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Experimental comparison of surface chemistries for biomolecule immobilization on paper-based microfluidic devices

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Abstract

Biomolecules (e.g. proteins and nucleic acids) as target analytes of microfluidic paper-based analytical devices (μPADs) are usually immobilized on a cellulose paper substrate (with intrinsically anionic surface) through physical adsorptions by van der Waals forces and electrostatic interaction thanks to cationic patches on the biomolecule. However, the physical adsorption could lead to weak biomolecule-substrate binding strength and thus low biosensing performance. Benefitting from the abundance of hydroxyl groups on the cellulose paper, chemical modification based on specific surface chemistries is capable of biofunctionalization on the μPADs by providing functional groups for covalent bindings with the target biomolecule. There are many previous reports on chemical modifications of cellulose surface for improvement of biomolecule immobilization. Nevertheless, no study has been performed on experimental evaluation of modification efficiencies of various biofunctionalization methods in the context of biosensing applications. In this paper, we compare five surface chemistries for protein immobilization on μPADs made from pure cellulose paper. For each chemical modification method, surface analyses were first conducted to monitor the surface modification process. Then, paper-based fluorometric experiments and colorimetric enzyme-linked immunosorbent assays (ELISA) were carried out on paper substrates modified by the five surface chemistries to compare their efficiencies of covalent protein immobilization. Finally, a stability experiment was carried out on the five types of surface-modified paper after 30 d storage. It was demonstrated that the potassium periodate (KIO_4)-modified cellulose paper has the best performance with 53% increase in the signal output and 59% decrease in background noise of the colorimetric ELISA, and only 13% bioactivity loss after the 30 d storage. The comparison results provide a valuable experimental guideline for selecting the suitable surface chemistry for protein immobilization on μPADs.

Keywords: paper-based microfluidics, surface chemistry, biofunctionalization, biosensing

(Some figures may appear in colour only in the online journal)

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1. Introduction

Microfluidic paper-based analytical devices (μPADs) have been widely used for disposable, pump-free, and low-cost disease diagnostics [1–7]. Various studies have demonstrated robust, sensitive, and simple assays of protein [8–10] or nucleic acid [11, 12] biomarkers on μPADs. The pure cellulose filter or chromatography paper (e.g. the Whatman Grade 1 filter or chromatography paper) is the most commonly used substrate for μPAD fabrication, and provides unique advantages such as intrinsic capillarity, biocompatibility, mature surface chemistries, and low cost [13].

The three-dimensional (3D) porous structure of the cellulose paper also serves as a high-surface-to-volume-ratio matrix for immobilizing biomolecules (e.g. proteins and nucleic acids) on a μPAD [14]. Physical adsorption is a common way to immobilize biomolecules on paper, which relies on the van der Waals and electrostatic forces between the biomolecule and the paper surface. Pure and clean cellulose contains an anionic surface with negative charges [15]. Thus, the cationic patches on biomolecules are able to form electrostatic interactions with the cellulose surface for physical adsorption [16, 17]. However, the physical adsorption on paper reveals weak binding strength, making the immobilized biomolecules easy to wash off [14]. Besides, the cationic cellulose surface also adsorbs interference molecules via the same van der Waals and electrostatic forces, leading to low selectivity and high background noise of the assays on μPADs [14]. Applying a blocking buffer (such as bovine serum albumin solution and fat-free milk) to the substrate after the biomolecule immobilization step is used to present non-specific adsorption of interference molecules [14, 18].

Because of the abundant hydroxyl groups (–OH) in the glucose-unit-based cellulose, surface chemistries have also been developed to modifying the hydroxyl groups into other functional groups (e.g. aldehyde group) to facilitate biomolecule immobilization on μPADs and minimizing the non-specific adsorption. Different from physical biomolecule absorption, chemical immobilization of biomolecules on cellulose paper via covalent bonds is a more efficient strategy to achieve strong molecule binding and high assay sensitivity/specificity [14]. After surface chemical treatment, the cellulose surface is activated with functional groups suitable for subsequent chemical immobilization of biomolecules on μPADs. Several chemical routes have been investigated for efficient surface chemical modification of cellulose paper [19–22]. However, to limit the reagent consumption and reduce the process time for device biofunctionalization, sophisticated methodologies consisting of too many chemical reaction steps are not recommended for practical applications.

