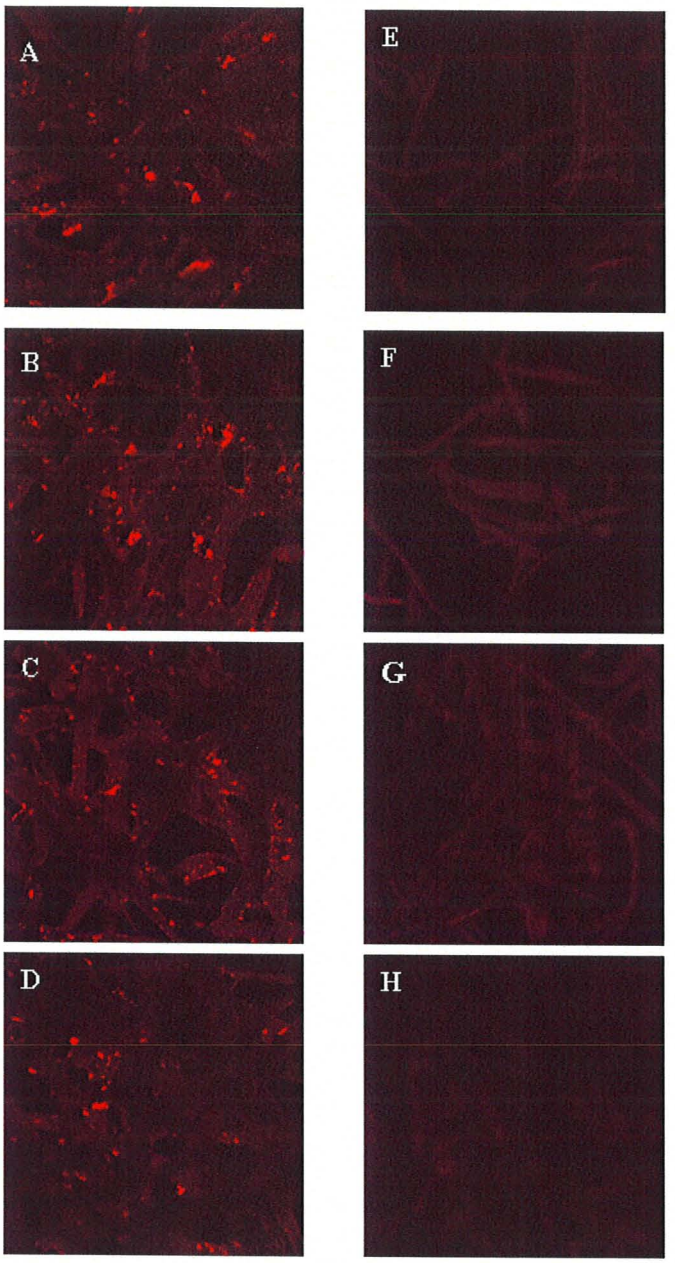


Figure 19 Confocal images of different paper samples with dye labeled Protein A (A)-(D): CBD-Ab on Untreated, 1.2% PAE, 19% PAE, 42% PAE-treated paper respectively; (E)-(H): Control experiments without CBD-Ab.

The reduction of antibody activity on PAE-treated paper at high PAE loading could be attributed to two possible factors. One of the factors was the decrease in

antibody adsorption due to the polymer coating which reduced the porosity and binding sites available for antibody binding. The other reason for the reduction of antibody activity was the possible denaturation of bound antibodies by the adjacent polymer resins. Such interaction could cause the antibody to lose its functionality to recognize its antigen. In order to validate the proposed reasons individually, two sets of experimental method were devised and discussed in the following sections.

4.4Antibody Binding

To investigate antibody adsorption on filter paper, a membrane chromatography system was utilized. The adsorption of antibodies was determined by measuring the amount of antibody permeating through a stack of filter paper discs. **Figure 20** shows the chromatographs of AR-Ab adsorption on untreated and PAE-treated papers. The peaks shown in the chromatographs represent the amount of AR-Ab permeated through the membrane. Large peak area indicates less AR-Ab adsorbed on the paper bed, whereas small peak area represents high AR-Ab binding on paper samples. To determine the amount of AR-Ab in the feed, a control experiment was performed with no paper discs installed in the membrane module.

