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TRENDS

Biomolecule immobilization techniques for bioactive paper fabrication

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Abstract Research into paper-based sensors or functional materials that can perform analytical functions with active recognition capabilities is rapidly expanding, and significant research effort has been made into the design and fabrication of bioactive paper at the biosensor level to detect potential health hazards. A key step in the fabrication of bioactive paper is the design of the experimental and operational procedures for the immobilization of biomolecules such as antibodies, enzymes, phages, cells, proteins, synthetic polymers and DNA aptamers on a suitably prepared paper membrane. The immobilization methods are concisely categorized into physical absorption, bioactive ink entrapment, bioaffinity attachment and covalent chemical bonding immobilization. Each method has individual immobilization characteristics. Although every biomolecule–paper combination has to be optimized before use, the bioactive ink entrapment method is the most commonly used approach owing to its general applicability and biocompatibility. Currently, there are four common applications of bioactive paper: (1) paper-based bioassay or paper-based analytical devices for sample conditioning; (2) counterfeiting and countertempering in the packaging and construction industries; (3) pathogen detection for food and water quality monitoring; and (4) deactivation of pathogenic bacteria using antimicrobial paper. This article reviews and compares the different biomolecule immobilization techniques and discusses current trends. Current, emerging and future applications of bioactive paper are also discussed.

Keywords Bioactive paper · Biomolecule immobilization · Paper-based analytical devices · Antimicrobial paper · Pathogen detection · Biosensor

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Introduction

This article discusses a research field called "bioactive paper", which targets new, simple, portable, disposable and inexpensive specialty paper products, aiming to improve the quality of life worldwide, especially in remote settings such as less industrialized countries. The research is multidisciplinary in nature and lies at the interface of analytical chemistry, paper chemistry, nanobiotechnology and biochemistry. The historical development of bioactive paper started when Martin and Syne invented paper chromatography and were awarded the Nobel Prize in Chemistry in 1952 [1]. The first paper-based bioassay was introduced in 1957 for the identification of glucose in urine [2]. In 1982, immunorecognition [3] was first introduced to dipsticks and commercially available paper strip tests for point-of-care diagnostics, including diabetes and pregnancy tests and the detection of biomarkers of pathogens and infectious disease [4].

One logical question is why bioactive paper instead of bioactive plastic is used. Plastic materials have replaced paper products for some food packaging, grocery bags and general packaging applications. However, paper offers unique advantages over plastic, including the following:

1. Paper is made from the naturally abundant material cellulose, and is biodegradable. In addition, lab-on-

paper is more energy efficient from the sustainable development standpoint because of the automatic capillary absorption of the fibre.
2. Cellulose is more protein and biomolecule friendly as most of biology is wet.
3. Paper itself has separation and "pumping" functions because the porous structure facilitates lateral-flow assays, and both simple paper strip tests and more complex paper-based microfluidic devices require no external power sources or require external power sources of low power.
4. Paper-based diagnostic devices are easy to use, inexpensive, low volume, easily adaptable and capable of rapid on-site detection. Paper-based diagnostic kits require a relatively small investment and can reach the market easily.

Bioactive paper research is currently dominated by three major research centres. The Sentinel Bioactive Paper Network of Canada was formed in 2005 [5]. The cornerstone of the Sentinel vision is bioactive paper that will detect, capture and deactivate water and airborne pathogens and high-speed manufacturing using inexpensive coating and printing technologies. The VTT Technical Research Centre of Finland [6] has a bioactive paper project aimed at developing a method of using printing technology to produce printed bioactive paper test strips. Whiteside's group [7] at Harvard University is also conducting research towards the development of paper-based diagnostic tools. Several smaller efforts are currently proceeding in Sweden [8] on antimicrobial activity of polyelectrolyte multilayer-treated cellulose films, in Australia [9] on paper-based microfluidic devices and in Japan [10] on ink-jet-printed microfluidic multianalyte chemical sensing paper.