During the past decade, facile and simple surface chemistry strategies have been developed for biofunctionalization of paper substrates. Araújo *et al* [23] developed an activated cellulose paper substrate for rapid hybridization-based detection of single stranded DNAs (ssDNAs). The cellulose paper was biofunctionalized with 1,4-phenylenediisothiocyanate (PDITC) to provide isothiocyanate groups for covalent binding of the animated ssDNA capture probes. Liu *et al* [24] proposed another modification strategy using 1,1′-carbonyldiimidazole (CDI) as a crosslinker to modify the cellulose substrate. The urethane (N-alkyl carbamate) linkages were covalently formed between aptamers and acylimidazole groups. Another modification strategy of cellulose using divinyl sulfone (DVS) was reported by Yu *et al* [25]. This method was demonstrated to form covalent bonds between electrophilic vinyl groups and biomolecules through nucleophilic addition. They also proved the compatibility of this functionalization method with inkjet printing for direct patterning of biomolecules. Wang *et al* [26] utilized cationic chitosan to first neutralize the anionic surface of cellulose for decreasing the non-specific binding caused by physical adsorption. Then, glutaraldehyde (GA) was used to modify the chitosan with residual aldehyde groups for covalent binding with biomolecules. To date, periodate oxides have been widely used to generate dialdehyde groups on cellulose [27, 28]; the simple modification process enables the practical production of functionalized cellulose paper. Although much efforts have been spent on examining the surface chemistries for cellulose modification, there is no systematic study on the evaluation and comparison of modification efficiencies of different chemistries for biomolecule immobilization on μPADs.

In this paper, we present the experimental comparison of five previously reported surface chemistries for cellulose modification and protein immobilization on μPADs. We implemented the five surface chemistries on pure cellulose Whatman No. 1 chromatography paper, and conducted surface analyses on the modified cellulose to confirm the expected chemical modifications. The modification efficiencies of these chemistries were calculated by the quantity ratio of the introduced functional groups and the unmodified surface groups. We then carried out colorimetric enzyme-linked immunosorbent assays (ELISAs) on the chemically modified cellulose paper wells to quantify the protein immobilization strength and specificity in terms of colorimetric signal output and background noise. Finally, we compared the stability of biofunctionalized cellulose paper after a long-term storage of 30 d. This study provides useful data on selecting suitable surface chemistry for protein immobilization on μPADs, and could thus improve signal-to-noise ratios of immunoassays conducted on these devices. Besides disease diagnostics, the experimental approach and results reported here could also have important implications in other fields such as environmental monitoring and food safety inspection.

2. Experimental methods

2.1. Materials and reagents

Whatman No. 1 chromatography paper, 10× phosphate buffered saline (PBS, P5368), rabbit immunoglobulin G (IgG, I5006), alkaline phosphatase conjugated (ALP-conjugated) anti-rabbit IgG (A4062), fluorescein isothiocyanate conjugated (FITC-conjugated) anti-rabbit IgG (F9887), BCIP®/NBT tablet (as ALP substrate, B5655), Tween® 20 (P1379), bovine serum albumin (BSA, A2153), potassium periodate (KIO_4, 99.8%, 210056), CDI (97%, 21860), DVS (stabilized

