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A zero-step functionalization on paper-based biosensing platform for covalent biomolecule immobilization

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ABSTRACT

As a potential platform for point-of-care clinical analyses and environment monitoring, paper biosensors have received considerable attention. In many cases, the conjugation of biomolecules onto paper surface is crucial for increasing the functionalities of paper-based bioanalytical devices. Until now, it is sometimes argued as in the literature that finding a surface chemistry for biomolecule covalent grafting to paper still remains a challenge. Here the study shows that at least to a certain extent some aspects of the argument involved is questionable, by demonstrating that paper without any modification could be utilized for the covalent conjugation of enzymes and serves as a tool for bioanalysis. Moreover, the detailed analysis of biomolecule immobilization strategies on paper through polysaccharide-coating chemistry has been offered as a contrast. We believe that the proposed method could provide a valuable perspective for paper-based biosensors.

1. Introduction

Biosensors as a diverse collection of bioanalytical devices for detecting and quantifying biomolecules, have been widely used in many areas, such as clinical and home-based diagnoses and environmental monitoring [1–3]. Typically, a biosensor consists of three main parts: a bio-recognition component, a signal transducing component and an output system. Among them, the bio-recognition component is responsible for the identification of the presence and quantification of analytes via specific interactions with analytes by using specific biological elements like proteins, nucleic acids and tissues [4,5]. Thus effective immobilization of these biological elements onto solid surfaces is an important step in biosensor fabrication. Considerable efforts have been devoted to endowing the supporting material surfaces with bio-recognition ability by attaching biomolecules covalently [6,7].

Nowadays, novel paper-based biosensors have emerged with potential as easy-to-use, rapid and inexpensive point-of-care devices [8,9]. Compared with commonly used substrate materials for biosensors, its advantages include low production cost, intrinsic water wicking ability and feasibility of patterning by printing technology [10,11]. So far, in paper-based sensor and paper-based ELISA plate design and fabrication the most used techniques for immobilizing biomolecules are based on physical adsorption. However, physical adsorption of biomolecules has an unavoidable weakness that it could not always promise reproducible results because biomolecules are weakly bound to paper fibers and could be easily washed off [12]. In order to significantly improve the performance of paper-based devices for quantitative bioanalysis, effective and chemically reliable methods for immobilizing a broad range of biomolecules on paper sensors, mostly immobilization via covalent bonding, need to be explored.

To date, a variety of surface chemistries have been proposed to facilitate the covalent immobilization of biomolecules onto paper, such as divinyl sulfone chemistry, diazonium chemistry or polymer chemistry [12–14]. Among them, long-chain and flexible polysaccharides with multiple functional groups have exhibited the potential to act as surface modifiers for paper sensor fabrication. In particular, Orelma et al. used the Langmuir–Schaeffer cellulose film as a model to investigate polysaccharide adsorption for biomolecule conjugation, which could give instructions to paper modification via polysaccharide coating technique [15,16]. However, to the best of our knowledge, the detailed study of using these polysaccharides for practical paper surface modification has not been assessed. Recently, Wang et al. reported the application of chitosan as the surface modifier to fabricate paper plates for paper-based ELISA; their work has demonstrated, partly, the potential of polysaccharide coating on paper [17].

Here comes the question, why bother doing paper functionalization? The reason is that paper is typically composed of cellulose fibers and hydroxyl groups in glucose are responsible for cellulose chemical activity

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[9]. Most published work took it for granted that hydroxyl groups need to be transformed to other functional groups to enable covalent immobilization of biomolecules by common bioconjugate techniques. However, it is neglected that paper have low concentrations of carboxyl groups, which could potentially facilitate the covalent immobilization of biomolecules and probably make paper ready for binding biomolecules covalently [9,18]. In other words, people may be distracted by the familiarity with cellulose and its derivatives; the functionalization strategies for paper they are looking for may not necessary.

