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BIOACTIVE PAPER – A PAPER SCIENCE PERSPECTIVE

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CONTENTS

INTRODUCTION

This paper reviews the initial developments in a new field we call "bioactive paper". At the interface between the enormous global biomedical-biotechnology research activities and the small, esoteric world of paper science research, bioactive paper research targets exciting new paper products, improving quality of life world wide. The most exciting potential implementations of bioactive paper involve leading edge concepts in genetic engineering, biochemistry, and microbiology. Current bioactive paper initiatives are summarized herein from a paper technology perspective. Elementary descriptions are given for key biological science aspects, assuming little prior knowledge, and with emphasis on the role of the paper substrate. The first task is to define bioactive paper.

The Sentinel Bioactive Paper NSERC Research Network, a large Canadian initiative to develop bioactive paper, defines bioactive paper as "inexpensive paper products which detect and repel or deactivate waterborne, food-borne and airborne pathogens". Key elements of this vision are: 1) detection must be instant without aid of equipment or a laboratory; and 2) bioactive paper must be manufactured with conventional papermaking, coating and printing technologies, ensuring widespread application. This definition encapsulates the specific goals of Sentinel and thus is rather restrictive.

In their working paper entitled "Bioactive paper and fibre products patent and literature survey", VTT defines bioactive paper as "paper-like products, cardboard, fabrics and their combinations, etc., with active recognition and/or functional material capabilities" [1]. Although sounding as if it originated from a patent lawyer, this definition is more encompassing than the narrow Sentinel vision. In their 2006 review of bioactive packaging, Lopez-Rubio et al. proposed the definition "bioactive packaging, in which a food package or coating is given the unique role of enhancing food impact over the consumer's health" emphasizing one potential use of bioactive paper [2]. The field is young and, in my opinion, it is too early to worry about definitions – it is the opportunities which are exciting.

Two key drivers are propelling the bioactive paper revolution. The first is capability. Much of the world's research funding is focused on biomedical-biotechnological-nanotechnological sciences. All the technology platforms described herein have come out of this unprecedented global research investment. There is much low lying fruit which can be exploited for bioactive paper products.

The second driver is need. Issues involving tainted food and water, resistant bacteria in hospitals, the global spread of disease, and the threat of bioterrorism, receive almost daily coverage in the Canadian media. Although Canada is a rich country with a good health care system and large fresh water reserves, poor water quality (Walkerton Ontario, May 2000) and tainted food (Ontario, October 2008) recently have killed Canadians. The SARS epidemic of a few years ago highlighted our vulnerabilities. Hospital workers following existing protocols died from SARS exposure.

The need for bioactive paper is obvious. – how close are we? Existing products include tissues which claim to kill viruses, and nonwoven fabrics with antibacterial photochemical elements – see examples in Table 1. On the other hand, aside from dipstick test kits and similar devices, I am not aware of any commercial paper products which indicate the presence of pathogens.

Table 1 Examples of commercial bioactive paper products.

Supplier	*Description*	*refs*
Ahlstrom	Photocatalytic nonwovens	[158]
Kimberly Clark	Ascorbic acid anti microbial Kleenex®	
Domtar	Silver based antimicrobial office paper.	[64]
Agion	Silver based antimicrobial food liners.	

However, based on the intensity of current research efforts, consumer bioactive paper products will appear.

Scope

The focus of this review is research leading to large-scale implementation of inexpensive bioactive paper products which can function without laboratories or significant instrumentation. The "paper" part of the bioactive paper is viewed in the broadest context to include nonwoven fabrics and coated and uncoated paperboard. Not considered herein are examples involving small cellulosic adsorption pads and nitrocellulose films which are widely used in "dip-stick" lateral flow devices such as over-the-counter pregnancy test kits.

The biochemical toolbox

Bioactive paper research is very interdisplinary. Paper scientists and engineers must work with biologists, microbiologists and chemists. Like building the Tower of Babel, a major challenge for members of multi displinary research teams is understanding each other. As a step in this direction, the following paragraphs very briefly summarize some key biological materials and biotechnological approaches relevant to bioactive paper. Polymer science is the core of my comfort zone, so many of the following descriptions are tainted with a polymer perspective.

Biosensors

Proteins, and in particular, antibodies and enzymes are important players in bioactive paper. From a polymer science perspective, proteins are linear polyamides based on specific sequences of amino acids. The shapes (tertiary structure) of proteins are critical for many biological functions. Compared to cellulose and most polymers used in paper technology, proteins are often fragile. They can easily be denatured, which is the irreversible change in the tertiary structure, resulting in the loss of biological function. In addition, proteins are readily decomposed by proteolytic enzymes. Nevertheless, proteins with exceptional stability can be isolated. For example, enzymes with remarkable stability at high temperature have been isolated from high temperature zones near underwater volcanoes [3].

Enzymes function as catalysts and they are currently used in the paper industry to modify fiber properties and to hydrolyze synthetic polymers in

recycled pulps [4]. Enzymes are routinely employed in pathogen detection, either because they can "recognize" a specific substrate or because they can catalyze color generating reactions. Examples will be presented in following sections.

Antibodies (Ab) are typically complexes (immunoglobulins) of four proteins generated by the immune system to deal with foreign bodies. As part of this function, antibodies specifically bind to their target, the antigen. Figure 1 shows the cartoon view of an antibody. The antigen binding sites are at the end of the Y-arms – the variable region. Antibodies are widely used in diagnostic schemes because Ab can have very specific and high binding affinities for their target, the "antigen". Since much of the antibody protein is not involved in antigen recognition, sometimes antibodies are degraded and the variable region is isolated for analytical applications – such derivates are called FAB (fragment, antigen binding). Finally, the immune system generates mixtures of antibodies when challenged. For diagnostic applications, however, it is necessary to employ monoclonal antibodies which have one specific structure tuned to a desired target. Finally, there are reports of using *Camelidae* (llamas, camels, and alpacas) antibodies which have no light chains and thus are smaller, stable, and can be produced in good yields in yeast [5, 6].

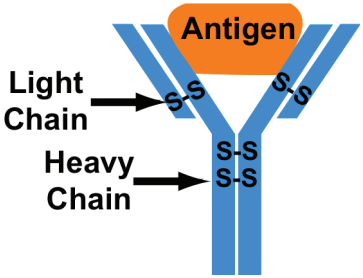


Figure 1. An antibody based on two heavy chains and two light chains.

DNA (deoxyribonucleic acid) derivatives are also likely to be important building blocs for bioactive paper. From a polymer science perspective, DNA is a linear anionic polyelectrolyte based on specific sequences of four monomers (nucleotides – adenine (A), guanine (G), cytosine (C), and thymine (T), condensed with phosphoric acid. In nature, every DNA chain has a complementary chain in which T is replaced with A and C is replaced with G. DNA

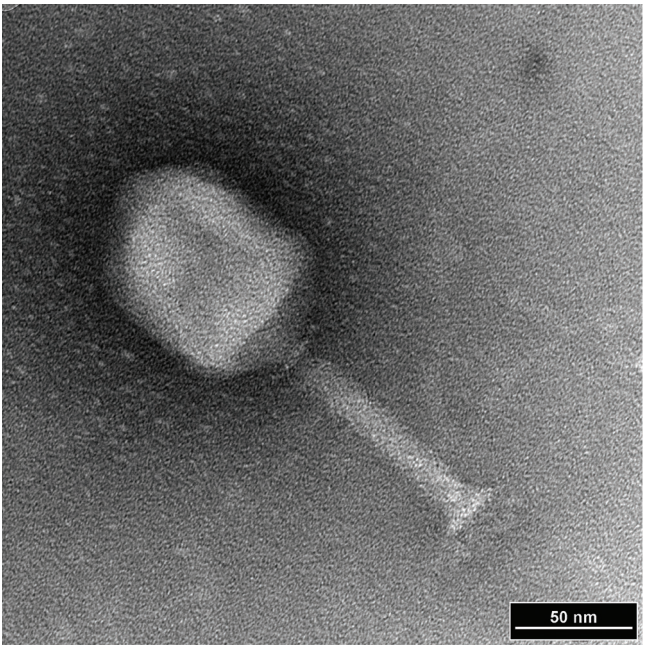
chains form the famous double helix structure with their complementary chain. Helix formation is driven by hydrogen bonding between complementary T-A and C-G pairs. Each T-A pair has two hydrogen bonds, whereas C-G pairs are stabilized by three hydrogen bonds.

Beyond the normal biological functions of storing genetic information, short manmade DNA chains offer the following unique properties which can be exploited in bioactive paper functions.

1. DNA chains are more robust than proteins with respect to heating, dehydration, oxidation, and enzyme catalyzed degradation.
2. DNA chains form specific complexes (duplexes) with their complementary chains. Examples of biosensors exploiting this behavior will be given in the following sections.
3. In the presence of metal ions, short, man-made DNA chains, called DNA aptamers, fold into specific three dimensional structures which can specifically complex with targets.
4. Infinitesimally small quantities of DNA can be amplified into much larger samples by the polymerase chain reaction (PCR). It is this ability which makes DNA analysis an important forensic tool.

Bacteriophages are one of the most fascinating tools in the biotechnology toolbox [7]. Phages are viruses that infect bacteria and not mammalian cells. A picture of a T4 phage is shown in Figure 2 – they look like a nano-scale lunar landing craft. Receptors on the long tails recognize and bind the phage to the target bacterium surface. The phage then injects DNA or RNA into the bacteria. Using the bacterial cell machinery, the injected genetic material is converted into up to a 1000 copies of the phage at which point the bacterium breaks open (lyses), releasing the next phage generation. Phages are very attractive components of bioactive paper because:

1. They can be very selective. Receptors on the longtail fibers recognize specific bacterial surfaces.
2. They kill the target bacteria.
3. By making multiple copies of themselves, there is built-in signal amplification
4. They are relatively robust. [8]
5. Edible commercial phage products are “generally recognized as safe” by the FDA and have been approved for food treatment to inhibit Listeria colonization, a problem with fresh vegetables and ready-to-eat meats [9, 10].