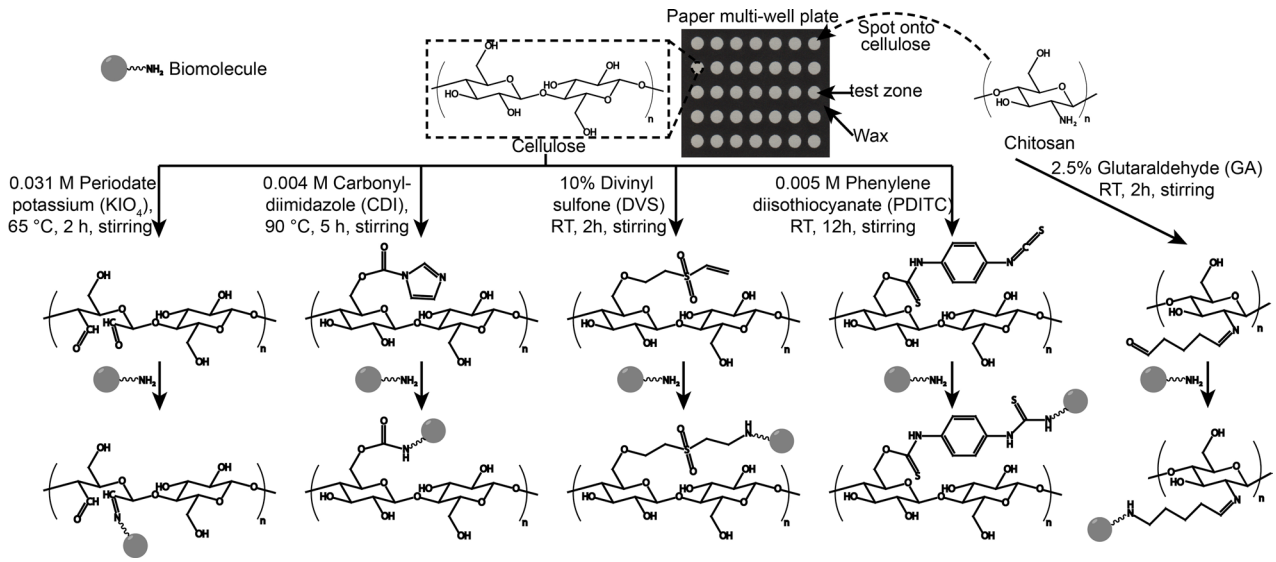

Figure 1. Schematics of five surface chemistries for chemical modification of cellulose paper and covalent immobilization of biomolecules. Potassium periodate (KIO_4), carbonyldiimidazole (CDI), divinyl sulfone (DVS), phenylene diisothiocyanate (PDITC), and chitosan/glutaraldehyde (GA) were utilized to modify cellulose paper and generate functional binding sites to covalently bind with amino groups on biomolecules (e.g. protein and aminoed nucleic acid).

with hydroquinone, 97%, V3700), PDITC (98%, 258555), chitosan (448869), GA (70% in H_2O, G7776), and dimethyl sulfoxide (DMSO, D8418) were purchased from Sigma-Aldrich and used as received without further purification. The 10× PBS was diluted using deionized water (diH_2O) to 1× PBS.

2.2. Surface chemistries and multi-well paper plate fabrication

A piece of 100 mm × 100 mm Whatman No. 1 chromatography cellulose paper was chemically modified by one of the following five surface chemistries (figure 1). (i) For the modification with KIO_4, a piece of cellulose paper was put into 50 ml aqueous solution of 31 mM KIO_4 at 65 °C for 2 h. After the activation, the paper was washed three times with diH_2O. (ii) For the modification with CDI, a piece of cellulose paper was put into 50 ml DMSO solution of 4 mM CDI at 90 °C for 5 h. After the reaction, the paper was washed three times with DMSO. (iii) For the modification with DVS, a piece of cellulose paper was put into 50 ml of 10% (v/v) DVS (in 0.1 M sodium carbonate at pH 11) at room temperature for 2 h. Then the modified paper was washed three times with diH_2O. (iv) For the modification with PDITC, a piece of cellulose paper was put into 50 ml of 5 mM PDITC (in DMSO) solution at room temperature for 12 h. After the reaction, the paper was washed three times with DMSO. (v) For modification with chitosan and GA, a piece of cellulose paper was first put into 50 ml of aqueous solution of 0.25 mg ml^{-1} chitosan at room temperature for 1 h. Then, the paper was transferred to 50 ml of 2.5% GA (in 1× PBS) at room temperature for 2 h. Finally, the modified paper was washed three times with diH_2O. During the dipping of the cellulose paper into different chemical solutions for reaction, heating (if the reaction temperature is above room temperature) and continuous stirring of the chemical solution were conducted using a hot plate. All the modified paper substrates were finally blotted with nonwoven wipers and dried in a lightproof desiccator overnight before fabrication of the multi-well plate.