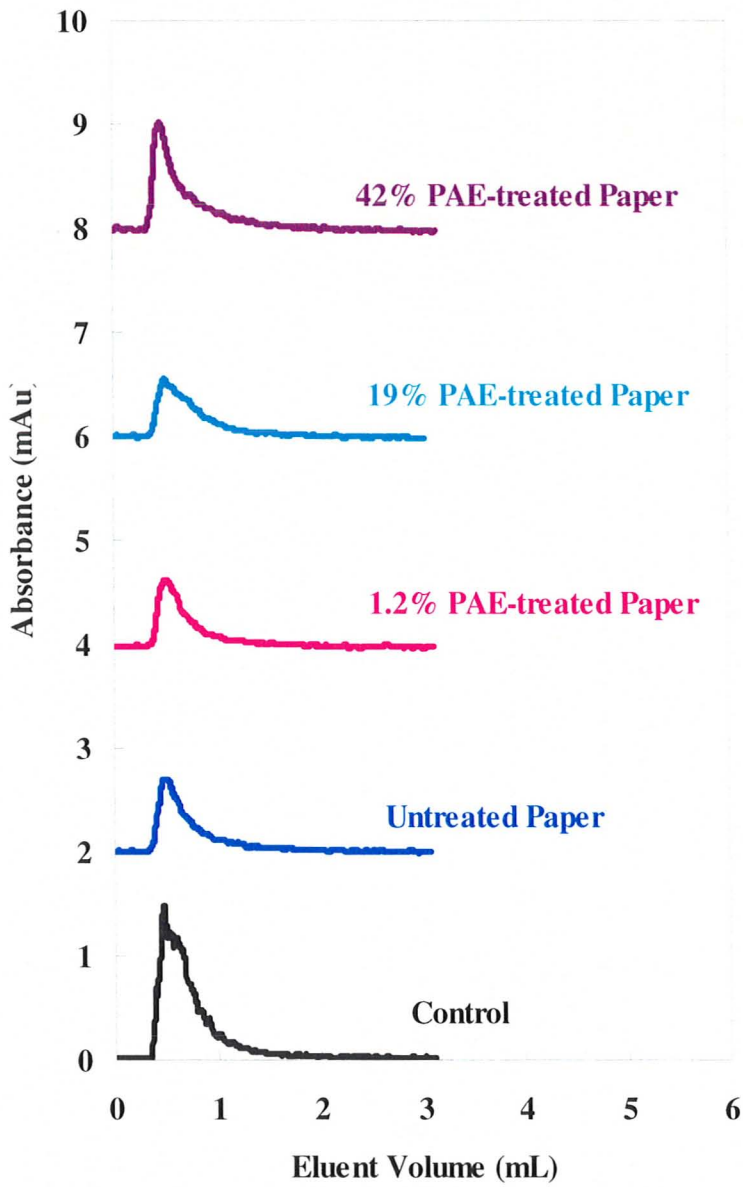


Figure 20 Chromatograph of AR-Ab on untreated and PAE-treated papers

The percentage of AR-Ab adsorbed on paper could be determined by calculating the peak area difference between control ($A_{control}$) and paper-loaded (A_{paper}) experiments. **Equation 3** was used to compute the percentage AR-Ab adsorption.

$$\% \text{ AR} - \text{Ab Adsorption} = \frac{A_{paper} - A_{control}}{A_{control}} X100\%$$

Equation 3

Table 5 Adsorption of AR-Ab and CBM-Ab onto untreated and PAE-treated papers

PAE (wt %)	AR-Ab Adsorption (%)	CBM-Ab Adsorption (%)
0	54	64
1.2	59	65
19	53	59
42	39	50

Table 5 summarizes the CBM-Ab and AR-Ab binding capacity of untreated and PAE-treated papers. Each antibody adsorption was investigated independently in the binding experiments. As shown in the table, approximately half of the AR-Ab and CBM-AB feed was adsorbed on untreated paper. When compared to untreated paper, 1.2% PAE improved antibody binding whereas higher PAE loading reduced antibody adsorption on papers. The slight antibody binding improvement in 1.2% PAE-treated paper was possibly due to the electrostatic interaction between positively charged PAE resins and negatively charged antibodies. However, the significant reduction in porosity and binding sites adversely affect the antibody binding at higher PAE content. Such trend was found to be similar to that of antibody activity experiments, discussed in previous section. Hence, the decrease in antibody activity at high PAE content could be justified by the reduction in antibody adsorption, as a result of polymer coating on paper surface.