In this study, unmodified cellulose fiber-based paper was investigated for covalent immobilization. An enzyme, alkaline phosphatase (AP) used as a model protein, was immobilized onto paper substrates. In a comparison study, two kinds of representative polysaccharides, chitosan and sodium alginate (NaAlg), were utilized for paper modification. They were deposited as surface modifiers on paper surfaces. The effects of these modification methods were evaluated with a colorimetric reaction of substrates corresponding to AP. To the best of our knowledge, the direct use of original paper for covalent linking of biomolecules has not been previously reported, that is, the surface activity of the residual carboxyl groups for immobilization has never been studied. Our results show that, despite of the low concentration, the residual carboxyl groups on unmodified paper can still provide a considerable capacity for AP immobilization. Furthermore, we also described the construction of a simple immunoassay on differently modified paper substrates. We hope that our results could make a contribution to future fabrication of paper-based bioanalytical devices.

2. Experimental

2.1. Materials and instruments

Whatman qualitative filter paper #1, cut into 10 mm × 10 mm pieces, was chosen as paper substrate throughput the work. All reagents and proteins used in this study were purchased from Sigma–Aldrich. Glutaraldehyde, *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were used for chemically crosslinking. Medium molecular weight chitosan (200–800 cP, 1% in 1% acetic acid) and medium molecular weight sodium alginate (⩾2000 cP, 2% in water) were chosen for the comparison study. 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) was used in conjunction with nitroblue tetrazolium (NBT) for the colorimetric detection of AP activity. Buffers and solutions, unless specially noted, were prepared with ultrapure water (18.2 MΩ cm, Mill-Q Gradient System, Millipore, USA).

The digital colorimetric images of the assays were generated by scanning the results using a desktop scanner (Epson Perfection 2450) set to color document scanning, 600 dpi resolution; the digital assay results were analyzed in grayscale using ImageJ. In order to correct the influence of the paper background color to assay results, background grayscale value of biomolecule modified paper without adding BCIP/NBT substrate was subtracted, unless otherwise noted. The color intensity values were obtained by taking the average of the quadruplicate readings, with the variation of values represented by error bars.

2.2. Preparation of unmodified paper with immobilized enzymes

2.2.1. Glutaraldehyde coupling

Typically, 20 μL of 3% glutaraldehyde in 0.01 M pH 7.4 phosphate buffered saline (PBS) was introduced to activate the original paper for 2 h in a wet box, and the paper was washed three times by adding 100 μL of the coupling buffer (1.5% (w/v) mannitol, 0.15% (v/v) glycerol and 0.01% (v/v) Tween 20) to the top surface of it, and then putting the bottom of the paper in contact with a piece of cotton wool to remove the excess buffer. Subsequently, 3 μL of an enzyme solution in the coupling buffer (100 μg mL^{-1} AP) was spotted onto the freshly activated paper square and incubated in the wet box for 2 h. After that, the paper was washed with 100 μL of Tris–HCl buffer (50 mM, pH 9.2, containing 0.1% (v/v) Tween 20) three times and allowed to dry for 30 min under ambient conditions. Finally, 9 μL of a colorimetric substrate solution for AP (i.e., BCIP/NBT) was added to the paper. After being incubated for 2 h, the paper square was scanned by the desktop scanner and the intensity of the color was measured using ImageJ.

2.2.2. NHS/EDC coupling

Typically, 20 μL of a mixed solution (0.1 M NHS and 0.1 M EDC) was introduced to activate the original paper in the wet box for 15 min, and then this activation step was repeated once more. Subsequently, the paper square was washed with the coupling buffer (3 × 100 μL). Afterwards, 3 μL of a 100 μg mL^{-1} AP solution in the coupling buffer was spotted onto the freshly activated paper square and incubated in the wet box for 2 h. The paper was then washed with Tris–HCl buffer (3 × 100 μL) and allowed to dry for 30 min under ambient conditions. Finally, BCIP/NBT was added to the paper; after 2 h, the image was recorded by the desktop scanner and then analyzed.

2.3. Preparation of polysaccharide-coated paper with immobilized enzymes

In a typical experiment (Fig. 1), 20 μL of chitosan solution (0.05%, w/v) in aqueous acetic acid solution or 20 μL of NaAlg solution (0.05%, w/v) was dropped onto one piece of paper. After being dried under ambient conditions overnight, polysaccharide-coated paper was ready for use. Enzyme could be covalently immobilized on glutaraldehyde activated chitosan-modified paper square or NaAlg-modified paper square activated by NHS/EDC. Based on the above-described procedures, a variety of reaction parameters like polysaccharide concentration were examined for polysaccharide coating.