The structure and surface chemistry of paper influence the immobilization of biomolecules on the paper membrane. The structure of paper influences the maximum quantity of biosensors that can be attached to cellulose. For bioanalytical applications, the surface chemistry of paper must facilitate biosensor immobilization, minimize non-specific adsorption and be compatible with reporting strategies. A summary of the structure and surface chemistry of paper related to biomolecule immobilization is given in Fig. 1.

Biomolecule immobilization on paper

The components of paper can be tailored to meet certain required quality demands. Paper can be functionalized at different levels of the manufacturing process. Functional chemicals can be added to the paper during and after the web formation, and functional components can be added to the finished paper product during the paper-making conversion processes or can be printed on the surface of the paper. This review focuses on the strategy and procedure for immobilization of biosensing molecules (such as antibodies, enzymes, phages, cells, proteins, synthetic polymers and DNA aptamers) on the finished paper.

Physical adsorption

Physical adsorption is a simple method for coating surfaces. It takes advantage of non-covalent interactions such as van der Waals forces, hydrogen bonding, and hydrophobic interactions. Molecules attached through adsorption tend to slowly leach from the surface. The adsorbed molecules form a randomly oriented, heterogeneous surface, which may lead to reduced activity, and the surface density is not always very high. The physical adsorption methods according to the biosensing molecules are summarized below:

1. *Physical adsorption of synthetic polymer on a paper membrane*. Clean cellulose is a hydrophilic, slightly anionic surface with a low, negative, surface-charge density [11]. Cationic polymers readily adsorb onto cellulose from aqueous solution, whereas anionic and non-ionic water-soluble polymers tend not to.
2. *Physical adsorption of protein on a paper membrane*. Electrostatic interaction between cationic patches on proteins and anionic cellulose is also an important driving force for protein adsorption onto paper and regenerated cellulose. Evidence of attractive interactions between tyrosine groups and cellulose has been reported [12], and these interactions may also contribute to binding. The properties of both proteins and paper substrates are sensitive to pH, ionic strength and specific ion

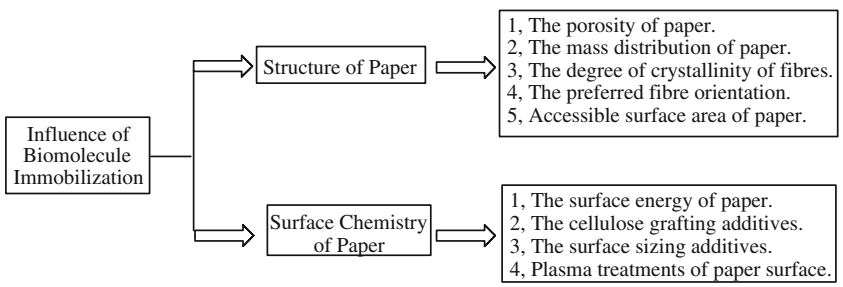

Fig. 1 The structure and surface chemistry of paper related to biosensor immobilization

effects. Halder et al. [13] reported that the ranking of proteins in terms of the number of molecules of adsorbed protein per mass of cellulose was gelatin >β-lactoglobulin>lysozyme>bovine serum albumin under one set of conditions [13]. Proteins are not strongly adsorbed onto pure cellulose, so protein-based sensors are likely to require a more aggressive immobilization strategy. Di Risio and Yan et al. [14] reported the influence of sizing on ink-jet-printed horseradish peroxidase. They showed that moderate sizing increased the colour intensity from reactions catalysed by horseradish peroxidase, whereas excessive sizing lowered enzyme efficiency. Presumably, excessively hydrophobic surfaces denatured the adsorbed antibody.