The modified paper substrates were fabricated into multi-well plates through wax printing. A Xerox 8570 inkjet printer was used to print solid wax patterns (well diameter: 6 mm, and pitch: 9 mm) on the modified 100 mm × 100 mm chromatography paper, and the paper was then baked on a hotplate at 120 °C for 30 s to melt the printed wax and form the paper wells. All the multi-well plates were stored in a lightproof desiccator before any experiment.

2.3. Surface characterization of modified cellulose paper

Physical properties of the modified cellulose paper were characterized first. To prepare the modified cellulose paper for scanning electron microscopy (SEM), the modified paper samples were first coated with 2 nm of platinum using a sputter coater (E14 RF/DC sputter, Denton). Then SEM micrographs of the modified cellulose paper were acquired with a SEM (Quanta 450, FEI) at a beam accelerating voltage of 10 kV.

For monitoring the chemical composition changes during the modification process and calculating the modification efficiency, attenuated total reflectance-Fourier transform infrared spectral analysis (ATR-FTIR) was recorded with a Perkin–Elmer spectrometer (UATR Single Bounce with ZnSe/Diamond Crystal) for characterizing the KIO_4-modified cellulose paper as the modification does not introduce additional chemical elements. In contrast, the CDI-, DVS-, PDITC-, and GA-based methods introduce nitrogen (N) and/or sulphur (S) elements to the cellulose surface, and x-ray photoelectron spectrometry (XPS) was thus utilized to characterize the cellulose paper modified by these four methods. The XPS analysis was performed using an XPS instrument (Thermo Scientific Kα XPS, Thermo Scientific). Three spots were positioned and analyzed for each sample. The reported data were averaged over the three spots. During the XPS analysis, an incident

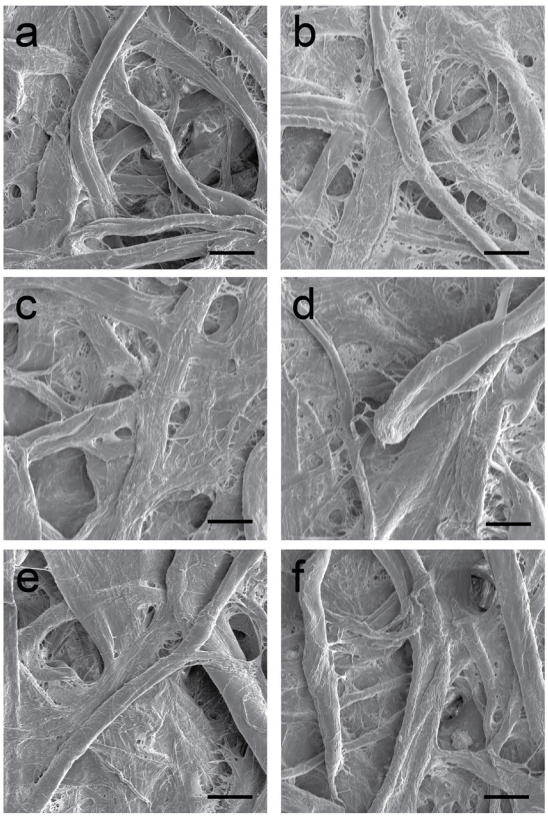

Figure 2. SEM photographs of: (a) unmodified cellulose paper, (b) KIO_4-modified cellulose paper, (c) CDI-modified cellulose paper, (d) DVS-modified cellulose paper, (e) PDITC-modified cellulose paper, and (f) chitosan/GA-modified cellulose paper. The scale bars indicate 25 μm.