2.4. Immobilized enzymes' stability assays

Different paper substrates with immobilized AP (0.05% chitosan or 0.05% NaAlg for paper coating, AP concentration of 100 μg mL^{-1} for immobilization) were stored at room temperature for various periods of time. Afterwards, the residual enzymatic activity of immobilized AP was evaluated by the color-producing enzymatic reaction as mentioned above.

2.5. Fabrication and processing of immunoassays

Rabbit IgG (3 μL, 100 μg mL^{-1}) was immobilized on different paper substrates for 2 h. After washing with 1 mL of PBST (0.1% (v/v) Tween-20 in PBS), the paper squares were immersed in a blocking buffer (5% (w/v) BSA in PBS) for 30 min, followed by washing with 1 mL of PBST to remove the free BSA. An AP-conjugated goat anti-rabbit IgG solution (3 μL, prepared by diluting the stock solution 1000 times in PBS) was added onto the test paper and allowed to incubate for 5 min. The test zones were then washed with 1 mL of PBST, and allowed to dry under ambient conditions for 30 min. Finally, 9 μL of a solution of BCIP/NBT was added to the paper, and after 30 min the results were recorded and analyzed by ImageJ.

3. Results and discussion

3.1. Demonstration of the paper-based bioassay format

In the current study, in order to simplify the experimental procedure, spotting assays were performed on paper squares. Specifically, the most widely utilized Whatman filter paper for bioassays was chosen as the substrate in our study. Then, after immobilizing a small amount of enzyme (3 μL) onto paper substrate, we would utilize 9 μL of enzyme

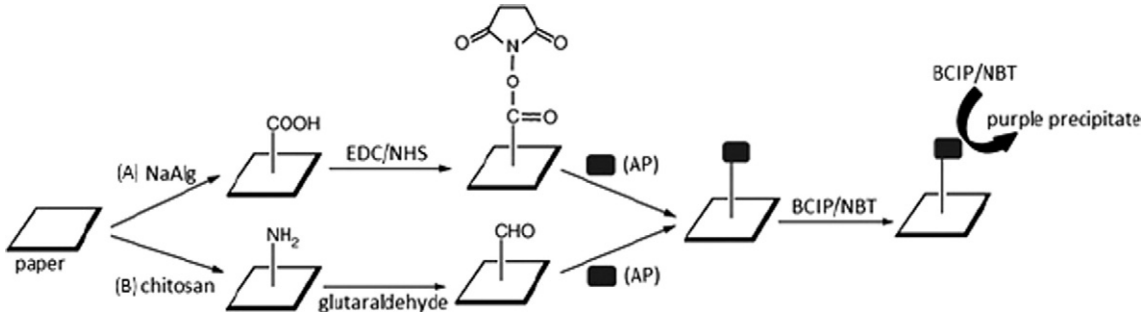

Fig. 1. Scheme of AP immobilization on polysaccharide-coated paper. (A) Coupling of AP to NaAlg-coated paper via NHS/EDC activation. (B) Coupling of AP to chitosan-coated paper via a Schiff base.

substrate to wet test paper completely for better understanding the accurate distribution of enzyme. We tested the efficiency of immobilizing proteins and evaluated the conservation of protein biological activity on different types of paper via using solutions of AP as a model protein, because AP is considered very sensitive particularly when using colorimetric detection and also less stable than other popular alternatives such as the enzyme horseradish peroxidase (HRP).

3.2. Covalent attachment of protein to unmodified paper

The amount of protein covalently immobilized on original filter paper could vary with different coupling chemistries (Fig. 2). Glutaraldehyde-mediated coupling on filter paper is not efficient for enzyme immobilization. Apparently, there are no primary amine groups on paper for covalent bonding of glutaraldehyde (Table S1, Supporting information). Because of the lack of primary amine on filter paper, any further covalent attachment of enzyme through the glutaraldehyde coupling would not be possible.