3. *Physical adsorption of DNA on a paper membrane*. High molecular weight DNA does not adsorb onto cellulose at pH 6 and pH 8, whereas adsorption is observed at pH 4. Su et al. [15] reported adsorption isotherms for adsorption of low molecular weight DNA aptamers onto microcrystalline cellulose. However, the utilization of direct DNA–aptamer application is limited because the aptamers are 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 tend not to adsorb onto cellulose. Polyamide–epichlorohydrin (PAE) wet-strength resin can influence both biosensor immobilization and activity. Cationic PAE promotes the adsorption of DNA aptamers, but bound sensors are denatured and thus inactive [16]. It seems that paper treated with wet-strength resin may be a good substrate for the direct immobilization of biosensors. A cationic surface will adsorb most biomacromolecules, so it may be necessary to use blocking chemicals to prevent non-specific adsorption. Common blocking chemicals include Tween 20, bovine serum albumin, casein and fat-free milk.
4. *Physical adsorption of antibodies on a paper membrane*. Wang et al. [17] evaluated the influence of wet-strength resins on antibody-based detection with more than 40 commercial filter media, and a few model surfaces were evaluated as potential supports for antibody detection. They found that normal loadings of reactive cationic wet-strength resins did not interfere with the antibody assays. Indeed, low PAE or polyvinylamine contents are actually helpful for antibody-based detection.
5. *Physical adsorption of phages on a paper membrane.* Tolba et al. [18] recently reported of the only direct application of phages to cellulosic surfaces. They showed that wild-type T4 phage does bind to cellulose, but the subsequent activity of the bound wild-type T4 phage is lower than that of genetically engineered T4, which binds via its head. They speculated that the wild-type T4 phage interacts with cellulose via the binding sites on the phage's long tail fibres, which are the bacterial binding sites. Protein, phages, and DNA aptamers are weakly bound on pure cellulose paper. In physical adsorption, there is no control of biosensor orientation, and highly cationic and reactive surfaces to denature protein-based and DNA-based sensors may be expected. The general sense from the literature is therefore that direct application is not a robust strategy because every biosensor–paper combination would have to be optimized before use.

Bioactive ink entrapment immobilization

Bioactive ink entrapment immobilization has been used with polymeric materials as the carrier particles. The carrier particles are key elements of the so-called bioactive ink (or bioactive pigments for bioactive inks). Compared with small, water-soluble biomacromolecules, it is easier to concentrate colloidal particles onto exterior surfaces of porous papers.

There are two methods of bioactive ink entrapment. The first method is to mix the biomolecules with the reactants before the polymerization is completed. When polymerization occurs, the biomolecules become entrapped in the polymer matrix. Studies have shown that entrapment may protect the proteins against proteolytic deactivation, enhance their thermal stability and improve their reusability [19]. However, the structure of the polymer network may reduce or completely block the diffusion of a large analyte. There might also be problems with leaching if the entrapped molecule is small, and the reactive prepolymers or the conditions during the polymerization may be harmful to the biomolecule. Chen et al. [20] entrapped lipase enzymes in organic–inorganic hybrid sol–gel polymers. By forming the gels within the pores of a non-woven polyester fabric, they obtained a novel immobilized biocatalyst in a sheet configuration. They also observed increased specific activity and thermal stability compared with a free enzyme. Ortega et al. [21] stabilized glucosidase entrapped in alginate and polyacrylamide gels against thermal and proteolytic deactivation.

The second method of bioactive ink entrapment is to covalently couple the biosensor to colloidal particles that can then be printed and coated. The coupling of the biosensor to a colloidal particle involves difficult, expensive and sensitive reagents and can be performed in suitable bioprocessing facilities far from the sites of paper-making, printing, coating or converting operations. The microenvironment around the biosensor is determined by the support-particle chemistry, not the paper surface. Thus, supported biosensors should be less

sensitive to variations in paper-substrate properties compared with small, water-soluble biosensors.