4.4 Antibody Specific Activity

In order to accurately quantify the antibodies which were active and bound on the paper surface, a particular variable, known as specific activity, was introduced. Specific activity was determined by computing the ratio of absorbance values **(i.e. Figure 18)** to the corresponding adsorption data **(i.e. Table 5)**.

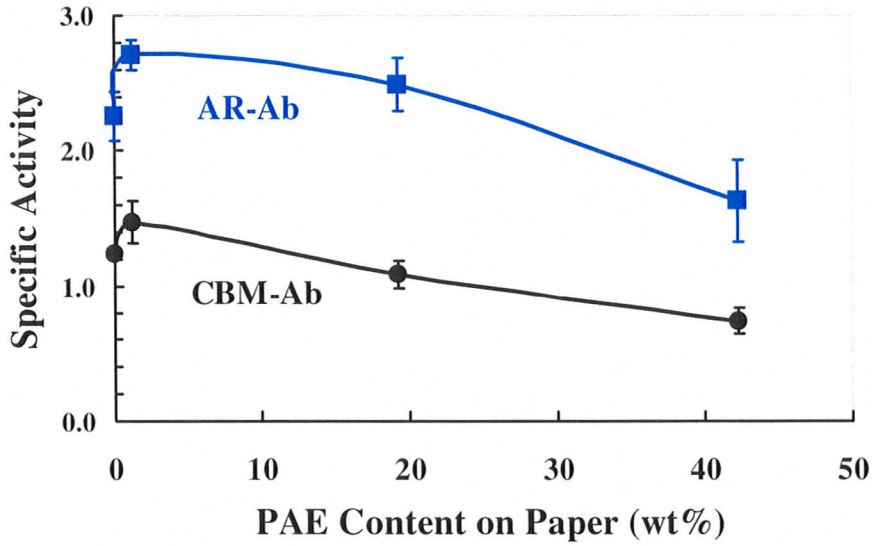


Figure 21 Influence of PAE on the specific activity of AR-Ab and CBM-Ab

Figure 21 shows the specific activity of AR-Ab and CBM-Ab as functions of PAE content of paper. The typical PAE loading range used in commercial papers (1 -2%) was found to improve the specific activity of both antibodies. Thus, there is no evidence of significant deactivation of antibody by the reactive and cationic surfaces. On the other hand, higher PAE loading resulted in diminishing specific activity.

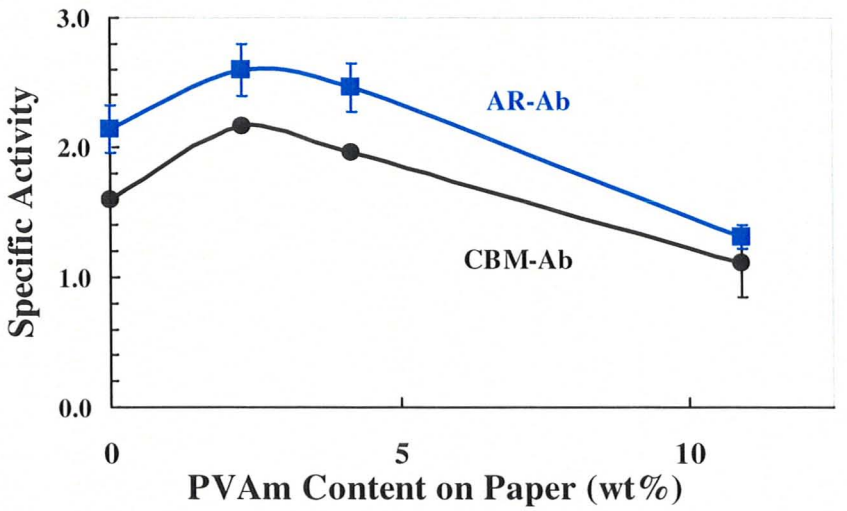


Figure 22 Influence of PVAm on the specific activity of AR-Ab and CBM-Ab

The influence of PVAm on the specific activity of two different antibodies is shown in **Figure 22**. PVAm is a relatively new papermaking chemical which is a linear polymer with primary amines on every repeat unit.[47] Similar to PAE-treated paper, the typical loading of PVAm in commercial paper was also found to improve the specific activities of both antibody assays. The specific activity profile for PVAm-treated paper was found comparable to PAE-treated paper. The results showed that the improvement of assay sensitivity at 2.3% PVAm treated paper was probably because the weakly charge surface did not denature the protein, but, in fact, preserve the biological activity of the antibodies. As the PVAm content increased, the specific activity of antibody decreased.