Surprisingly, NHS/EDC activation strategy displays different behavior, depending on diverse crosslinking mechanisms. The results obtained from NHS/EDC activation are exactly opposite to those from glutaraldehyde activation method (Fig. 2). Our data show successful enzyme immobilization on NHS/EDC-activated paper, which indicates the possibility that carboxyl groups on paper are sufficiently active to react with EDC and further with NHS to form the activated surface for protein immobilization. It is a known fact that paper surface is slightly negative charged, which is partly caused by the presence of carboxyl groups [9,18]. To further evaluate this possibility, we performed direct enzyme immobilization on filter paper without the NHS/EDC activation. Once eliminating the activation step, paper surface would only have a low concentration of carboxyl groups, and would not be able to acquire the more reactive NHS ester groups from the NHS/EDC coupling chemistry; we therefore expected insignificant enzyme immobilization on the unmodified paper. Colorimetric measurement of paper samples treated with direct enzyme deposition followed by Tris–HCl buffer rinsing shows insignificant color change. This result supports our reasoning that the native residual carboxyl groups on original paper surface may provide a certain level of NHS/EDC coupling for biomolecule immobilization.

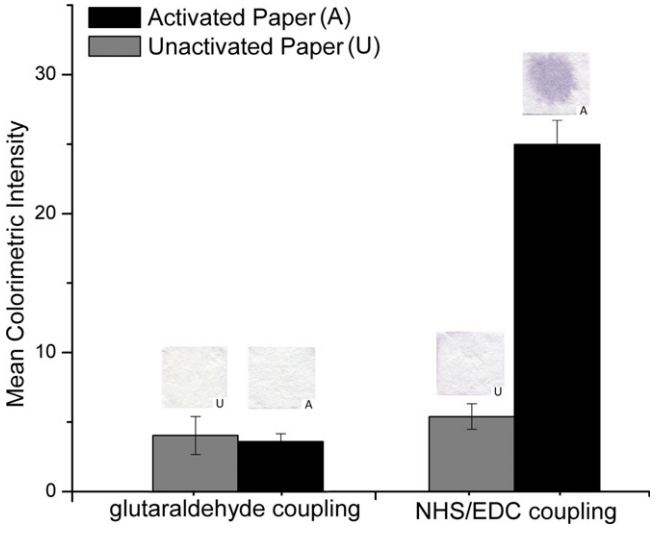

Fig. 2. Detection of immobilized AP on differently treated unmodified paper: glutaraldehyde coupling (left) and NHS/EDC coupling (right).

3.3. Covalent attachment of protein to polysaccharide-modified paper

Polysaccharide-modified paper was contrasted with activated original paper by NHS/EDC for examining the performance of protein immobilization. The reason why polysaccharide-coated paper was taken as a reference is that polysaccharides as an important class of biopolymers have been successfully used as surface modifiers to functionalize paper-based analytical devices. Meanwhile, there is a lack of detailed analysis of polysaccharide modification strategies for paper-based biosensing platform, which could increase our knowledge about the chemistries of these bioconjugation systems and their bioassay performances.

Some studies on cellulose model surfaces (i.e., Langmuir–Schaeffer cellulose films) have reported that polysaccharides' structural similarity to cellulose makes these macromolecules possible to engage in hydrogen bonding interactions with cellulose, which could lead to irreversible polymer adsorption [15,16]. Here, when it comes to practical cellulose fiber-based paper, XPS surface analysis was performed to verify paper functionalization via polysaccharide coating. As shown in the XPS elemental composition data (Table S1, Supporting information), nitrogen was not detected on unmodified paper. As for chitosan-coated paper, however, even after thorough washing with water, the similar appearance of a nitrogen signal in the XPS spectrum indicates the successful irreversible adsorption of chitosan on paper. Similarly, after the coating of NaAlg, sodium could be observed by XPS on NaAlg-coated paper, which demonstrates the rinsing–resistant adsorption of NaAlg. The results validate that polysaccharide has similar affinity with cellulose fiber-based paper just as it has with Langmuir–Schaeffer cellulose films.

3.3.1. AP immobilized on chitosan-modified paper

Chitosan-coated paper was exploited for enzyme immobilization. Two major mechanisms contributed to the biomolecule immobilization – electrostatically driven adsorption mechanism and a covalent

coupling mechanism with Schiff bases. The abundant primary amine functional groups in chitosan enable the polymer to carry positive charges under acidic conditions, with a pKa of ~6.5 [19]. AP was used in this study as the model biomolecule with a pI range of 4.4–5.8, which allows chitosan and the enzyme to have electrostatic interactions.