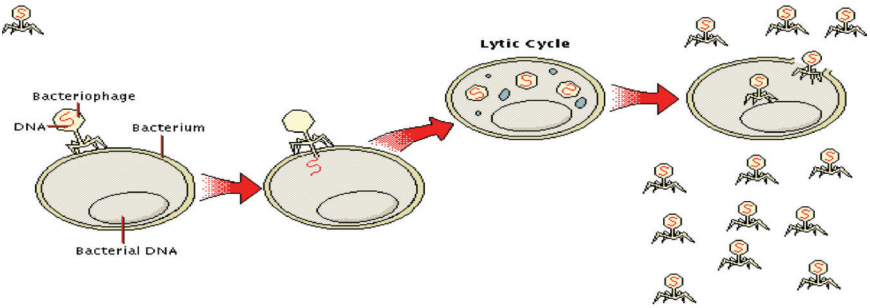


Figure 2. A transmission electron micrograph of a phage and a schematic illustration of the bacterium lyses (provided my Mansel Griffiths) [176].

Biochemical coupling strategies

Biochemists have also developed tools for coupling biomacromolecules to surfaces or other biomacromolecules – there are journals dedicated to this topic, as well as excellent texts [11]. Most conventional organic chemical reactions are not suitable for the specific attachment of sensitive biomolecules in water. Many of the tools described below employ biologically derived molecules which display specific binding. For example, cellulose binding modules (CBM) are proteins and function as the cellulose-sticky part of cellulase enzymes [12–15]. A molecule or particle bearing a CBM will spontaneously adhere to cellulose. Specific examples will be given in later sections.

CBM technology appeared in the early nineties and potential applications were highlighted in a couple of reviews by Levy and Shoseyov [16, 17]. One of the first proposed applications in papermaking technologies of CBM was as a paper strengthening agent [18, 19].

Another example of a biologically derived coupling platform is variations of the biotin-streptavidin complex. Biotin (vitamin B_7) is a small molecule which forms an essentially irreversible complex with streptavidin and related proteins such as streptavidin. The biotin binding proteins have as many as four binding sites, so a single streptavidin molecule can couple two or more biotins. In a typical application, biotin is attached to a molecule or surface of interest by either chemical coupling of a reactive biotin derivative, or by genetically engineering biotin into proteins. In a second step, the biotinylated molecule or surface is exposed to streptavidin to give spontaneous, specific, and irreversible binding. Specific examples will be given in the pathogen detection section.

Two other biochemical coupling pairs, which have already appeared in bioactive paper research, are Protein A-antibodies and streptag-streptavidin. Protein A binds to a wide range of antibodies without blocking the active sites. Strep tag is a peptide sequence which can be engineered into proteins to give binding to streptavidin.

Finally, and far beyond the scope of this review, the central role of genetic engineering is recognized in development of the tools described above. Enzymes, antibodies, and bacteriophages can be modified by genetic engineering approaches to generate active agents not found in nature. For example, antibodies can be engineered to carry a color producing enzyme in ELISA (explained below) detection.

Another example of the application of genetic engineering to bioactive paper involves the genetic isolation of the cellulose binding modules, CBM, (sometimes called cellulose binding domains). These cellulose-sticky proteins

can be fused to antibodies, enzymes and phages to give biosensors which spontaneously bond to cellulose. These are described in the biosensor immobilization section.

Genetic engineering approaches are also key to the production of large quantities of low cost reagents required for the large scale deployment of bioactive paper. For example, Hall's group have genetically engineered tobacco plants to produce antibody fragments. [20–22].

Potential bioactive paper formats

One can imagine a number of formats in which a pathogen detecting bioactive paper might function. In direct contact format, the bioactive paper would report the presence of a pathogen coming in contact with its surface. Applications could include protective clothing which could warn the user of contamination.

Filters are another important format for bioactive paper. Bacteria are easily trapped on filters which is a form of amplification (actually concentration). A filter with built in pathogen detection could demonstrate the presence of dangerous bacteria in water.

Finally, lateral flow bioactive paper devices are also likely to be important. The idea is that an aqueous sample is introduced onto one end of a dry sample of bioactive paper. Capillary forces pull the liquid along the paper strip. Many bioanalytical assays employ lateral flow devices. Over the counter pregnancy tests are an example. Lateral flow offers the following advantages which are unique to paper and similar porous substrates:

a) Liquid will move whereas larger particles will be trapped in the paper matrix, a form of sample filtration. Chromatographic separation of soluble components on the paper surface is also possible.
b) The paper can be treated to give hydrophilic channels which can split the sample into two or more parts which are transported to different locations on the paper surface. Whiteside's group have recently illustrated this concept [23].
c) An important application of lateral flow is the ability to expose a sample consecutively to a series of binding sites along the eluted surface.
d) Finally lateral flow can be used to remove unbound components from a region of paper with surface capture groups.

Paper versus plastics

Over the decades, plastic has replaced paper products for some food packaging, grocery bags and general packaging applications. This begs the

question – why bioactive paper instead of bioactive plastic? Paper offers unique advantages over plastic including: paper is very inexpensive and is manufactured locally in nearly every part of the world from renewable and recyclable resources; paper has a long and successful history performing as filter media and barrier media and can even function as sterile packaging; paper is easily printed, coated and impregnated; and cellulose is particularly protein and biomolecule friendly. Thus, outside the bioactive paper landscape, cellulose and its derivatives have long been important substrates for separations (i.e. paper chromatography and cellulose membranes) and as supports for biosensing elements. Nitrocellulose film is widely used for analysis. For example, in a western blot, proteins are transferred from an electrophoresis gel to a nitrocellulose film for subsequent identification.

In another example, cellulosic microspheres with and without magnetic cores are commercially available as dispersed solid supports for bioseparations and solid phase synthesis. Nanoscale magnetic cellulose has also been proposed as a protein isolation platform [24].

An intriguing application close to bioactive paper is the SPOT technology [25–27]. Filter paper sheets are divided into arrays of mm scale spots with a plastic manifold giving an array for multiple, simultaneous analyses. Although much larger than chip-based analytical arrays, the inexpensive and controllable nature of paper makes it an attractive substrate for traditional analytical assays. This widespread use of cellulose in traditional biochemical assays underscores the protein friendly nature of cellulose.

The following sections summarize bioactive paper research under headings related to end-use: antiviral/antimicrobial (no pathogen detection), pathogen detection, and bioseparations.

ANTI-VIRAL AND ANTIMICROBIAL PAPER

Introduction

The goal of antimicrobial paper is to deactivate viruses and prevent microbial colonization on or near the paper surface. Disinfecting tissues, filters, and food packaging offer many potential advantages, although there is some indication that widespread antimicrobial environments will promote far more resistant and potentially dangerous microbes [28].

The scientific and patent literature contains many examples of antibacterial surfaces bearing an active agent which inhibits bacterial growth [29]. A technology area which is close to bioactive paper is "active packaging" which mainly involves building functionality into plastic films.

Suppakul et al. have published an excellent summary of antimicrobial film packaging, including lists of commercial products [30]. The active agents on the various types of antimicrobial surfaces fall into two groups – those permanently attached to the surface and those which are mobile and can leave the surface and enter a liquid or vapor phase. For example, antibacterial polymers [29] will be immobilized, whereas silver nanoparticles or small molecule bactericides will have the potential to diffuse into an aqueous phase.

Both scientific studies and antimicrobial product development activities require test methods to quantify efficacy. Tappi [31], the Environmental Protection agency [32], ASTM, and ISO [33], have defined standard methods for measuring microbial contaminants in paper products. Variations of these tests are described in various publications [34–44]. On the other hand, we have found no general methods for accessing the performance of antimicrobial paper. Most of the publications employ some form of incubation followed by counting of colony forming units. Sample preparation varies widely: the paper is directly placed on the media [45]; the paper is disintegrated and added to culture media [44, 46]; or the bacteria are washed from the paper surface, cultured and counted [47]. In yet another variation, which mimics some food packaging situations, an air gap separates the antimicrobial paper and growth medium requiring the bactericide to diffuse through the vapor [48]. Finally, genetic identification of microbes following PCR amplification is an important tool in mainstream microbiology which has been applied to paper [42]. Note that all of the above procedures take hours to days to be performed by a skilled technician in a laboratory.

The following paragraphs describe four classes of antimicrobial papers – fixed antimicrobial agents, labile agents, light-activated agents and biological agents. Fixed agents should inhibit bacterial colonization on the paper surface, whereas labile agents can enter a contacting aqueous or vapor phase to kill nearby bacteria. Light-activated agents can be fixed or labile and require light to function. Finally, biological agents are natural materials with antimicrobial efficacy.

Bioactive paper with fixed antimicrobial agents

Table 2 summarizes examples of recent publications describing antimicrobial paper surfaces with fixed active agents. Many of the agents are cationic polymers with hydrophobic chains attached to quaternary nitrogen atoms. In some cases the cationic polymers are covalently coupled to cellulose [46], whereas others exploit the high affinity binding of cationic polymers to

Table 2 Examples of publications describing antimicrobial paper surfaces in which the active agent is fixed to the paper surface.

Substrate	*Active*	*At*	*Ref*	*Target*
Copy paper	Hydrophobic gallates grafted to chitosan	Y	[49]	*S. aureus* and *Listeria innocua*
Microfibrilated cellulose	octadecyldimethyl(3-trimethoxysilylpropyl)-ammonium chloride	Y	[51]	*S. aureus*, *E. coli* and *P. aeruginosa*
Whatman 5 Filter Paper	C6 quaternary ammonium plasma grafted		[159]	*S. aureus* and *K. pneumoniae*
Bleached sulfite pulp	guanidine polymer		[46, 160] [161]	*E. coli*
Unspecified	Fatty acid methyl ester – no covalent grafting		[45]	many
Whatman 1 Filter Paper	Hexyl bromide quaternized 4-polyvinylpyridine		[162]	*E. coli*
Regenerated cellulose	Hydrophobically modified polyvinylamine		[50]	*E. coli*
Semibleached kraft pulp handsheets	Laccase polymerized phenolics		[163]	*S. aureus*, *E. coli*, and *Bacillus subtilis*

anionic cellulose [49]. Wågberg's group adsorbed alternating layers of hydrophobically modified polyvinylamine and polyacrylic acid onto oxidized cellulose films. The amount of adsorbed polyvinylamine increased linearly with the number of layers, whereas after six layers, there was no further improvement in the antimicrobial effect [50]. Finally, investigations employing more than one type of bacterium showed that the effectiveness of surface treatments depended upon type [49, 51].