Blocking and reporting functions can be built into the carrier particles. Polystyrene latexes are available as monodispersed particles, which have clean surfaces and could be magnetic. Streptavidin adsorbs spontaneously and irreversibly, giving particles that will bind biotinylated biosensor. Pichot et al. [22] reported extensively on the preparation and the characterization of a wide variety of polymer colloids as potential support particles for biosensors. They concluded that colloidal microgels based on poly(*N*-isopropylacrylamide) (PNIPAM) were superior because PNIPAM results in little non-specific protein binding. Su et al. [16] first coupled streptavidin to a carboxylated PNIPAM microgel in the presence of *N*-ethyl-*N*-(dimethylaminopropyl)carbodiimide, then covalently coupled biotin-terminated DNA aptamers or biotin-terminated antibodies to the above-mentioned streptavidin-substituted PNIPAM microgel. They observed that simply air-drying the microgels after spotting them on filter paper could immobilize the gels. They did not come off or move when the dried paper was subsequently immersed in buffer or the contents were eluted with buffer. They also described laccase encapsulated in cross-linked polyethyleneimine and deposited on paper. Heikkinen et al. [23] prepared bioink from the heterobifunctional cross-linker 6-maleimidohexanoic acid *N*-hydroxysuccinimide, which was first reacted with 3-aminopropyltriethoxysilane to form the silane linker 6-maleimide-*N*-(3-(triethoxydilyl) propyl)hexanamide. Carboxy-terminal cysteine genetically engineered to chimeric avidin was reacted with the maleimide group of the silane linker in methanol/phosphate-buffered saline solution to form a suspension, which was printed on sol–gel-modified poly(methyl methacrylate) film.

Bioaffinity attachment

Bioaffinity attachment between certain molecules can be applied to biomolecule immobilization. The cellulose-binding domain (CBD) can be used as a suitable affinity tag and then fused with the protein to be immobilized on cellulose paper. This fusion protein is easily attached to appropriate surfaces [24], whereas the CBD spontaneously binds to cellulose. Cao et al. [25] developed a novel method for immobilizing antibodies on magnetic cellulose microspheres using a CBD–protein A linkage. The protein A end of the bifunctional protein specifically binds to a wide variety of antibody fragments, whereas the CBD spontaneously binds to cellulose. The biospecific connection between antibodies and magnetic cellulose microspheres resulted in convenient and simple preparation, elimination of toxic compounds and highly efficient antibody utilization. This can create effective affinity chromatography matrices for the separation of proteins from complex mixtures.

Lewis et al. [26] described the evaluation of llama V_{HH}–CBD fusion proteins. Fusion proteins were shown to be stable at high pH and in the presence of a detergent base. Craig et al. [27] developed a fusion protein incorporating a CBD and an antibody binding domain, protein LG, to provide an adaptor molecule for cell separation with regenerated cellulose hollow fibre arrays. CBD–protein LG efficiently captured CD34+ cells labelled with an IgG_1 antibody (HPCA-2). The development of an adaptor molecule to display recombinant domains at the surface of hollow fibres will be an effective tool to investigate cellular ligand–receptor interactions.

Minikh et al. [28] reported that wild-type T4 bacteriophages and recombinant T4 bacteriophages displaying CBD on their heads were immobilized on microcrystalline cellulose. Infectivity of the immobilized phages was investigated by monitoring the phage-mediated growth inhibition of bioluminescent *Escherichia coli* B and cell lysis using a bioluminescent ATP assay. The results showed that phage immobilization resulted in a partial loss of infectivity when compared with the free phage. Because steptavidin bind biotin with high affinity (K_d of ~10-15 mol/L) and specificity, the covalently biotinylated TiO2 nanoparticles were attached to the streptavidin-coated paper. Finally, there are reports of DNA aptamers designed to specifically bind to cellulose [29]. Complex aptamers with binding and detection functions were designed and used as modular components in the engineering of complex functional nucleic acids. The optimized aptamer exhibits robust binding of cellulose in the paper form. By fusing the aptamer to a glmS ribozyme sequence (the minimal cellulose-binding RNA aptamer), one can also graft the glmS ribozyme sequence onto other RNAs to permit the isolation of RNAs from complex biochemical mixtures via cellulose affinity chromatography, and selectively elute ribozyme cleavage products from cellulose using glucosamine 6-phosphate to activate ribozyme function.