In addition to the electrostatic interaction, chitosan activated by glutaraldehyde is able to immobilize biomolecules with amino groups through the Schiff reaction. Fig. 3A shows that the trends of enzyme immobilization through physisorption and covalent coupling were similar, that is, the quantity of the immobilized protein increased with the increase of chitosan proportion used for paper surface treatment. The quantity of enzyme immobilization through covalent bonding was higher on paper treated by low to medium concentration of chitosan solution (⩽0.5%, w/v); but became almost the same as the chitosan concentration further increased to 1% (w/v). The results reveal that even non-activated chitosan can immobilize the enzyme strongly enough to withstand rinsing by Tris–HCl buffer that could lead to the deprotonation of chitosan. Although the electrostatic charge of chitosan could be weakened, the hydrophobic interactions between chitosan and enzyme molecules could still contribute to enzyme physisorption [20].

From the perspective of paper bioassays based on popular colorimetric analysis, paper should ideally not acquire any background color after the surface modification with polysaccharides. However, as shown in Fig. 3B, paper modified with chitosan solution concentration above 0.1% does acquire an unwanted level of background color after being activated by glutaraldehyde. It is possible that the adsorbed chitosan could go through a phase inversion in weak alkaline solution (PBS) and become conducive to form the interpenetrating polymer network, which might allow, although could not be confirmed, the subsequently introduced glutaraldehyde to bring about crosslinked chitosan [21]. In order to retain the high brightness of the paper and to achieve a practically significant covalent coupling ability to biomolecules, low concentrations of chitosan solution (not more than 0.05%) is more suitable for the fabrication of chitosan-coated paper biosensing platform, which is in some agreement with previous report by Wang et al., where they used 0.025% chitosan for paper modification [17]. In that case, the enzyme immobilization capacity of NHS/EDC-activated original paper was considerable, just slightly lower compared to that of chitosan-coated paper.

3.3.2. AP immobilized on NaAlg-modified paper

As another commonly-utilized macromolecule, NaAlg has a large number of carboxyl groups distributed along the polymer backbone, which enables covalent immobilization of biomolecules via the well-understood NHS/EDC chemistry. Unlike the chitosan immobilization system, the amount of protein immobilized on NaAlg-modified paper varied with different methods (Fig. 4A). In the case of NaAlg-modified paper with NHS/EDC activation, the amount of immobilized AP exhibited a low dependence on the concentration of the NaAlg solutions employed for surface treatment, which may be attributed to the fact that the residual carboxyl groups on unmodified paper can react with biomolecules bearing primary amine groups. The results show that NaAlg-coated paper has similar enzyme immobilization capacity as NHS/EDC-activated original paper.

It is observed in Fig. 4A that the physisorption of AP on non-activated NaAlg paper was weak, particularly in the low NaAlg concentration range (below 0.1%). Electrostatic repulsion could be responsible, since both NaAlg and AP carry negative charges. The reason for the increased AP physisorption in higher NaAlg concentration, however, is unclear. Possibly, the increased NaAlg concentration that could change the pore size of paper to some extent made the adsorbed enzyme difficult to be washed off, which can affect the reproducibility of paper-based analytical device signal data. Unlike chitosan modification, NaAlg modification does not raise the background signal, which is a desirable performance for paper-based colorimetric bioassays (Fig. 4B).