Bioactive paper with labile anti-viral and antimicrobial agents

Surfaces which release soluble antimicrobial agents have the potential to kill bacteria in solution, near the paper surface. In addition, one would suspect that mobile bactericides can more rapidly move to bacterial surfaces giving faster killing. On the negative side, such surfaces will become depleted with time and may not be appropriate for food contact. Simple impregnation of paper sheets with bactericidal agents has been described in the patent literature [52–57] More sophisticated approaches employ nanoscale bactericide depots. For example, Sun and Lindsay [58] describe the immobilization of cyclodextrins onto cellulose to serve as a depot for labile bactericides. In a similar approach, Xiao's group showed that cationic polymers bearing cyclodextrin groups served as depots for Triclosan and Butylparaben bactericides [59]. These approaches exploit the affinity of hydrophobic bactericides for interior cavities in the cyclodextrin moieties. Similarly, van de Ven's group has investigated bactericide loading and release from polymer micelle depots [60].

Metallic silver and silver salts are another common class of bactericides which have been incorporated into paper and regenerated cellulose fibers [61–65]. No mechanistic studies of silver-treated papers have been published.

Most of the current commercial bioactive paper products have anti-virial/antimicrobial properties and employ labile active agents – see Table 1. Kimberly-Clark's Kleenex® Anti-Viral is one of the prominent examples. The tissue is impregnated with citric acid and sodium dodecyl sulfate which are claimed to kill viruses and bacteria, making used tissues less of a threat for disease transfer. Other examples of products include papers designed to prevent mold when used as wall paper, methicillin-resistant *Staphylococcus aureus* packaging [40], and packaging which minimizes food (milk) spoilage [66].

Bioactive paper containing light-activated antimicrobial agents

Some of the commercial bioactive paper products in Table 1 employ photocatalysts which can kill and decompose bacteria. Anatase, a form of TiO_2, is

one of the most studied catalysts since its discovery in 1972 [67]. UV irradiation of wet anatase produces reactive oxygen species in the water, including hydroxyl radicals, superoxide and peroxide species. These very reactive chemicals have a short lifetime because they readily oxidize virtually any organic material in their path. Self-cleaning glass has been the hallmark implementation of photocatalysis. Exterior windows with a low anatase content are activated by ambient UV, decomposing oil and biofilms before they become established on the window surfaces.

Over the last decade there has been much interest in photocatalytic paper, with commercial products from Nippon Paper and Ahlstrom – see Table 1. Also there have been a few scientific publications describing photo disinfection with paper [68–71]. We reviewed the photocatalytic paper literature in 2006 [70]. In recent work, we showed that the timescale for disinfection is hours, maximum disinfection rates occur when TiO_2 is on the exterior surfaces, and that photocatalytic disinfection efficiency drops off dramatically when the water content of the paper is lowered from 40% [72].

One reason for the slow disinfection kinetics with TiO_2 based photocatalytic paper is that the ambient UV flux is low, particularly indoors in a Canadian winter. Many publications have described antimicrobial dyes which can be activated by visible light [73–81]. Griffith's group have very recently published an initial investigation of photocatalytic dyes for killing food-borne pathogens [82].

Bioactive paper containing biological antimicrobial agents

Living organisms, including you and I, have mechanisms for recognizing and killing bacteria. High concentrations of lysozyme in our tears protect our eyes [83] and proteins called defensins attack bacteria in tissues [84, 85]. In 2005 the antibiotic nisin was the only antimicrobial protein (a bacteriocin) which was approved as "generally recognized as safe" by the FDA and the World Health Organization [86]. This protein is produced by the bacterium *Lactococcus lactis*. The food science literature contains a number of publications describing packaging, including paperboard and cellulose film, coated with nisin [66, 86–88] and other antimicrobial proteins [89].

In a previous section we introduced the bacteriophage as both a bacterium recognition agent and a killing agent. Indeed, phages have been approved as bactericidal agents for vegetables. Griffith's group has published the first publication suggesting the use of phages in bioactive paper [90]. In this work they genetically engineered phage so that the head was coated with cellulose binding module protein. The engineered phage spontaneously adhered to cellulose surfaces presumably in the desired configuration with the head down and

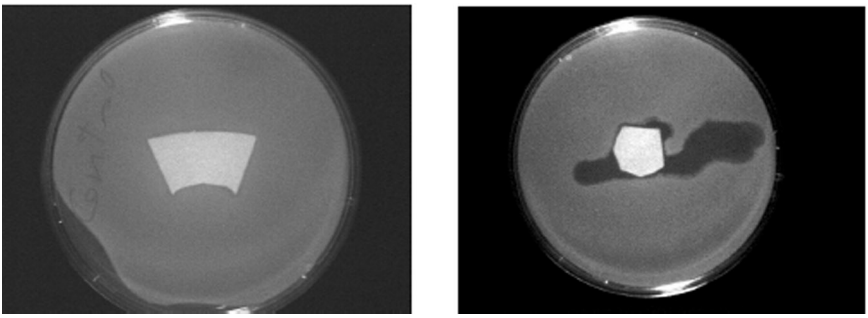


Figure 3. Killing bacteria with filter paper impregnated with wild T4 phage (left), and filter paper with genetically engineered T4 phage with cellulose binding module (right) giving spontaneous adhesion of the phage head to cellulose [90].

the long tail fibers pointed out to capture target bacteria. Figure 3 shows photographs comparing the ability of two filter paper samples to kill *E. coli*. The left sample shows paper impregnated with wild T4 phage with no specific immobilization and there is no evidence of infection of the bacteria by the phage. Since the papers were washed before testing, the wild T4 phage simply washed off of the filter paper. By contrast, the right hand photo shows significant infection of *E. coli* (the clear areas in the media) by the phage, immobilized on the cellulose by the CBM protein on the phage head.

Summary

Antimicrobial and antiviral paper products are available in the marketplace. All the commercial products are based on synthetic killing agents which may or may not be fixed to the paper surfaces. Most of the scientific studies focus on material preparation with simple and limited antimicrobial testing. In particular, there are virtually no examples of the measurement of fundamental kinetic parameters for the disinfection processes. An exception is the work of Lee et al. who published a comprehensive investigation of killing kinetics from paperboard coated with nisin, an antimicrobial polypeptide [66].

None of the current commercial antimicrobial papers, nor any described in the literature, include any form of microbial detection. In addition, we have found no discussion of the recycling implications of antimicrobial paper. However, since bactericides are widely used in papermaking operations to prevent slime buildup, recycling is unlikely to be an important issue.

Finally, there does not seem to be any specific requirements for antimicrobial paper from a paper structure/property perspective. Of course virtually all applications of bioactive paper will involve wet paper because there are virtually no significant biological processes in dry conditions. Bacteria, like human cells, require water. Therefore most antibacterial papers need to fulfill their functions when wet, which may require wet-strength resins [91], or other types of treatments to maintain physical integrity.

PATHOGEN DETECTING PAPER

Introduction

The detection of pathogens in our food, water and air is receiving a lot of attention because of the enormous public health implications [92] [2]. The challenge is to achieve sensitive and selective detection using rapid inexpensive assays. Similarly, point-of-care biomedical diagnostics require the same characteristics – sensitivity, selectivity, speed while being inexpensive. In my opinion, it is in these applications that bioactive paper offers the greatest promise. Diagnostic food packaging, disposable medical protective coverings, and consumer products would have a big positive impact in the developed world. Most exciting are the potential impacts of simple, inexpensive point of care diagnostics for the developing world.

Litmus paper is a spectacular example of a sensing paper – it is inexpensive, requires no amplification, requires no equipment, requires little training, has a long shelf life and is very sensitive. A litmus paper indicating a pH value of 11 is reporting a hydrogen ion concentration of 10^{-11} moles per liter which is picomolar sensitivity. Pathogen detecting paper with these characteristics does not exist yet, however, I believe it will.

In some respects, rapid pathogen detection, with paper or without, is an unsolved problem, even with the resources of a fully equipped laboratory. For example, a useful bacterial assay for hamburger must be sensitive enough to detect 1 bacterium in 25 grams of product [93]. Therefore sampling is the first problem – one bacterium in 25 grams is a needle in a haystack. Once a representative sample is obtained, the sample must be amplified. The traditional approach was to amplify the sample by culturing the sample to grow a detectable quantity of bacteria in Petri dishes. This procedure took days. The modern approach is to use essentially the same techniques as used in forensic science [42]. DNA isolated from the sample is amplified by multiple cycles of the polymerase chain reaction (PCR). DNA fragments are compared to genetic profiles of known pathogenic microbes. This method provides the identity of bacteria [42]. However, it is slow,

requiring a day. The development of a pathogen-detecting litmus paper is a significant challenge.

A pathogen detecting paper must perform two important functions, biorecognition and reporting. Biorecognition refers to the capture of the target pathogen, or a chemical marker indicating the presence of a dangerous bacterium or virus. To be useful, the capture must be specific – there are many benign microbes which are ignored by a useful biosensor. Note, there exist dyes which report the presence of bacteria on paper, however, these are nonspecific giving no information about the nature of the microbe [94]. Biorecognition is a critical component of pathogen sensing bioactive paper.

The biorecognition agents, herein called biosensors, likely to be important for bioactive paper are antibodies, enzymes, bacteriophages and DNA aptamers – examples of each will be described below. The mainstream analytical literature describes other potential capture/recognition agents, including molecular imprinted polymers [95] and whole cells [96], which have not yet been applied to paper. In addition, this review does not consider paper-supported electronic devices.

Reporting is the second step in pathogen detection. When the target is captured by an antibody, or one of the other types of capture/recognition agents, the paper must signal (report) the occurrence of the capture event to the human user. In my opinion, the development of robust, sensitive reporting is the greatest challenge in the development of bioactive paper.

Biosensor immobilization on paper

Introduction to immobilization

Described in this section are the various approaches to attachment of the biosensing agents to paper. The goal is to control: a) the location of the biosensors in or on the paper structure; b) the density of biosensors; and, c) the tertiary structure and orientation of the immobilized sensor molecules.

The immobilization process can be considered as two steps – transport and attachment. Transport is the process by which a buffer solution of sensor molecules is brought to the surface. Because of the importance in printing and coating, the transport of aqueous solutions into paper structures is much studied and well understood [97–100]. Capillary force driven liquid flow is the major process. Paper surface chemistry, controlling contact angle, and the pore structure distribution in the paper influence both the rate and the extent of penetration of water into paper.