Covalent chemical bonding immobilization

Covalent chemical bonding immobilization is the most stable and uniform immobilization on a paper membrane. To covalently link biomolecules onto paper, both the biomolecules and the paper need to contain functional chemical groups through which the immobilization occurs. The biomolecules and the paper are derivatized with a homobifunctional or heterobifunctional chemical linker that acts as a bridge between the paper and the biomolecule. Ideally, chemical coupling reactions should achieve very high yields in water under mild conditions with few side reactions and

little denaturation of biomacromolecules. Pure cellulose has 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 C-6 hydroxyls and the oxidizing end of the cellulose chains are the only available functional groups on pure cellulose. 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. Several typical activation reactions are listed below:

1. *Periodate oxidizes the surface hydroxyl of cellulose to give aldehyde groups.* Su et al. [15] reported that a cellulose membrane was oxidized to give surface aldehyde groups. The fluorescein-labelled DNA strand (fluorescein labelled at the 5′ end) of an ATP-binding aptamer was coupled via the terminal amino group at the 3′ end using a Schiff base reaction plus reduction to give a stable covalent bond (Fig. 2). The efficiency of coupling of the fluorescein-labelled DNA strand to the membrane was approximately 25%, determined by measuring the fluorescence in the supernatant before and after coupling.
2. *Epichlorohydrin reacts with the surface hydroxyl group to give epoxy groups.* Cellulose membranes were modified by treatment with epichlorohydrin to provide homogeneous epoxy functionalization. The activation level increased with the increase of NaOH concentration and the amount of epichlorohydrin used. The number of moles of epichlorohydrin must exceed that of NaOH because excess NaOH could react further with the epoxy groups in the beads, resulting in a decrease of activation level.
3. *A photoreactive cellulose membrane* [30] *was prepared by the reaction of the fluoro group of 1-fluoro-2-nitro-4-azidobenzene and the hydroxyl group of cellulose in an alkaline medium.* The photoreactive cellulose membrane thus prepared is capable of forming a covalent bond with a biomolecule in the presence of UV light of wavelength 365 nm. On exposure to UV light, the azido group of the photoreactive membrane transforms into highly reactive nitrene, which binds with a protein. The efficacy of the activated membrane was checked by immobilizing glucose oxidase on it in the presence of light.
4. *Tiller et al.* [31] *prepared a cellulose architecture with redox-chromogenic properties and anchor groups for the immobilization of biomolecules.* The cellulose architecture was prepared by the reaction of *p*-toluenesulfonic acid esters of cellulose with 1,4-phenylenediamine (PDA) in dimethyl sulfoxide solution at 100 °C. The degree of substitution of the PDA groups and the remaining *p*-toluenesulfonate groups can be adjusted by changing the number of PDA molar equivalents and the reaction time. Most PDA celluloses are soluble in dimethyl sulfoxide and *N*,*N*-dimethylacetamide and form transparent films. The suitability of the PDA units as reactive anchor groups was shown by the immobilization of enzymes such as glucose oxidase, peroxidase and lactate oxidase. Most of these approaches involve multiple chemical steps, and they are not very attractive as a route to immobilize biomolecules on paper. However, chemical coupling could be effective in the preparation of bioactive pigments for bioactive inks.