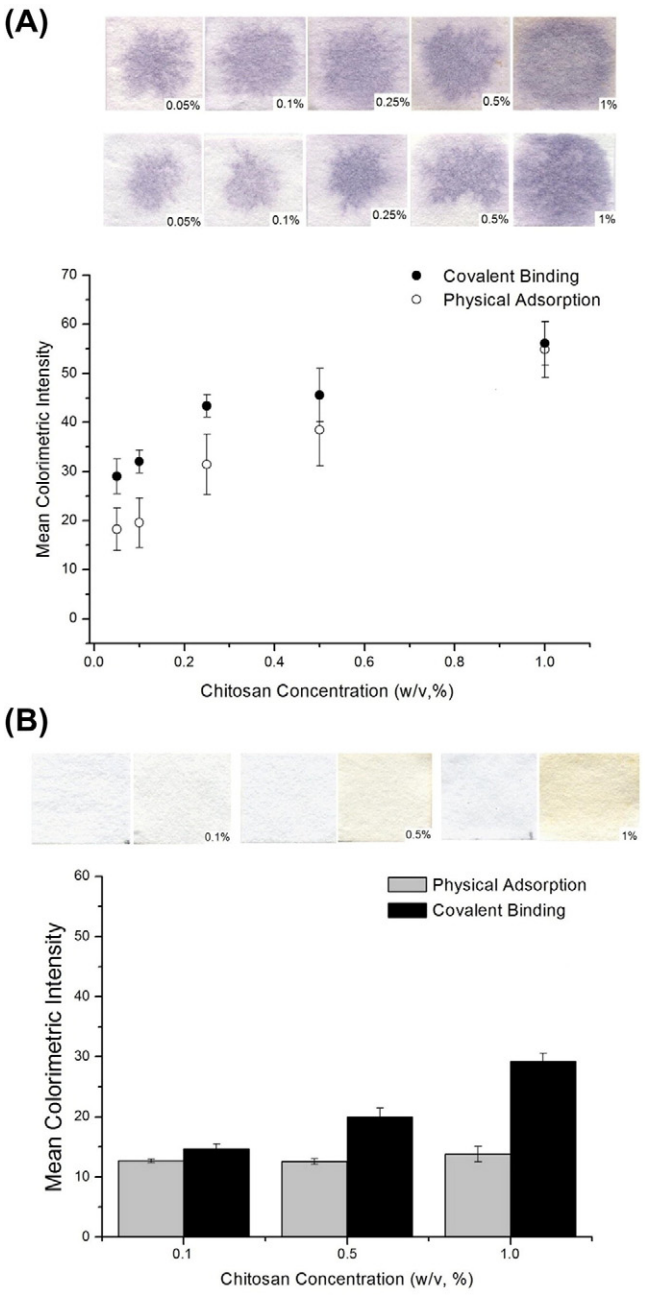

Fig. 3. Detection of immobilized AP on chitosan-modified paper. (A) Colorimetric intensity of AP assay as a function of chitosan solution concentration. Insets: Images of colored product from AP assay on glutaraldehyde-activated paper (top row) or non-activated paper (bottom row). (B) Background intensity for chitosan-modified paper with immobilized AP before BCIP/NBT incubation. Insets: images of pairs of paper with immobilized AP with (right) or without glutaraldehyde activation (left).

3.4. Comparison of immobilization methods

Since the high biomolecule immobilization capacity could improve the performance of bioassays, it is of importance to quantify the amount of biomolecules that could be immobilized on the substrates. Here, the covalent immobilization of AP on three paper samples with different treatments (i.e., 0.05% chitosan-modified paper activated by glutaraldehyde; 0.05% NaAlg-modified paper and original paper activated by NHS/