Paper structure also influences the maximum quantity of biosensors which

can be attached to cellulose. Specifically, the maximum biosensor content is $\Gamma.\sigma$ where Γ is the maximum density of immobilized sensor and σ is the specific surface area of the paper structure accessible to the biosensor. Most polymers and proteins have Γ values between 0.1 and 1 mg/m^2 [101]. Paper structures can have a wide range of specific surface areas, σ. For non-porous paper such as glassine, only the macroscopic external surface is accessible. In this case the $\sigma = 2/bw$ where bw is the basis weight which is the mass of paper per square meter. Thus for a glassine with a bw of 50 g/m^2, the corresponding accessible specific surface area is $\sigma = 0.04$ m^2/g. By contrast, Hong et al. recently measured the accessible specific surface area of various forms of cellulose to proteins using a probe protein which was a fusion of cellulose binding domain and green fluorescent protein [102]. They found that the accessible specific surface area of Whatman No. 1 filter papers was 9.5 m^2/g. Of course smaller proteins will access smaller pores, giving a higher specific area and vice versa.

Consider two extreme cases – a biosensor with a low Γ of 0.1 mg/m^2 coated on glassine, described above, gives a maximum biosensor coating of 0.004 mg of sensor per gram of paper. Whereas a more compact biosensor, giving a higher Γ of 1 mg/m^2, can be taken up by the Whatman No. 1 filter paper to give 9.5 mg of biosensor per gram of paper. Thus we see that the capacity of conventional paper substrates to take up biosensors can range over four orders of magnitude.

Attachment is not a strict requirement for incorporation of biosensors or any other water-soluble component into filter paper. A dry filter paper will sorb more than its dry mass of aqueous solution when immersed in a bath. Indeed, the cellulose fiber walls typically adsorb between 0.5 to 2.5 grams of water per gram dry fiber. Removal of the paper and drying will leave all of the nonvolatile components of the bath solution in the paper structure. However, impregnation without attachment is not recommended because it is difficult to control the distribution of the biosensor molecules in the paper structure. Furthermore, subsequent exposure to water is likely to leach the biosensors.

The location of the biosensing elements in the paper structure is important – biosensing molecules are expensive and must be used efficiently. For example, an antibody hidden in a cellulose fiber pore will never be able to contact the surface of a micrometer diameter bacterium. Thus, although filter paper may have the capacity to adsorb a lot of biosensor, much of it could be inaccessible to the target.

In summary, we face a wide range of potential paper substrate types, biosensor types, and immobilization strategies. Table 3 attempts to simplify the immobilization landscape by defining four categories: 1) physical

Table 3 Four approaches to immobilizing biosensors onto dry or wet cellulose.

	IMMOBILIZATION			
Biosensor	*Physical*	*Chemical*	*Biochemical*	*Carrier Particles**
Antibodies	[128]	Film [164]	[6, 125, 126, 128] [165]	Microgel [138] Silica [129]
Enzyme	[116, 117, 144] [146] [107] Extruded with regenerated cellulose film [166] Layer-by-layer [121]	Film [164] [27] [167] Paper [168] [169]	[126]	Silica [139]
Phage	[90, 170]		Phage [90]	
DNA Aptamer	[112, 150]	Paper [112] [171] Nitrocellulose film [172]	[131]	Microgel [138]
Cells		[173]	[127]	
Biotin, streptavidin		[174]		

* there are many examples of biomacromolecule immobilization on particles – these references are restricted to cases where the particles are subsequently put onto paper

immobilization where the biosensor adheres to the paper surface because of van der Waals and electrostatic forces; 2) chemical immobilization where covalent bonds fix the biosensor to the paper surface; 3) biochemical coupling where CBM or other biochemical binding agents are employed; and, 4) bioactive pigments where biosensors are coated on colloidal particles which are then printed or coated onto the paper. Each category is now described.

Physical immobilization – Direct application to wet or dry paper

Printing and coating technologies allow application of almost any fluid onto dry paper. Aqueous solutions are particularly easy because capillary forces and the hydrophilic nature of cellulose promote rapid sorption. Antibodies, enzymes, aptamers and phages can be spotted or printed onto dry filter paper without denaturation. However, in most cases the biosensors are not firmly

anchored. The following paragraphs briefly review the adsorption behaviors of synthetic polymers, proteins, DNA aptamers and phages onto pure cellulose. Adsorption experiments indicate whether physical forces are sufficient to fix the biosensor to cellulose.

There have been many studies of the adsorption of synthetic polymers onto both pure cellulose [103] and to papermaking fibers [104]. Clean cellulose is a hydrophilic, slightly anionic surface with a low negative surface charge density [105]. Cationic polymers readily adsorb onto cellulose from aqueous solution, whereas anionic and nonionic water-soluble polymers tend not to. Electrostatic interactions between cationic patches on proteins and anionic cellulose are also an important driving force for protein adsorption onto paper [106] and regenerated cellulose [107]. There is some evidence of attractive interactions between tyrosine groups and cellulose [108, 109] which may also contribute to binding. Halder et al. reported adsorption isotherms for a number of proteins on cellulose powder, and found that ranking of proteins in terms of moles of adsorbed protein per mass of cellulose was gelatin > β-lactoglobulin > lysozyme > BSA under one set of conditions [110]. Note that the properties of both proteins and paper substrates are sensitive to pH, ionic strength and specific ion effects so the details are important.

Many researchers have investigated blood plasma protein adsorption onto regenerated cellulose, a potential membrane material for hemodialysis. For example, Brash showed that while fibrinogen did adsorb onto cellulose, the rate and extent of adsorption were low compared to hydrophobic surfaces such as silicone, PVC and polyethylene [111]. In summary, proteins are not strongly adsorbed onto pure cellulose, and so protein based sensors are likely to require a more aggressive immobilization strategy. Other types of biosensors do not adsorb strongly either.

Halder et al. showed that high molecular weight DNA did not adsorb onto cellulose at pH 6 and 8, whereas adsorption was observed at pH 4 [110]. Su et al reported adsorption isotherms for low molecular weight DNA aptamers onto microcrystalline cellulose (see Figure 4) [112]. However, the utility of direct DNA aptamer application is limited because the aptamers were easily washed off. The low affinity of these low molecular weight oligonucleotides is consistent with the synthetic polymer adsorption literature which shows that anionic polymers do not adsorb onto cellulose [113].

Tolba's recent publication is the only report of the direct application of phages to cellulosic surfaces [90]. They showed that wild type T4 phage does bind to cellulose, however the subsequent activity of the bound wild T4 phage is lower than genetically engineered T4 which binds via its head. They speculated that the wild phage interacted with cellulose via the binding sites

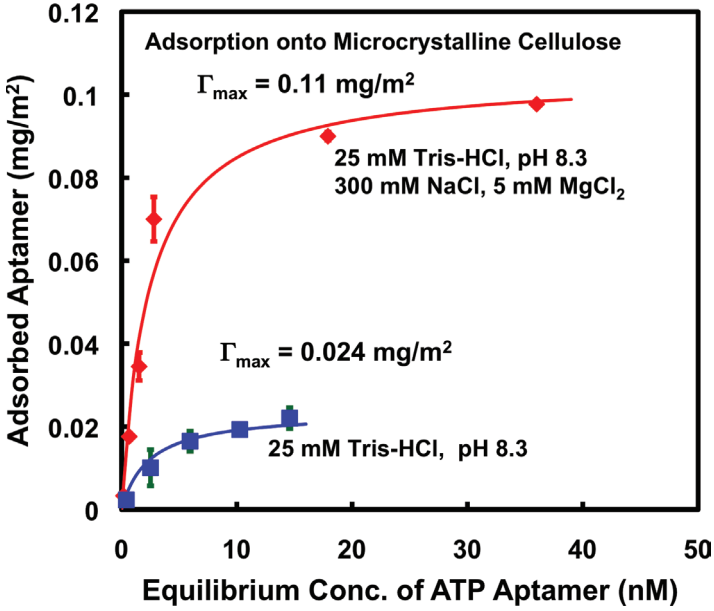


Figure 4. DNA aptamer adsorption onto microcrystalline cellulose (adapted from Su et al. [112]).

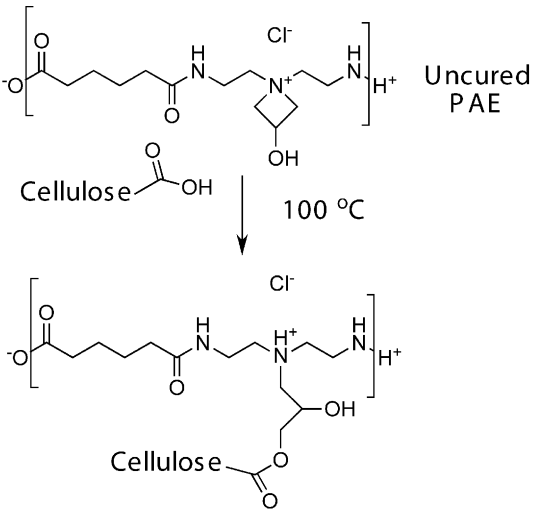


Figure 5. The chemical structures of polyamine epichlorohydrin (PAE) wet-strength resin.

on the phage's long tail fibers, which are the bacterial binding sites (see Figure 2).

Most laboratory filter papers are pure cellulose which is slightly anionic and very hydrophilic. By contrast, paper products which are expected to function while wet are usually treated with wet-strength resins which are reactive polymers added to maintain paper strength in water [91]. Kitchen towels and coffee filters are everyday examples of paper with high wet strength resin contents. Figure 5 shows the chemical structure of polyamide-epichlorohydrin (PAE), the most widely used wet-strength treatment. Paper treated with PAE has a net positive surface charge [91] and there may be residual chemically reactive groups which can couple to proteins. Polyvinylamine is another polymer used in papermaking, which renders cellulose cationic and reactive due to the large number of primary amine groups.

