Digital Fabrication of Bioactive Paper

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Abstract

Seventy Canadian graduate students, post docs and university professors have spent the last three years on a collaborative project called Sentinel – The Bioactive Paper Network. Sentinel's goal is to develop technology platforms for the production of bioactive paper which can detect and deactivate pathogens. The detection of pathogens without laboratory equipment is the major challenge. Biorecognition agents attached to paper surfaces must specifically bind to target pathogens and binding must trigger a signaling mechanism (reporting) for the human user. Ultimately, we aim to produce paper surfaces much like litmus paper where color changes indicate dangerous bacteria or virus.

Digital printing is a particularly promising approach to the application of biodetection agents to paper surfaces. This presentation overviews our efforts on the development of bioactive ink-jet inks containing biorecognition agents based antibodies, DNA aptamers and bacteriophage. Important issues include effective coupling to paper surfaces while maintaining activity after drying and storage.

Introduction

For more than a century, paper-based food packaging, facemasks and protective clothing have played an important role in protecting us from pathogens. These paper-based products reflect the fact that paper is inexpensive, disposable, sterile, and can have well defined porosity. Nevertheless, in most applications, paper functions simply as a passive barrier or filter. To expand the utility of paper in the health sector, food and biotechnology industries, a group of us have been making efforts in creating and applying 'bioactive paper" that has the capability to detect and/or deactivate biological substances such as bacteria and virus.

The widespread application of bioactive paper requires detection assays that are inexpensive, sensitive, robust, and ideally, operational without instrumentation. Most candidate assays involve specific molecular interactions such as antibody-antigen recognition, DNA aptamer-target binding, or nucleic acid hybridization. Of these, the aptamer technology has recently attracted considerable interests mostly due to the fact that aptamers can be conveniently created in test-tube evolution experiments for virtually any target of interest. In recent years, significant progresses have been made to explore aptamers as biosensors. Our on-going interest involves implementing some of the sensing strategies on conventional paper surfaces.

Paper is frequently employed in assays either as a separation medium or as a sample capture and transport media for dipstick assays. In many assays, it is necessary to fix the biodetection agent (i.e. antibody, aptamer, bacteriophage etc.) to the paper surface. Many assays involving paper employ very pure cellulosic paper for the coupling experiments. However, our vision of bioactive paper requires approaches that can work on standard paper surfaces. This requirement presents a significant challenges including: a) the need to function on a wide range of paper surface chemistries; b) the need survive dehydration and long storage times; and, c) the need for useful sensitive reporting without instrumentation.

In this paper we summarize the results of two recent studies aimed at depositing DNA aptamers onto paper by direct application, covalent coupling [1] and by ink-jet printing aptamers covalently coupled to microgels [2].

DNA aptamers are short DNA oligonucleotides that can undergo structural rearrangement when in the presence of a specific target, resulting in the capture of the target. We used employed an aptamer developed by Nutiu and Li which signals ATP binding – see Figure 1 [3].

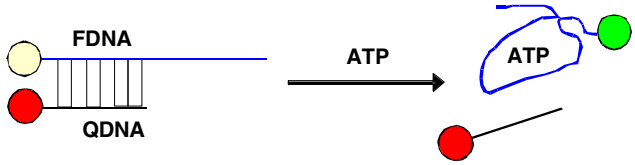

Figure 1. Principle of the ATP-binding structure-switching signaling aptamer: A duplex is formed between a fluorescently labeled DNA (FDNA) that contains a motif for ATP recognition, and a short DNA molecule that is 3' labeled with a quencher (QDNA), so that the quencher and the fluorophore are in close proximity. In the presence of ATP, the FDNA strand switches its configuration due to its high affinity for ATP, the QDNA strand leaves the duplex and a fluorescence signal can now be detected. Figure adapted from Nutiu and Li [3]

Herein we report the use of poly(N-isopropylacrylamide), PNIPAM, microgels[4, 5] as support particles for protein and oligonucleotide-based biosensors for paper-supported applications. This approach follows the pioneering work of two groups. Kawaguchi and coworkers who have studies PNIPAM microgels as support for proteins,[6, 7]and Pichot and coworker who have reported microgel-supported oligonucleotides.[8, 9] In both cases, PNIPAM is a benign support that produces minimum nonspecific binding and denaturation. Since the early work, there have been a number of reports of biomacromolecules coupled to microgels – Lyon's work using microgels as bio-detecting micro-lenses is particularly interesting.[10, 11]

PNIPAM microgels must contain reactive function groups for bioconjugation. We recently reported a novel type of PNIPAM microgel formed by copolymerization with vinylacetic acid.[12] These microgels are unique because most of the carboxyl groups are located on the end of PNIPAM chains (hairs) on the microgel surface,[13, 14] making them ideal supports for bioconjugation.