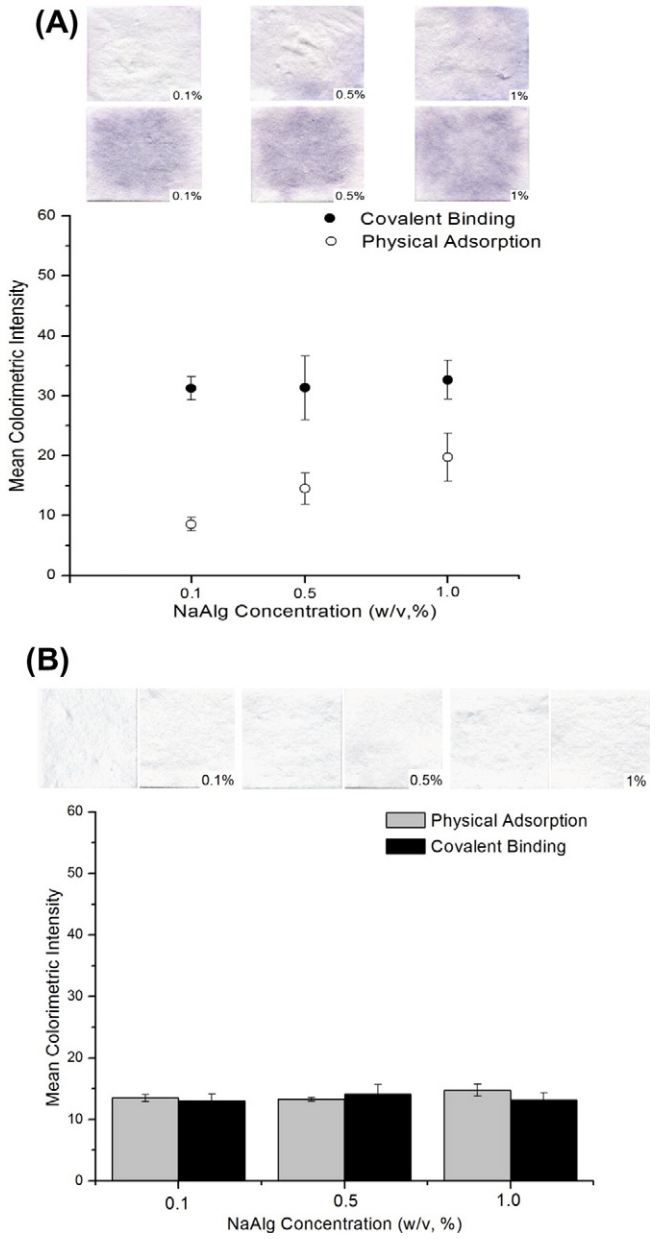

Fig. 4. Detection of immobilized AP on NaAlg-modified paper. (A) Colorimetric intensity of AP assay as a function of NaAlg solution concentration. Insets: Images of colored product from AP assay on non-activated paper (top row) or NHS/EDC-activated paper (bottom row). (B) Background intensity for NaAlg-modified paper with immobilized AP before BCIP/NBT incubation. Insets: images of pairs of paper with immobilized AP with (right) or without NHS/EDC activation (left).

EDC) was studied by spotting solutions of AP with concentrations varying from 0 to 1 mg mL^{-1}. As expected, the quantity of immobilized enzyme increased as a function of AP concentration (Fig. 5). The colorimetric analysis of these paper samples that had been modified by chitosan or NaAlg did not show significant difference from that of original paper treated with by NHS/EDC chemistry. The above results suggest that original paper has the potential to simplify the route of covalent biomolecule conjugation onto it. The long-term stability on various paper substrates was also investigated. Our results (shown in Fig. 6) indicate that AP retained some activity over a period of at least one month when stored at room temperature. To some extent, covalent coupling could help to maintain the active conformation of the biomolecules. Intuitively, covalent immobilization could reduce the likelihood of enzyme aggregation by conjugating them with supports to restrict their movement. Unfortunately, the results in Fig. 6 demonstrate that paper modified with polysaccharides did not offer a significant advantage over the unmodified paper treated with NHS/EDC chemistry in preserving the bioactivity of AP. During storage, low-concentration chitosan and NaAlg may not offer effective protection for biomolecules.

3.5. Assessment of bioassays on different papers

In order to demonstrate the capability and versatility of different types of paper in conjugating biomolecules, we performed a simple-form paper-based immunoassay, that is, the interaction and recognition between rabbit IgG and AP-conjugated anti-rabbit IgG. As illustrated in Fig. 7, for both original and NaAlg-modified paper, there is an obvious signal difference between NHS/EDC-activated and non-activated paper, in accord with the role played by carboxyl groups during the process of biomolecule immobilization. The relatively weak signal observed on the non-activated paper can be attributed to a low level of the physical adsorption of rabbit IgG onto the paper. However, in the case of chitosan-modified paper, even non-activated paper had a strong signal, which means that the physisorption of rabbit IgG could be resistant to the stringent washing steps. Perhaps it is of some value to verify the feasibility of utilizing unactivated chitosan-modified paper for bioassays in the future. Besides, it is worth noting that the activated original paper showed no significant difference with activated chitosan paper, which, once again, demonstrated the ability of NHS/EDC conjugation strategy to covalently immobilize biomolecules bearing amino groups onto the unmodified paper.