The potential impacts of wet-strength resins on protein immobilization are illustrated in Figure 6, which shows the results of paper chromatography

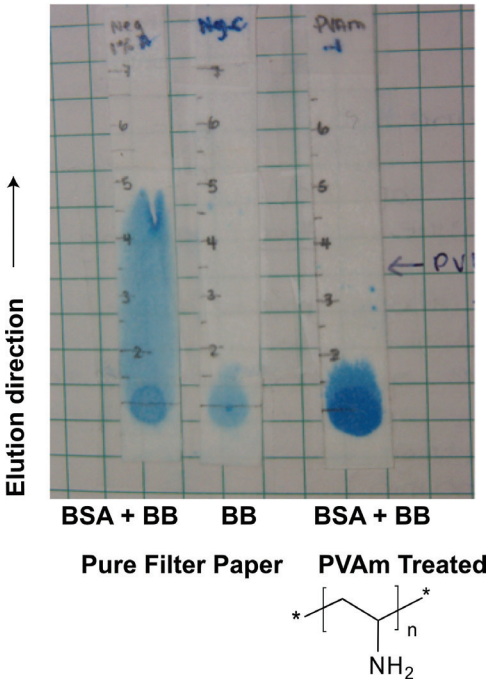


Figure 6. Influence of filter paper treatment with PVAm on BSA chromatographic mobility. The Rf value is the distance the protein traveled relative to the solvent front during chromatographic elution (adapted from Davies et al. [114]).

experiments in which bovine serum albumin, a negatively charged protein labeled with blue dye, was eluted with buffer [114]. With untreated filter paper, the protein migrated with the buffer, whereas protein moved very little on paper pretreated with the cationic polymer, polyvinylamine. In other work we showed that BSA had little intrinsic adhesion when spread onto wet cellulose [115]. Similarly, horseradish peroxidase (HRP) could not be eluted from polyamide-epichlorohydrin (PAE) treated paper (see Figure 5 for PAE structure). Whereas some proteins tend not to adhere strongly to pure cellulose, they do adhere to paper impregnated with wet strength resin.

Paper surfaces are also often covered with a grafted layer of hydrophobic chemical called "size" in the paper industry [100]. Recently Yan's group has reported the influence of sizing on ink jet printed HRP [116, 117]. They showed that moderate sizing increased the color intensity from HRP catalyzed reactions, whereas excessive sizing lowered enzyme efficiency. Presumably excessively hydrophobic surfaces denatured the adsorbed antibody.

Finally, a variation of physical immobilization is Decher's [118] layer-by-layer assembly, where surfaces are consecutively exposed to oppositely charged polymer solutions, followed by washing to give multilayer adsorbed structures. The driving force for sorption is usually electrostatic, however, hydrogen bonding and other interactions can drive assembly [119]. Layer-by-layer assembly can be used to embed particles, viruses [120] or cells onto surfaces. Lvov's group has demonstrated that layer-by-layer assembly can be used to fix enzymes onto cellulose surfaces while maintaining enzyme activity [121]. As mentioned in a previous section, layer-by-layer assembly has also been used to deposit large quantities of antimicrobial polymers on surfaces [50]

In summary, proteins, phages and DNA aptamers are weakly bound on pure cellulose paper. It seems that paper treated with wet-strength resin may be a good substrate for the direct immobilization of biosensors. There are two caveats. The cationic surfaces will adsorb most biomacromolecules so it may be necessary to use some kind of blocking to prevent nonspecific adsorption [122]. Common blocking chemicals include Tween 20 (a nonionic surfactant), bovine serum albumin, casein or fat free milk [123]. Second, there is no control of biosensor orientation and we might expect very active surfaces to denature protein and DNA based sensors. Thus, the general sense from the literature is that direct application is not a robust strategy because every biosensor/paper combination would have to be optimized before use.

Chemical immobilization (covalent coupling)

Bioconjugation is a large, mature field which has been summarized in an excellent text. [11] Ideally, chemical coupling reactions should achieve very

high yields in water under mild conditions with few side reactions and little denaturation of biomacromolecules. Herein we summarize some examples relevant to bioactive paper fabrication.

Pure cellulose offers few functional groups for direct bioconjugation. The backbone hydroxyl groups are too unreactive for specific reactions in water at low temperature. Low concentrations of carboxyl groups from inadvertent oxidation of the C6 hydroxyls and the oxidizing end of cellulose chains are the only available functional groups on pure cellulose. Of course, practical paper surfaces may also have hemicellulose, lignin and other extractives offering a wider range of potentially reactive centers.

The lack of reactivity means that most cellulose substrates need to be activated by reaction with a small molecule or polymer to give surface functional groups suitable for a subsequent bioconjugation reaction. For example, we oxidized regenerated cellulose to give aldehyde groups which reacted with amine groups on a DNA aptamer to form a Schiff base which was reduced to give a stable covalent bond – see Figure 7 [112]. Note, the selective oxidation of the C6 cellulose hydroxyl to the corresponding aldehyde and acid has received much recent attention in the cellulose literature [124] – this should have applications for bioconjugation.

Table 4 gives examples of the coupling of biomacromolecules to activated cellulose surfaces. The literature contains many more examples, particularly for coupling to nitrocellulose. However, I believe these methods offer little to practical bioactive paper production. Since most of these approaches involve multiple chemical steps, they are not very attractive as a route to commodity

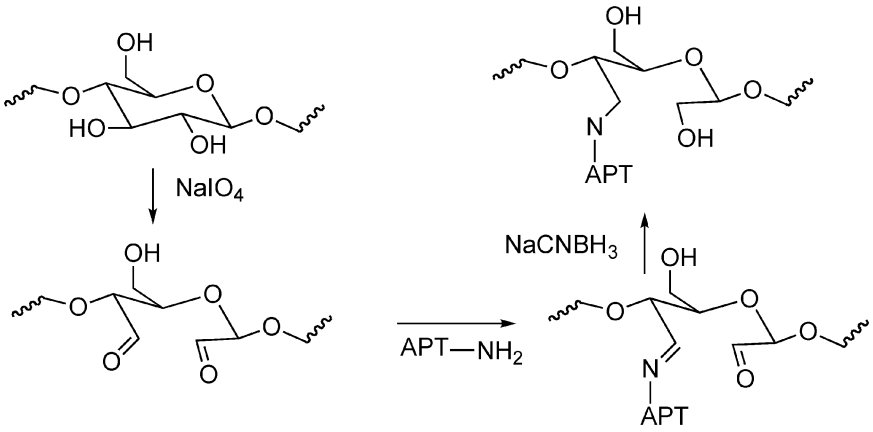


Figure 7. Oxidation of cellulose for covalent coupling to a DNA aptamer.

Table 4 Examples of conjugation to activated cellulose surfaces.

Surface Activation	*Biosensor*	*Surface*	*Reference*
Epichlorohydrin reaction to give epoxy groups	DNA for antibody removal from blood	Regenerated cellulose	[171]
Periodate oxidation to give aldehyde groups	DNA aptamers	Regenerated cellulose and MCC	[112]
1-fluoro-2-nitro-4-azidobenzene photo-activated linker	Antibodies	Regenerated cellulose	[164]
Epichlorohydrin followed by pentaethylenehexamine	Invertase	Cellulose regenerated from diacetylcellulose	[168]
1,4-diaminobenzene reaction with C6-tos	Glucose oxidase, HRP, and lactate oxidase	Regenerated cellulose	[167]
1,4-butanediol diglycidyl ether	Glucoamylase	Bacterial cellulose	[175]

paper products with inexpensive pathogen detection. On the other hand, chemical coupling could be effective in the preparation of bioactive pigments for bioactive inks – see below.

Biochemical immobilization

Genetic engineering approaches have been used to couple cellulose binding modules, CBMs, to antibodies [125] [126], enzymes, bacteriophages [90], or cells [127] which adhere spontaneously to cellulose and/or hemicellulose. For example, Cao described a fusion protein consisting of CBM bound to protein A [125]. The protein A end or the bifunctional protein, specifically binds to a wide variety of antibody fragments, whereas the CBM spontaneously binds to cellulose. In another example, Lewis and coworkers described an elegant approach in which cellulose binding modules were engineered onto llama antibodies to give a construct which spontaneously bonded to cotton, a form of nearly pure cellulose [6]. In their experiments, the llama antibody chain bound to antigen coated coacervate spheres which then spontaneously deposited onto cellulose. This is an excellent paper, which gives much information regarding the strength of CBM-cellulose adhesion.

Wang et al. recently reported the influence of PAE treatment on the activity of paper supported antibodies [128]. They evaluated two types of

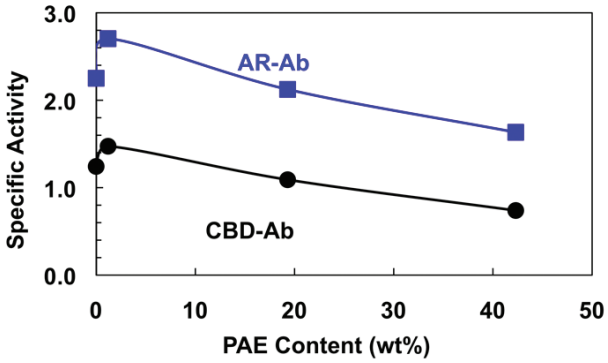


Figure 8. The influence of PAE wet-strength resin on paper supported ELISA (adapted from Wang et al [128]). AR-Ab is a conventional anti-mouse Ab immobilized by non-specific adsorption. CBD-Ab is complex structure with 5 antibodies fused to 5 cellulose binding domains.

antibodies – a construct consisting of 5 llama antibody chains fused to 5 cellulose binding domains, and a conventional anti-mouse antibody with no specific binding sites for cellulose. They reported the influence of wet-strength resin on the efficiency of the antibody immobilization and function. Using an ELISA assay (see reporting section below for an explanation of ELISA), they showed that antibody activity was only slightly decreased by high loadings of PAE – see Figure 8. Indeed, low PAE loadings typical of commercial papers actually improved the antibody activities. This is a surprising result; one would anticipate catastrophic denaturation of the antibody on the cationic polymer.

A recent publication from Ye and coworkers demonstrates the simultaneous application of three types of biological immobilization in a process used to attach TiO_2 nanoparticles to cellulose [129]. Figure 9 summarizes their approach. In the first step, cellulose was coated with a bifunctional fusion protein based on CBM and strep tag. The CBM ensured irreversible binding. The surface was exposed to streptavidin in a second step. In a final step, exposure to biotinylated TiO_2 [130] gave specific attachment of the nanoparticles to cellulose.