Results and Discussion

The ATP-binding DNA aptamer was mixed with microcrystalline cellulose (MCC) suspension in buffer and the adsorption isotherm was measured at two ionic strengths. The results are shown in Figure 2. The solid lines were fits to the Langmuir isotherm using the same binding constant for both data sets, suggesting that both adsorption and desorption rate constants had similar ionic strength dependencies. The maximum adsorption values were 0.105 mg/m2 for buffer A (higher ionic strength buffer) and 0.024 mg/m2 for buffer B (lower ionic strength buffer). Under these conditions the MCC was slightly negatively charged (electrophoretic mobilities of -0.29±0.19 ×10-8 m2/Vs and -0.73±0.04 ×10-8 m2/Vs in buffers A and B respectively). Thus, electrostatic interactions opposed adsorption of anionic oligonucleotide, and the amount of adsorption increased with ionic strength.

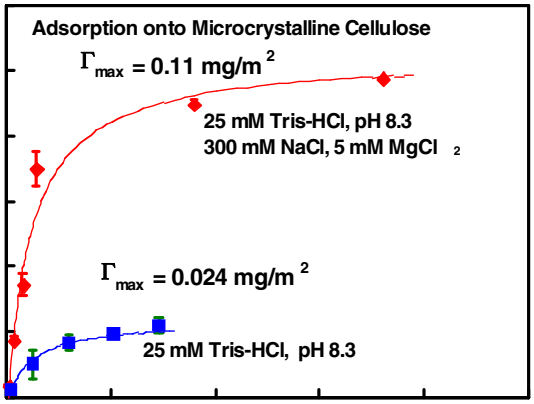

Figure 2. Adsorption isotherms of an ATP-binding DNA aptamer onto microcrystalline cellulose. Both curves were calculated from the Langmuir equation using a binding constant of 0.42.

The strength of the aptamer-cellulose interaction was probed by washing experiments. Aptamer-treated MCC was washed 5 times (0.3 ml each) with buffer using a centrifuge. Fluorescent analysis of the supernatant confirmed that the bound aptamer was completely removed. We also performed confocal fluorescence microscopic imaging to obtain visual confirmation of the removal of the ATP-binding aptamer from the surface of the MCC powder during washing. All the fluorescence initially present in the sample was lost after washing. **Physical adsorption is too weak to be a useful strategy for attaching short oligonucleotides to cellulos**e.

Covalent coupling was evaluated as an alternative. Cellulose membrane (dialysis tubing) was oxidized to give surface aldehyde groups. The FDNA strand (fluorescein labeled at the 5'-end) of an ATP-binding aptamer was coupled via the terminal amino group at 3' using a Schiff Base reaction plus reduction. The coupling efficiency of the FDNA strand to the membrane was found to be approximately 25%, determined by measuring the fluorescence in the supernatant before and after coupling. The coupled aptamer was effective and could not be washed off.

Thus physical adsorption is not a suitable strategy for imparting biological specificity to cellulose surfaces using aptamers. On the other hand, we demonstrated that the activity and specificity of an ATP-binding structure-switching signaling aptamer is maintained after covalently coupling it to the cellulose surface with no spacers. Apparently the amorphous hydrophilic nature of regenerated cellulose is particularly suited for maintaining DNA aptamer activity. Preliminary work with paper surfaces has shown that aptamer activity can be maintained after drying when the paper is re-wetted with buffer. However, we do not believe that chemically coupling to directly to paper is practical because of the range of paper surface chemistries and because the papermaking/printing/coating processes are not suitable for these reactions. Instead, we proposed making bioactive ink based on aptamers coupled to support particles.