4. Conclusions

On the grounds that popular covalent methods of attaching biomolecules to substrates for biosensing platforms include amine chemistry and carboxylic chemistry, this work provides detailed data about the application of chitosan and NaAlg on cellulose fiber-based paper modification, which could offer some valuable suggestions for other applications using bioactive paper. Besides, there is a common view that for covalent biomolecule immobilization, paper must be functionalized at first. However, our results indicate that efforts may not be required for the step of functionalization by using residual carboxyl functional groups on paper (i.e., zero-step paper functionalization for covalent biomolecule immobilization). We believe that the work reported here would provide a

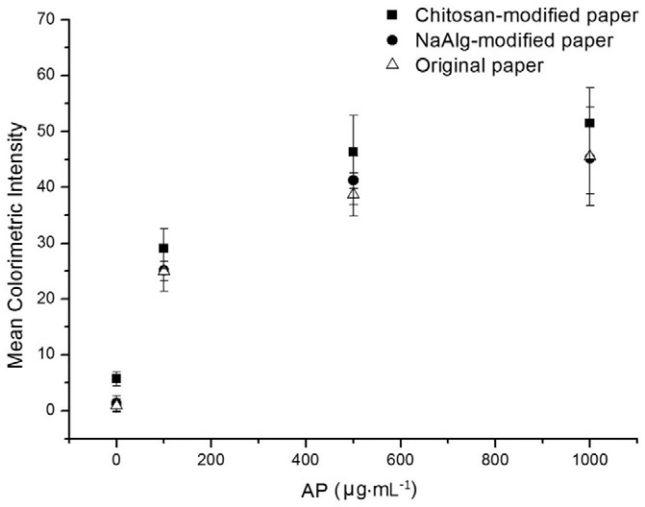

Fig. 5. Colorimetric assays for covalently immobilized AP on different types of paper squares.

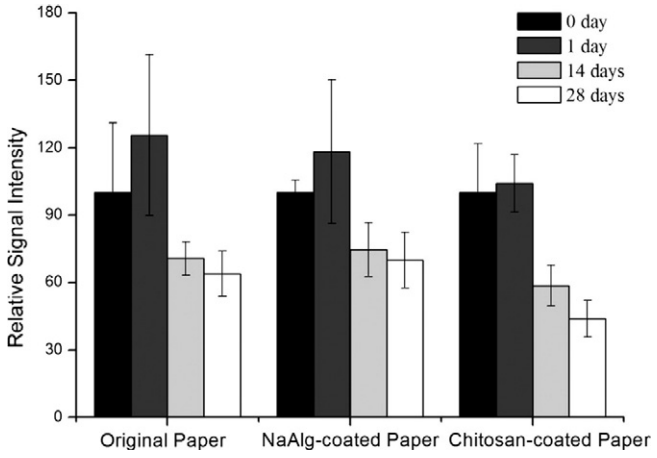

Fig. 6. Stability of immobilized AP on different paper squares with varying storage time.

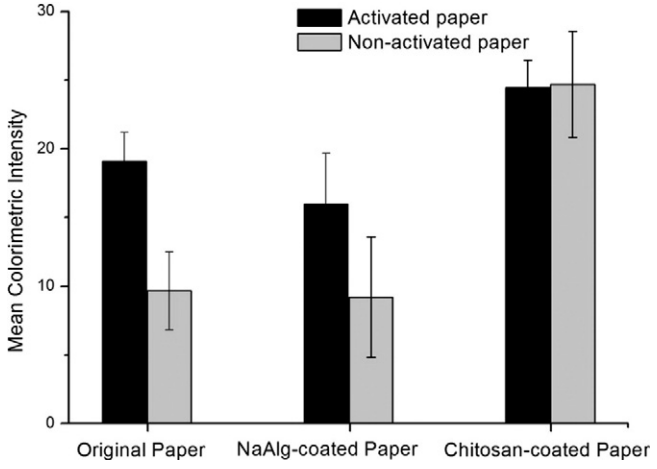

Fig. 7. Paper-based immunoassays for the detection of rabbit IgG immobilized on different paper substrates.

valuable perspective for the fabrication of paper-based biosensing platform.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.sbsr.2015.09.002.

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