Finally there are reports of DNA aptamers designed to give specific binding to cellulose [131]. Presumably complex aptamers with binding and detection functions could be designed.

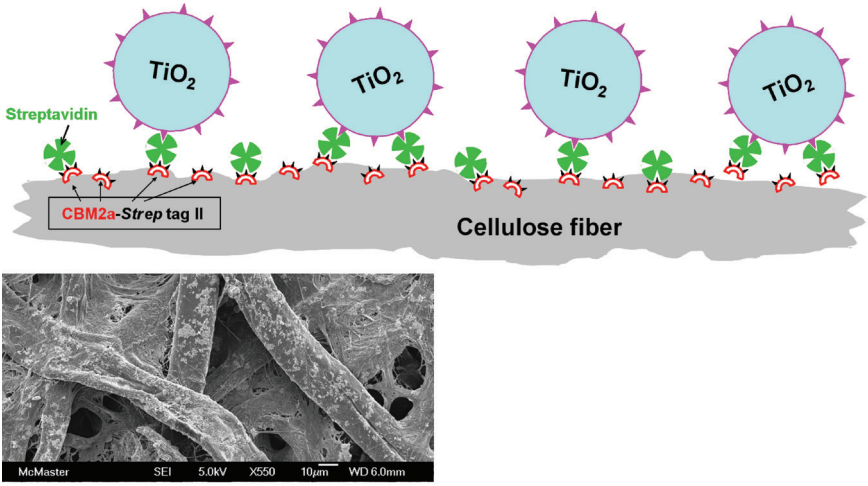


Figure 9. CBM containing protein used to attach TiO_2 to cellulose (adapted from Ye et al. [129]).

Carrier particles (bioactive pigments for bioactive inks and coatings colors)

In my opinion, a very good approach to biosensor immobilization is to covalently couple (conjugate) the biosensor to colloidal sized particles which can be then printed, coated or even added during the papermaking process. The advantages of this approach include:

- Coupling processes involving difficult, expensive and sensitive reagents can be performed in suitable bio processing facilities far from papermaking, printing, coating or converting operations.
- Compared with small, water-soluble biomacromolecules, it is easier concentrate colloidal particles onto exterior surfaces of porous papers.
- The microenvironment around the biosensor is determined by the support particle chemistry and not the paper surface. Thus, supported biosensors should be less sensitive to variations in paper substrate properties compared to small, water-soluble biosensors. Blocking and reporting functions can be built into the carrier particles.

Attaching biosensors to particles is an old subject. For example, latex agglutination assays have been commercial for decades. When dilute suspensions of latex particles, coated with antibodies, are exposed to antigen, antibody-antigen binding causes the latex dispersion to aggregate giving a visible response [132]. The early work involved polystyrene latexes, available

as monodisperse particles with clean surfaces, and which could be magnetic. Streptavidin adsorbs spontaneously and irreversibly, giving particles which will bind biotinylated biosensors [133, 134]. Pichot's group reported extensively on the preparation and characterization of a wide variety of polymer colloids as potential support particles for biosensors [135]. They concluded that colloidal microgels based on poly(N-isopropylacrylamide), PNIPAM [136], were superior because PNIPAM gives little nonspecific protein binding [137]. Following from Pichot's work, we covalently coupled DNA aptamers and antibodies to carboxylated PNIPAM microgels [138]. The conjugation chemistry is shown in Figure 10. We were surprised to observe that simply air-drying the microgels after spotting them on filter paper immobilized the gels – they did not come off or move when the dried paper was subsequently immersed in buffer or eluted with buffer. Figure 11 shows examples of elution experiments – the microgels did not move. Examples of signals given by the microgel supported sensors will be given in reporting section below.

Silica is also a convenient surface for biosensor immobilization and silica is available as either solid or porous nanoparticles. Voss et al. described the

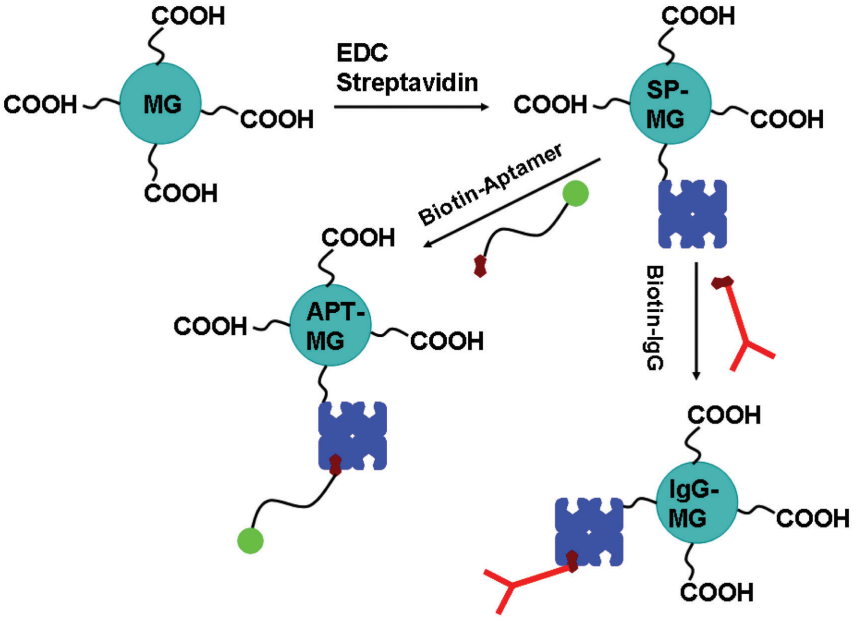


Figure 10. The coupling of antibodies or DNA aptamers to carboxylated microgels. (adapted from Su et al. [138]).

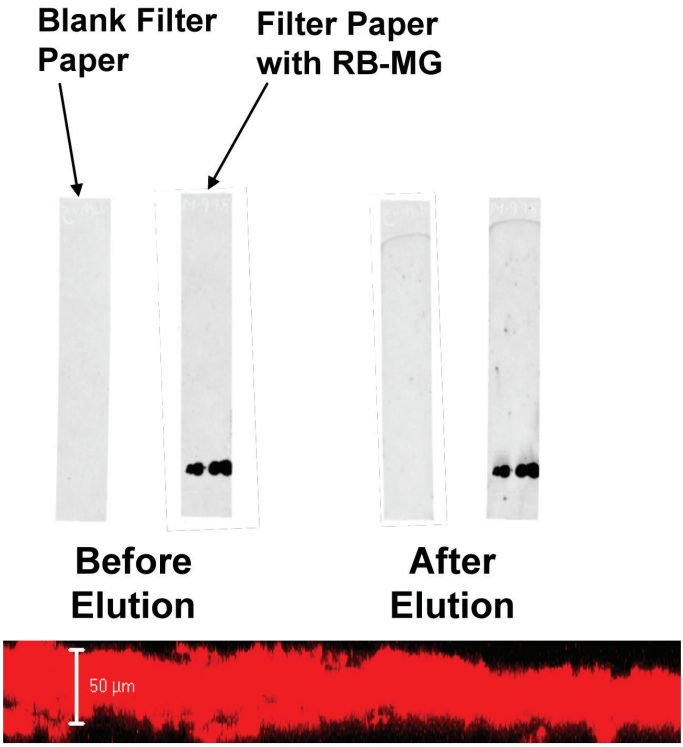


Figure 11. Chromatographic elution of filter paper spotted with fluorescently labeled microgel (adapted from Su et al. [138]).

preparation of porous silica particles with active horse radish peroxidase in the pores [139]. Thus, one can imagine support particles with the biosensor functions on the exterior surface and the reporting chemistry embedded in interior pores.

Phages are much larger than enzymes and antibodies so there may be less advantage to immobilizing phage on carrier particles. Phage have a tendency to adsorb onto silica [140] or clay [141]. Adsorption is promoted by electrostatic attraction between oppositely charged surface and areas on the phage.

Microcapsules have a long history of use in paper products. Carbonless paper is one of the oldest and most famous applications. Microcapsules could be used to load paper with bioactive materials, perhaps to protect fragile components or to isolate reagents. To date there are no reports of microcapsule use in bioactive paper.

Reporting

It is relatively straightforward to attach a biosensor, such as an antibody, to a paper surface and then capture a specific target. The challenge is to report the capture event to the human observer. Bodenhamer's recent patent describes an interesting displacement assay for transparent plastic packaging which has many of the desired attributes for bioactive paper [142, 143]. Figure 12 illustrates their approach. A heat killed or facsimile antigen is immobilized in a printed pattern such as an "√". The surface is then saturated with an antibody bearing a pigment giving a √ when the excess is washed away. Upon exposure to pathogenic antigens in contacting liquor, the dyed antibodies are released from the surface in favor or forming stronger complexes with the pathogenic target. The disappearance of the √ is the reporting event.

The following paragraphs give examples of other reporting systems that either have been implemented on paper or are potentially suitable for bioactive paper. Approaches involving significant instrumentation or complex steps are not included in the summary. For more information in these areas, an excellent summary can be found in Leonard's review, which describes a range of electrochemical sensors, including surface plasmon resonance (SPR), amperometric, potentiometric, and acoustic wave sensors. [92] In most cases, antibody capture agents are coupled to the sensor surface and

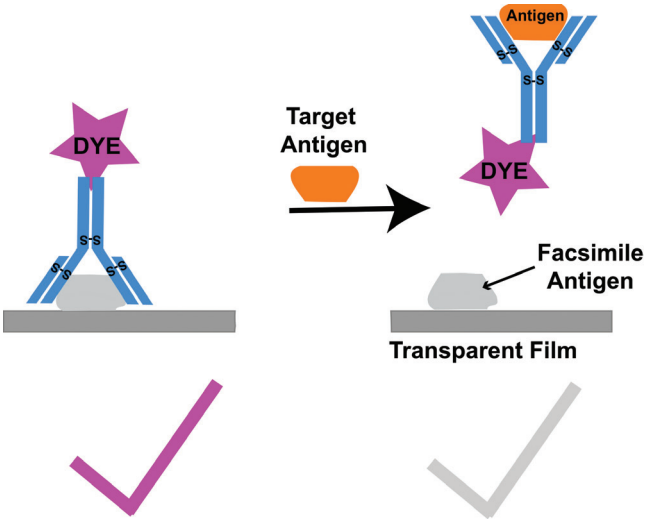


Figure 12. Bodenhamer's displacement assay for pathogen detection on transparent films [142, 143].

capture produces a signal. Although developments in printed electronics may one day facilitate the widespread application on paper substrates, the following sections will focus on strategies which may be applicable to paper.