Streptavidin was coupled to the PNIPAM-VAA carboxylated microgels which were decorated with either antibodies or DNA aptamers – the overall scheme is shown in Figure 3. Streptavidin-coupled microgel (SP-MG) was prepared with a content of 7.5 µg of protein per mg of dry microgel (i.e. 0.75wt%). Assuming all of the streptavidin is located on the exterior surfaces of the microgels and that the water content is 45% (low swelling conditions), 7.5 µg of protein per mg of dry microgel corresponds to a coverage of ~0.2 mg/m2, which is an order of magnitude less than the 5 mg/m2 value that Caldwell reported for streptavidin physical adsorption onto polystyrene latex. The electrophoresis results shows that streptavidin modification rendered the microgels slightly positive with an isoelectric point of ~ 8 which is high compared to the isoelectric point of streptavidin which is 6-6.5. It is possible that side reactions from the EDC coupling agent introduced some additional cationic groups.

Our goal was to demonstrate the activity of the APT-MG on paper surfaces. For this 1 µl aliquots (6.5 mg/ml) of microgel (quenched with QDNA in advance) were printed as a band on filter paper strips with a Fuji-Dimatix Materials inkjet printer giving coverage of approximately 3.25×102 mg dry microgel per square meter of paper. After room temperature drying, the paper strips were eluted with either ATP or GTP in binding buffer and the strips were scanned. Figure 4 shows images of the strips after elution with ATP or GTP in binding buffer, followed by room temperature drying. The fluorescing microgels appear as black bands in these monochrome images.

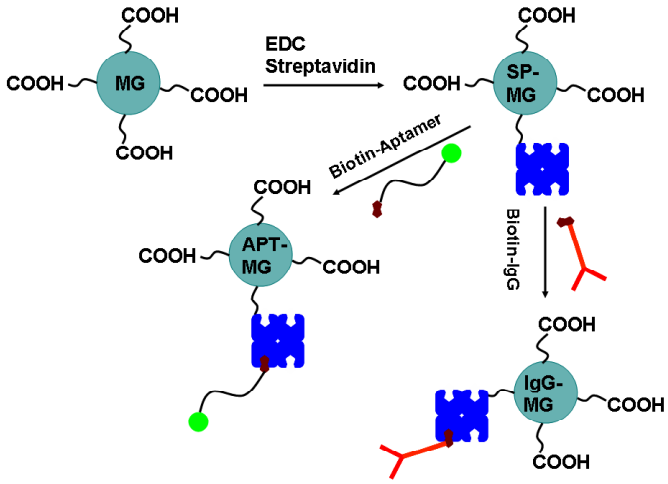

Figure 3 Schematic illustration of PNIPAM-VAA microgel derivatization

The microgels exposed to ATP gave greater fluorescence than the GTP control. This result shows not only that the APT-MG is active on the paper surface, but also that ATP infiltrates the microgels during the elution. Obviously there is much scope for improving the discrimination between ATP and GTP, nevertheless, these results are an important proof of concept.

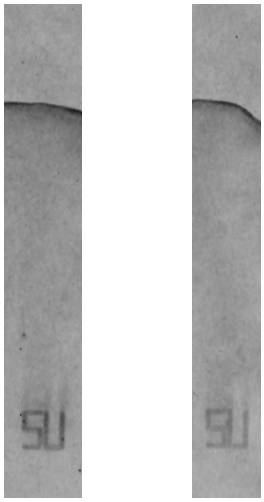

Figure 4 Ink-jet printed microgel-ATP aptamer after elution with ATP or GTP (the control).

Conclusions

In this work, we have shown that microgels, large enough to isolate the biosensors from the paper surface, are sufficiently hydrophilic to be wetted during chromatographic elution, exposing the gel-supported biosensors to their targets. Although the assays in Figures 4 do not display spectacular sensitivity, they are an important first demonstration of microgel-supported antibodies and DNA aptamers on paper surfaces.

The main conclusions from this work are:

1. Poly(N-isopropylacrylamide-co-vinyl acetic acid) microgels can be immobilized in paper due to mechanical entrapment. A key aspect for successful immobilization is to allow the applied microgel to dry before re-wetting the paper, so that capillary forces during drying force the microgel particles to penetrate the fiber network.
2. Antibodies and DNA aptamers retained their recognition activities when coupled to carboxylated poly(N-isopropylacrylamide) microgels.
3. The functionalized microgels retain activity when dried onto paper surfaces.
4. The colloidal-sized microgels protect DNA-aptamers from denaturation on cationic paper surfaces.
5. The microgel-supported aptamers can be applied to paper surfaces by ink-jet printing.

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