The mechanisms which promoted the increase in specific activity in low resins loading case and the decrease in specific activity at high resins loaded paper were not

clearly understood. Lin[89] has published his work about the influence of PAE and PVAm on bacterialphage binding on treated papers. In his work, he showed that PAE- and PVAm-treated papers exhibited high phage binding ability. However, most of the phages were deactivated by wet-strength resins due to the electrostatic interaction. Similarly, Su et al.[39] also found that cationic PAE facilitated the adsorption of DNA aptamers but it deactivated the aptamers. Hence, it is possible that the decrease in specific activity at high polymer loading was due to the strong attractive force between antibody and charge surface which deactivates the antibody.

However, the proposed reason cannot be validated because of the inconsistent reports on the electrostatic charge of the sub-regions of antibodies at neutral pH. Bergkvist et al.[90] used a tapping mode-atomic force microscopy (TM-AFM) to study the orientation of IgG adsorbed onto a negatively charged mica surface. He found that the IgG, with isoelectric point of 7.4, adsorbed onto negative surface by the positively charged Fc region while the Fab regions pointed away from the surface to maintain its activity at neutral pH. On the other hand, Zhou et al.[91], who also investigated the orientation of antibodies adsorbed on charged surfaces by simulation, suggested that the Fab and Fc regions of the whole antibody carried positive and negative charge, respectively, at the isoelectric point. Consequently, the antibodies preferred to bind on positively charged

surface by Fc regions and improve their activity. Therefore, the lack of consensus led to the inability to confirm the decrease of specific activity was attributed to the strong electrostatic interaction between resins and antibody. In order to understand the interaction between antibodies and charged surface, it is critical to know the charge of Fc and Fab regions of the studied antibodies.

4.5 Preliminary Study: Influence of AKD on Contact Angle Measurement and Antibody Activity

The percentage AKD contents in filter paper were found to be 1.3%, 3.6%, 11.2% and 26.1% when treated with 0.5%, 1.5%, 5%, 12.5% of AKD solution, respectively. The contact angle results, as presented in **Figure 23**, show that 1.3% AKD-treated paper has a very hydrophobic surface with contact angles of 124.0 ^{o}, whereas untreated paper has 1.5^{o}, indicating that untreated paper is completely hydrophilic. Also, there was a slight difference of contact angles between papers with 1.3% and 26.1% AKD content.

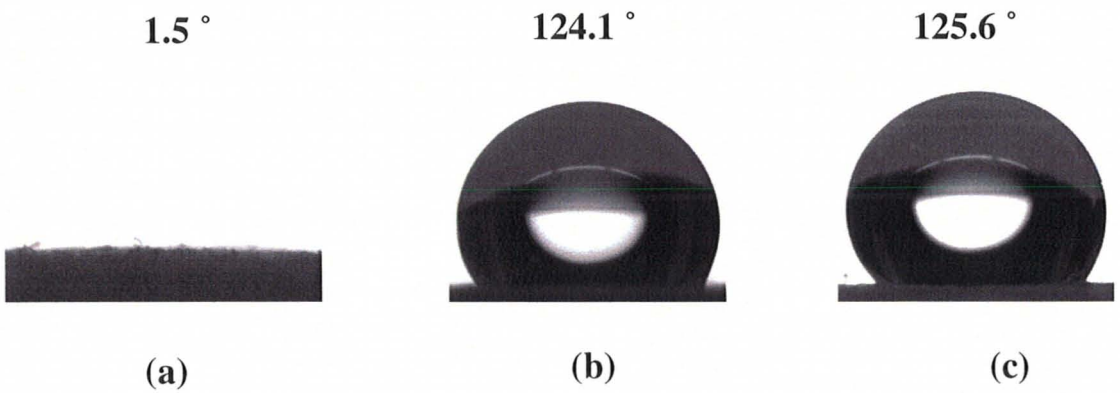


Figure 23 Contact angles of different paper samples: (a) Untreated Paper; (b) 1.3% AKD-treated Paper; (c) 26.1% AKD-treated Paper