The Enzyme-Linked Immuno Sorbent Assay (ELISA) is one the most important detection/reporting combinations. Figure 13 illustrates the main features of an ELISA assay. The biosensor (capture agent) is usually an antibody or antibody fragment which is immobilized on a support surface. Exposure of the test solution leads to antigen capture. After washing, a second antibody (secondary antibody) bearing an enzyme is introduced. Finally, the test surface is exposed to the substrate solution for the immobilized enzyme which catalyzes the production of a colored or fluorescent product. With care and some form of instrumentation for accessing color or fluorescence, the ELISA can be quantitative. To avoid high backgrounds it is often necessary to block the support surface by adsorbing a polymer, a biomacromolecules and or a surfactant to prevent the non-specific binding of the antigen or the secondary antibody. Although, it will be shown below that ELISA detection of paper works well, the multiple steps doom ELISA to "offline" applications where the exposed bioactive paper must be processed to generate a signal – not the best solution. For example, our paper-supported

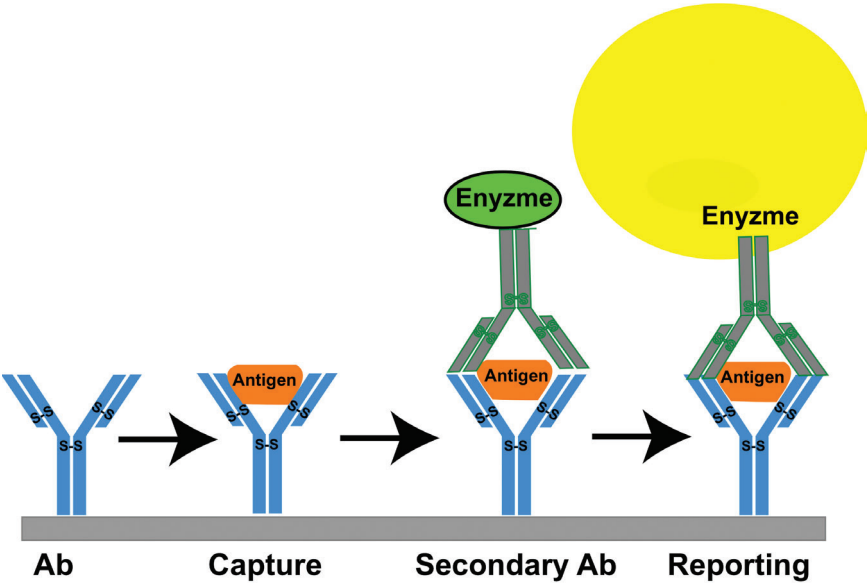


Figure 13. ELISA scheme for detection and reporting.

Table 5 A comparison of Ahlstrom commercial filter papers for an ELISA filtration assay [128]. Results are expressed as a percentage of earlier A-55 data.

Filter Media	*% of control*	*std dev*	*Filter Media*	*% of control*	*std dev*
A-55	105.1	25.5	A-111	104.9	53.0
A-75	95.9	34.3	A-121	127.5	106.3
A-95	120.8	29.7	A-141	84.7	42.0
100% cotton	88.1	29.5	A-151	124.8	53.3
A-54	270.6	35.4	A-161	48.9	38.2
A-74	115.6	81.6	A-142	82.5	28.2
A-94	301.7	124.0	A-162	26.5	14.4
A-601	89.1	33.5	A-184	15.5	6.4
A-610	84.6	20.5	A-193	–9.3	17.7
A-222	82.9	18.5	A-156	15.2	7.5
A-237	86.9	19.5	A-111	74.5	28.6
A-238	80.1	19.8	A-8964	83.7	61.3
A-631	93.8	12.1	A-8975	113.8	35.4
A-642	95.6	19.0			
A-320	122.5	28.2	100% polypropylene	19.7	31.0
A-205	70.6	24.9	100% polyester	73.8	70.3
A-319	119.5	28.4	100% rayon	83.1	17.7
			A-6613	256.9	73.7
HPZ-700	86.5	16.5	C-700	61.2	5.1
high α-cellulose wood pulp	159.6	61.6	A-501–5	60.5	8.6
A-613	99.3	34.1	C-510	52.2	11.2
A-615	27.9	10.6			
A-4002	43.3	41.0			
A-8613	30.5	20.0			
A-243	72.5	12.5			
A-204	89.2	29.8			
A-8301	114.1	29.1			
A-950	78.2	10.8			
A-901	75.1	11.2			
cellophane (100% cellulose)	17.1	1.6			
waterleaf before parchment	29.0	9.6			
vegetable parchment	13.0	5.6			

ELISA work, summarized previously (see Figure 8) involved many washing or treatment steps to generate the color after exposure to the target solution.

The effectiveness of an ELISA assay was shown to be very sensitive to nature of the paper substrate. Wang et al. compared a range of commercial papers in a filtration capture, ELISA detection assay and the results are summarized in Table 5 [128]. The signal intensity varied by more than an order of magnitude amongst the filter papers. Because the assay involved antibody immobilization, blocking, bacterial capture, secondary antibody capture and an enzyme catalyzed color development, it was difficult to determine which steps were sensitive to which paper properties.

The use of enzymes to generate a color is a common approach to reporting. Whiteside's group have published a series of papers demonstrating the use of paper to segregate a sample into different chambers where a different target is probed in each chamber using enzyme reporting [23, 144, 145]. The describe hydrophobic paper with a hydrophilic channel feed three chambers. The channels and the three chambers were created by printing a hydrophobic pattern onto filter paper. Color developing enzymes were spotted and dried onto the chambers, one for glucose detection and the other for protein. Capillary forces were used to carry the sample solution into the three chambers. Such devices could be very inexpensive and suitable for both point-of-care and developing world applications. Whiteside's work and that of others [146] emphasize an important feature of paper – the ability to generate complex "macro" fluidic devices useful for sample conditioning, separation and transport prior to the actual pathogen detection step.

Fluorescence based reporting is the workhorse of the modern bioanalytical laboratory. When used with suitable instrumentation, fluorescence is very sensitive. From a bioactive paper perspective, fluorescence reporting is a challenge for two reasons. First, to avoid using instruments, high fluorofors concentrations generating visible output are required. Second, many commercial papers fluoresce, giving a high background. It is common practice to include fluorescent agents in papermaking to increase the appearance of whiteness.

Li's group have developed DNA aptamers with built in fluorescent reporters [147]. Figure 14 shows an example. Initially the DNA aptamer is present as a duplex with a short DNA molecule labeled with a quencher (Q). The aptamer is also made to form a second duplex with another short DNA molecule labeled with a fluorescent group. Thus the fluorescence of the aptamer in the unbound state is quenched. When the target, ATP in this case, is introduced, the duplex dissociates in favor of aptamer-ATP complex

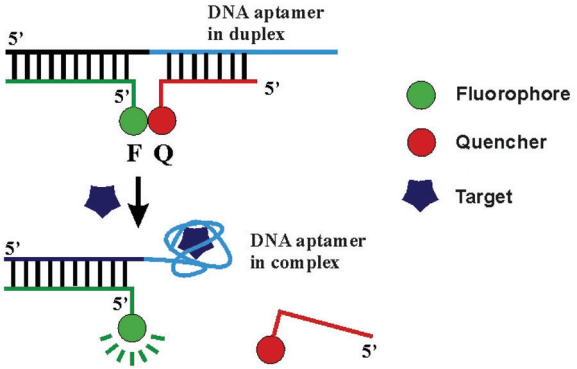


Figure 14. DNA aptamer with built-in fluorescent reporting (adapted from Nutiu and Li [147]).

formation, which does fluoresce. Su et al have shown that Li's aptamers can be immobilized on microgels which can be ink jet printed onto paper, giving an aptamer based sensor-reported combination [138].

Nanoparticle reporters show some promise. Gold nanoparticles in size range 10–50 nm have an intense red color when the particles are separated by a distance equivalent to a few particle diameters. The color changes to purple

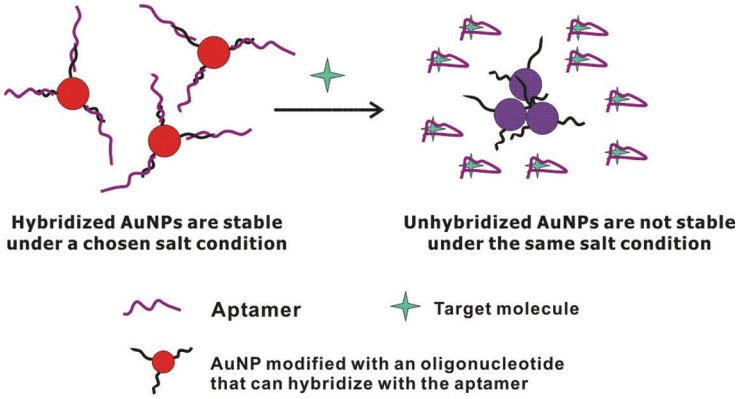


Figure 15. Gold nanoparticle reporting with a DNA aptamer biosensor (adapted from Zhao et al. 2007).

when aggregation brings the particles closer together. In a typical sensing application, the particles are coated antibodies or aptamers [148] and a color change is observed when the target antigen induces aggregation of the gold particles. This is an attractive approach because the colors are very intense, typically nanogold extinction coefficients are more than a thousand times greater than organic dyes [149]. In addition, the ability of thiols to chemisorb onto gold provides a simple route to immobilization. Figure 15 shows a schematic representation of DNA aptamer biosensor employing a nanogold sensor [149].