Biomolecule immobilization strategy in the mass manufacturing of bioactive paper

Currently, bioactive paper products are typically niche products, but a preliminary discussion on the biomolecule immobilization strategy in the mass manufacturing of bioactive paper is helpful for bioactive paper industry development. The methods and technologies involved in paper manufacturing and printing are mature and the interactions covering

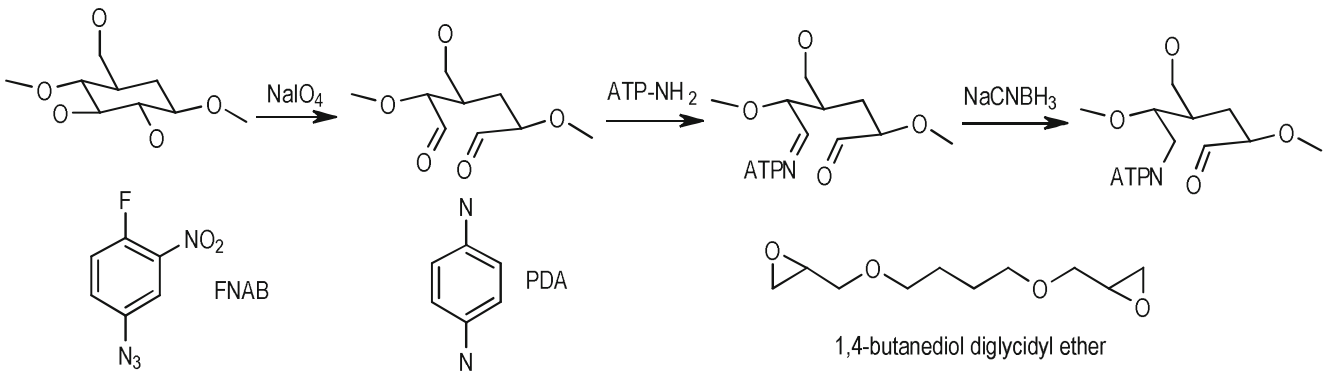

Fig. 2 Coupling a DNA aptamer to oxidized cellulose and the chemical structures of 1-fluoro-2-nitro-4-azidobenzene (*FNAB*), 1,4-phenylenediamine (*PDA*) and 1,4-butanediol diglycidyl ether

their performance are well understood. On the other hand, modern paper machines are typically designed to manufacture products in large batches and with as little variability in the product quality as possible. However, some mass-production methods, such as ink-jet printing and coating, can be used to make products with more variable properties, but this reduces the manufacturing capacity. Active components can also be embedded inside the paper. This kind of device can be used to transmit information on the changes created by bioactive substances present in the paper web. Addition of active materials by coating and/or converting onto paper includes surface sizing, spraying, coating, plasma treatment, hybrid sol–gel coatings, lacquering, laminating and dispersion coating. Printing of functional substances onto paper includes gravure printing, flexography printing, screen printing and ink-jet printing. Readers can refer to [5] for further details of these operations.

Application of biomolecules immobilized on paper

Embedded systems of bioactive paper are going to have an impact on the way we live by making them easier and safer. The most common applications of biomolecules immobilized on paper are summarized below.

Paper-based bioassay is the most direct application of bioactive paper. Li's group [32] have developed DNA aptamers with built-in fluorescent reporters. Initially, the DNA aptamer is present as a duplex with a short DNA molecule labelled with a quencher (Fig. 3). The aptamer is also made to form a second duplex with another short DNA molecule labelled with a fluorescent group, so the fluorescence of the aptamer in the unbound state is quenched. When the target, ATP in this case, is introduced, the duplex dissociates in favour of forming an aptamer–ATP complex which fluoresces. Su et al. have shown that Li's aptamers can be immobilized onto microgels that can be ink-jet printed onto paper, giving an aptamer-based sensor–reporter