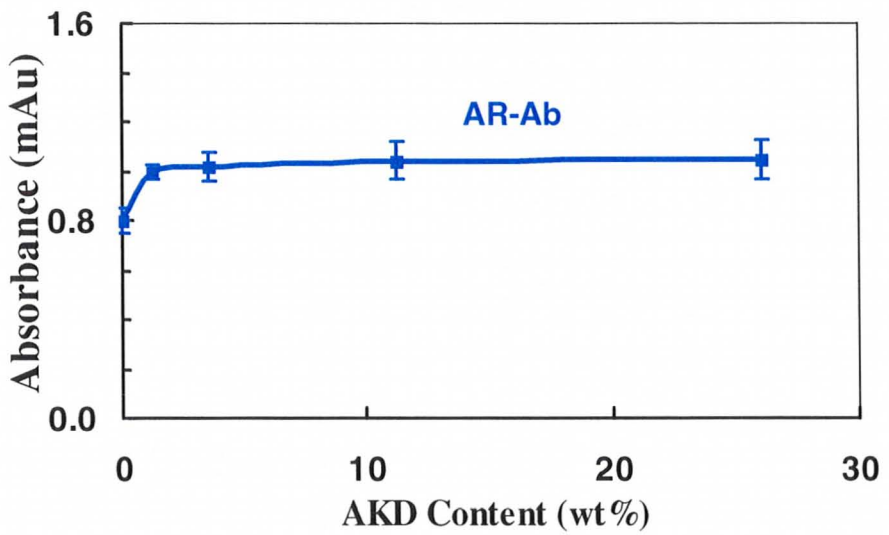


Figure 24 AR-Ab activity on untreated and different AKD-treated papers

The results of activity study of AR-Ab on untreated and AKD treated papers are presented in **Figure 24**. The antibody activity was found to increase with diminishing effect as the AKD content in paper increased. The reason for the increment of activity on AKD-treated papers could be attributed to the increasing hydrophobic interaction between

AKD and antibody as the polymer loading increased. Brash and Ten Hove[59] studied the adsorption of fibrinogen onto different polymeric materials and found that more proteins were adsorbed on hydrophobic substrates, such as polyethylene (PE) and polydimethylsiloxane (PDMS) than on hydrophilic substrate, like cellulose. The increase in antibody activity on AKD-treated paper might also due to the better orientation of antibodies on hydrophobic surface. Laseen and Malmsten[92] studied the IgG adsorption onto the hydrophobic hexamethyldisiloxane (HMDSO) plasma polymer modified silica surface. The result of the study showed that IgGs mainly adsorbed on HMDSO surface by Fc regions with little reorientation or conformation change to help keep the antibody functionally active.

Chapter 5 Conclusions and Recommendations

The objective of this work is to study the influence of paper chemistry on antibody activity. Two kinds of antibodies were chosen: commercial AR-Ab (whole molecule) and specially engineered CBM-Ab (single domain antibody). Paper surfaces were treated with wet-strength resins (PAE and PVAm) to give positively charged surfaces and sizing agent (AKD) to create hydrophobic surfaces. Then, antibodies were immobilized onto paper to observe how they behave on different paper surfaces. The conclusions are summarized as follows:

1. PAE and PVAm do not have a dramatic positive or negative influence on the activity of paper-supported antibodies and their associated enzyme reporting systems. Thus, wet-strength paper is a suitable support for antibody assays.
2. 1-2 wt% PAE or PVAm, which is typical dosage of commercial papers, actually improved antibody performance, whereas much higher polymer contents interferes with assay efficiency.
3. Preliminary study shows that AR-Ab worked well on AKD-treated paper. 1% AKD content could already make the fibre surface hydrophobic enough to improve AR-Ab activity onto paper surface.

4. Blocking of the paper surfaces to prevent non-specific adsorption of the reporting antibody-enzyme is critical for assay efficiency and skim milk worked very well on wet-strength paper with two different types of antibodies.
5. The conventional antibody AR-Ab , and the engineered CBD-Ab, demonstrated good binding to cellulose. Furthermore, small quantities of cationic polymer increased the level of antibody sorption to fibers. The strong and reproducible binding of AR-Ab onto papers would be open a new path for antibody application without CBM for binding of Antibodies to paper.

The following aspects are recommended for further studies:

1. It may be interesting to study the mechanism of antibody binding onto paper fiber without cellulose binding domain. If the mechanism is clearly understood, it would be reasonable to eliminate cellulose binding domain when immobilizing antibody onto cellulose fibers.
2. The study of antibody performance on AKD-treated paper is still at the early stage and therefore should be completed to fully understand the effect of AKD on antibody.
3. The antibody activity on original, wet-strength and sizing papers under the different temperature and humidity can also be investigated. These factors are both important and useful for transportation, storage and handling of bioactive papers.

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