Most publications describe nanogold sensors, which are dispersions in buffers. For gold to be useful on bioactive paper, the nanoparticles must function after drying and subsequent wetting of the sensor. Zhao et al. recently describe a paper-supported gold biosensor capable of detecting the presence of DNase 1, an endonuclease, and adenosine, a small biomolecule [150]. For both targets, the biosensor functions by causing the dissociation of a gold nanoparticle aggregate to give an intense red color – see Figure 16. The DNase I sensor functions by degrading the DNA chains bridging the nanoparticles, whereas with the adenosine sensor, the gold particles are weakly aggregated with an adenosine aptamer. The presence of adenosine strips the aptamer from the gold, causing the particles to disperse. This is an important publication because it demonstrates that a sensitive biosensor can be dried on paper, heated, and stored while retaining activity upon subsequent exposure to the target solution. From a paper science perspective, Zhao's work is important because it shows that the details of the paper substrate are important. Papers coated with hydrophilic or hydrophobic polymers were suitable for the nanoparticle assay, whereas untreated filter paper was not because capillary forces caused the spotted nanoparticles to bleed over too great an area.

Although our work on the development of a bioactive paper pathogen sensor has focused on instrument-free analysis, the use of cell phone cameras to capture images for analysis, on site or off site, is viable in most parts of the world due to the expansion of mobile phone networks [1, 145]. One can image quantitative analysis of inexpensive spot arrays such as those shown in Zhao's work-Figure 16.

Quantum dots are semiconductor nanoparticles and are intensely fluorescent, offering many advantages for biosensing [151]. One example, taken from a patent application, is shown in Figure 17 [152]. The biosensor consists of quantum dots with immobilized antibodies on the surface, which, in turn, are weakly bound to a surrogate facsimile antigen bearing a fluorescent quenching molecule. The quencher prevents the fluorescent emissions from the quantum dot. Reporting occurs when the target antigen displaces the

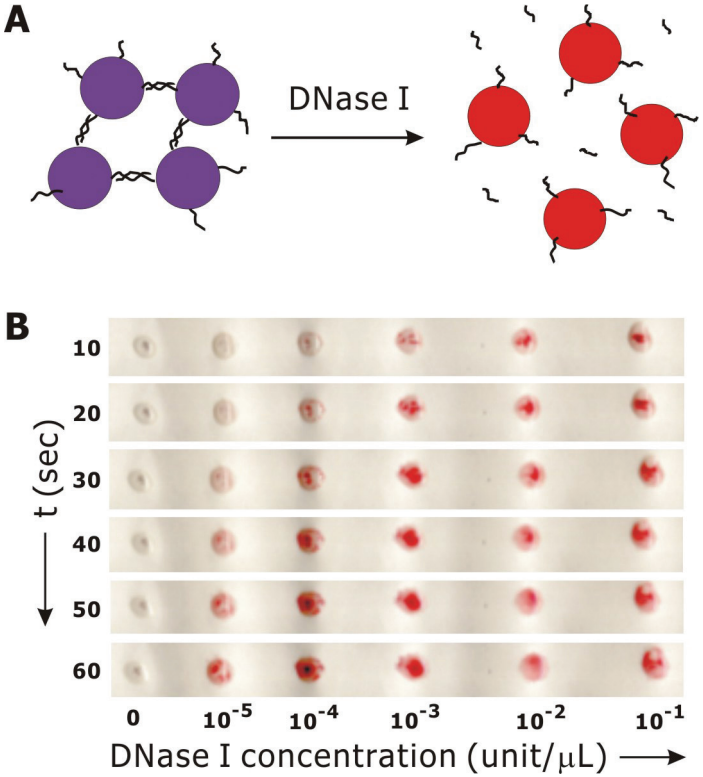


Figure 16. A paper supported sensor measuring the presence of DNase (adapted from Zhao et al. [150]).

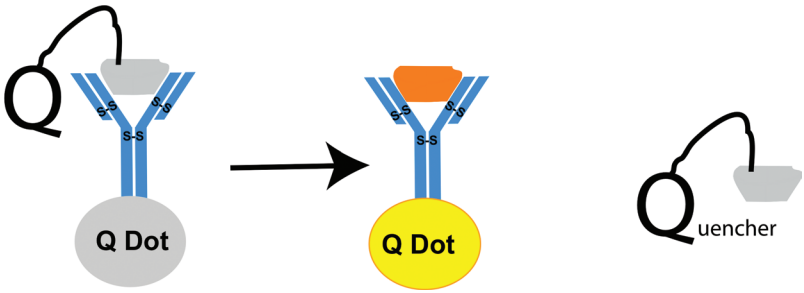


Figure 17. Antigen displaces facsimile antibody-quencher from quantum dot producing fluorescence [152].

surrogate, separating the quencher from the quantum dot, which is now free to fluoresce.

Whenever I am suspicious of meat which has lived too long in my refrigerator, I smell it before I eat it. Our olfactory senses are quite sensitive (not compared to my dog, of course) and offer a potential reporting mechanism which could be particularly helpful for face masks and other air borne applications. Arguably one of the most important sensing applications has been the doping of domestic natural gas with chemical which permit us to smell gas leaks. A recent patent application by Nicklin et al describes biosensors that are based on single living cells which report by emitting a smell [96]. It might be a challenge to maintain cell viability on dry paper. In spite of their appeal, there is very little other work describing olfactory (smell) based reporting. By contrast, there have been many reports describing chemical-electronic noses [153, 154].

In summary, there are promising approaches which could lead to bioactive paper which can detect pathogens. Although, it is early days, it seems that bioactive paper fabrication and biomolecule stability are not major problems. Immediate, sensitive detection and reporting without instrumentation or a laboratory environment remain a significant challenge.

OTHER USES FOR BIOACTIVE PAPER

Paper is an amazing, versatile material which impacts virtually every human activity. The increased functionality of bioactive paper will lead to new applications. The following sections briefly mention obvious applications, whereas I suspect completely unanticipated uses for bioactive paper will arise in the future.

Separations

Paper and nonwovens are widely used as filtration media. Filtration is a mature technology which is widely practiced and is well understood. What could bioactive paper offer filtration technology? Two obvious answers are pathogen detection and disinfection. The direct visualization of dangerous microbes on a filter have obvious applications. Similarly, having filters which self sterilize before they are handled could offer some advantage in very dangerous environments. Detection and disinfection were summarized above, including examples involving filters.

Bioseparation is another important opportunity for bioactive paper. One of the major challenges of the genetic engineering revolution is the efficient

separation of biomacromolecules from complex mixtures. One of the workhorses of biotechnology is affinity chromatography, where specific interactions such as antibody-antigen binding are used to separate specific targets during chromatographic elution. For example, Hayne's group has described the use of CBM-cellulose interactions to separate target biomacromolecules on cellulose columns [12–14]. In another example, Yu et al. have shown that filter paper supported grafted polyethylene glycol specifically captured a monoclonal antibody in 1.6 M ammonium sulfate [155]. Under these electrolyte conditions, the filter is relatively hydrophobic, enabling it to capture the antibody. The captured antibody was released simply by lowering the electrolyte concentration.

Lab-on-a-chip, inkjet devices etc. have spawned much research in the area of microfluidics. By analogy, we are just beginning see publications describing what might be called "paperfluidics" where hydrophilic channels in a hydrophobic paper can guide and separate liquid samples – e.g. see [23, 144–146]. I anticipate many more examples of paperfluidic devices in the future, including chemical logic gates.

Finally, bioactive paper may have future applications as tissue scaffolds. Both bacterial cellulose [156] and electrospun regenerated cellulose [157] have shown promise as substrates for promoting cell growth. Why not bioactive paper?

THE FUTURE OF BIOACTIVE PAPER

Commercialization issues are not normally addressed in scientific summaries, however, bioactive paper is a new field and it is important for those planning new research in this area to appreciate the barriers and recognize the opportunities. In my opinion, the development of useful bioactive paper products remains the biggest challenge. Specifically, demonstrating sensitivity, selectivity, and stability of biosensing paper remains an unanswered challenge, although I am very optimistic. There are other challenges.

The scale of the paper industry and the global production of paper products is enormous. The widespread implementation of bioactive paper will require very large quantities of inexpensive biological reagents. A glance at a biochemical catalog or website, where researchers purchase their supplies, reveals that routine materials such as standard antibodies or biotin derivatives are very costly for milligrams of material. Biotechnology has proven that if the need is there, the economies of scale take effect, dramatically lowering costs. For example, the use of enzymes in laundry detergents and as

aids for papermaking is routine because large scale bioprocessing has lowered enzyme costs. Similar processes will have to be developed before engineered proteins or DNA aptamers find commercial, large scale application.

Regulatory issues are also a barrier. Consumer products bearing biological components will require approval, particularly if food and water contact are involved. Many of the biosensors under development are protein-based, giving the potential for adverse allergenic responses. As we get closer to actual products, work will be required to prove both efficacy and safety.

All review articles are obsolete the day after they are published. Paper science and technology are mature research areas in which real change is slow. In contrast, biotechnology continues at frantic pace of development. Very aggressive research efforts underway in Canada, Scandinavia, the United States, Japan and Australia are, in my opinion, likely to spawn second-generation bioactive paper products which include pathogen detection.

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Transcription of Discussion

BIOACTIVE PAPER – A PAPER SCIENCE PERSPECTIVE

Robert Pelton

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Gil Garnier Australian Pulp and Paper Institute

Great review, Bob, I have one question. You presented paper as an inert medium: is it possible to develop some feature, some functionality, by taking advantage of the porosity, the hydro/hygro properties, and the structure?

Bob Pelton

Yes, I think the whole microphylitics area, which I do say something about, is an area where you are exploiting the porous nature of paper, obviously from which filtration is derived. I think that is where the paper industry can really be part of the value chain: when it starts building functionality inside the paper structure. That is not something you can do easily just with the printing operation.

Joel Pawlak North Carolina State University

When you talk about detecting bacteria – for example *E. coli* where there are certain *E. coli* that are harmless and other ones that are highly virulent – is there any work done on being able to distinguish these bacteria and their degree of virulence when they are detected?

Bob Pelton

The short answer is yes and, to be useful, any bacteria detection system has to be very specific. You need monoclonal antibodies for a specific bacteria because there are lots of benevolent bacteria around. This is a problem when

the bacteria are evolving very quickly, because it takes time to generate these monoclonal antibodies. So, there is a time step for generating these things, but certainly all the detection systems we're working on, are specifically targeted. Otherwise, I do not think they are a whole lot of use.

Bill Sampson University of Manchester

Just a comment, Bob. We have actually grown mouse cells on handsheets. We modified the surface with various amino acids. The work is preliminary, but it works to an extent.

Bob Pelton

I think more will people do such things; I am sure they will.