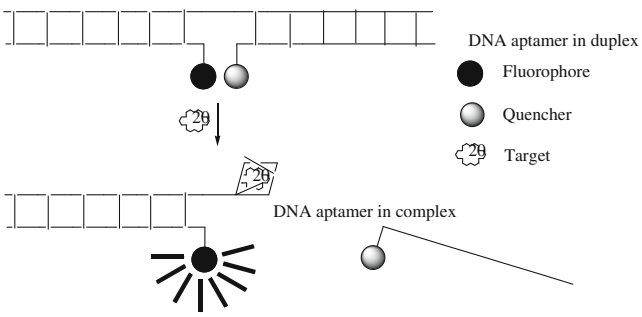

Fig. 3 DNA aptamer with built-in fluorescent reporting. In the initial duplex, the fluorescent aptamer is quenched. Upon exposure to the target, the duplex dissociates, giving a fluorescent signal

combination. Enzyme-linked immunosorbent assay on paper [17] includes the biosensor (usually an antibody or antibody fragment) which is immobilized on a paper surface. Whiteside's group [33] 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 a variety of assays, some using enzymes to generate a colour. Zhao et al. [34] recently described a paper-supported gold biosensor capable of detecting the presence of DNase I, an endonuclease.

The application of bioactive paper to the packaging and construction industries is increasingly important. A recent patent [35] described an interesting displacement assay for transparent plastic packaging, which has many of the desired attributes for bioactive paper. The UK-based company Stanelco [35] is launching a new intelligent PULSLine label that protects foods and high-value goods from counterfeiting and tampering.

Antimicrobial paper is an attractive application of bioactive paper. Dankovich and Gray [36] described a method to deactivate pathogenic bacteria by percolation through a paper sheet containing silver nanoparticles. Anany et al. [37] developed a novel method for the oriented immobilization of bacteriophages on a cellulose membrane, and these membranes were shown to effectively control the growth of *Listeria monocytogenes* and *E. coli* O157:H7 in ready-to-eat and raw meat.

Pathogen detection with bioactive paper is currently a hot research topic. The detection of pathogenic bacteria is key to the prevention and identification of problems related to health and safety. Pelton et al. [38] immobilized recombinant T4 bacteriophages on microcrystalline cellulose and strepavidin magnetic beads, the recombinant T4 bacteriophages displaying biotin-binding peptide (biotin carboxyl carrier protein) and CBD on their heads. Some researchers [39] believe that, soon, pathogen detection will undoubtedly benefit from the integration of biosensors into microdevices, although there may need to be a compromise between the time and sensitivity in its performance.

Outlook

Bioactive paper research has been attracting considerable attention for the past 10 years and is motivated by fundamental, biomedical and industrial interests. The permanent immobilization of biorecognition molecules on paper surfaces is a crucial step in the development of any sort of biosensor materials and devices. It provides the core of the biosensor and gives it its identity. Towards this end, there are a few requirements that biomolecule immobilization should meet:

1. The immobilized biomolecule needs to keep its original functionality as far as possible in order for the biosensor to work. Another common reason for biosensor failure or underperformance is chemical inactivation of the active/recognition sites during the immobilization stages. This means that care must be taken so that the recognition sites are not sterically hindered.
2. There is no universal immobilization method suitable for every application imaginable. When it comes to choosing the immobilization method, there are other important factors that need careful consideration, e.g. the type of transduction used, the nature and composition of the sample and the possibility of multiple use of the biosensor.
3. To facilitate potential large-scale application and to lower the cost, it is necessary to develop protein immobilization methods that are compatible with automated coating and/or printing methods and retain the biomolecule at the surface of the paper substrate.
4. Innovative products such as bioactive paper require multidisciplinary know-how in many areas—low-cost manufacturing methods for integration of bioactive components, printing, coating, grafting, spraying, manufacturing of nanoscale structures on large surfaces and their integration with microdevices and macrodevices, development of biocompatible materials, biomaterial immobilization into and on paper and nonwovens and other substrates, modification of substrate properties, new detection methods, and integration of electronics and optics onto large-area substrates.
5. To develop a commercial technology around the bioactive paper platform such as developing multianalyte and rapid assay sensor strips with good signal generation methods, with emphasis on integrating the individual technologies with functional components and devices, and take into account usability and low-cost manufacturing of the bioactive paper.

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