monochromatic x-ray beam (with 50 eV pass energy) from the Al target was focused with a spot size 100 μm to the modified cellulose paper surface.

2.4. Fluorometric testing of the protein immobilization strength

To examine the protein immobilization strength on paper wells modified with different surface chemistries, fluorophore-tagged antibodies were immobilized on the paper wells and then washed with a large amount of washing buffer, and the remaining amount of antibodies immobilized on paper wells modified by each chemistry was measured through fluorescence imaging to quantified the protein immobilization strength. First, 2 μl of 50 μg ml^{-1} FITC anti-rabbit IgG (in PBS) was added to each chemically modified paper well and incubated for 10 min to immobilize the antibodies onto the cellulose surface. Then, 500 μl of washing buffer (0.05% v/v Tween-20 in PBS) was added to each paper well to remove unbound antibodies. During the washing process, the multi-well paper plate was placed on top of a vacuum suction filter with a vacuum pressure applied by a pump (Gast High-Capacity Vacuum, Cole-Parmer). The paper wells were imaged by a fluorescent microscope (IX-83, Olympus Canada Inc., Toronto, ON) with a cooled complementary metal-oxide-semiconductor (CMOS) camera (EXi Blue, QImaging, Surrey, BC). The excitation and emission wavelengths were

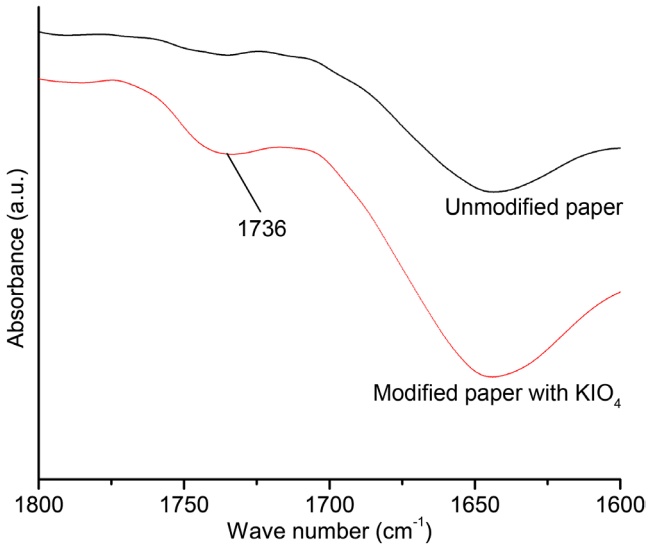

Figure 3. Infrared spectra of unmodified cellulose paper and modified cellulose paper with KIO_4. The characteristic absorption peak of the aldehyde group at 1736 cm^{-1} was observed on the infrared spectra. The unmodified paper was used as benchmark control.

set at 490 nm and 520 nm, respectively, and the average fluorescence intensity of each paper well was quantified using ImageJ (NIH, Bethesda, MD). The test was also performed on unmodified paper wells as the control.

2.5. Colorimetric ELISA on the modified paper

Colorimetric ELISA was performed on the surface modified paper multi-well plates using rabbit IgG as a model analyte. Following a paper-based ELISA protocol [29], we first immobilized 2 μl of 1 mg ml^{-1} rabbit IgG antigen (I5006, Sigma-Aldrich) to each paper well. 1× PBS was used as a negative control. After 10 min incubation, the paper wells were blocked with 2 μl of blocking buffer (0.1% (w/v) BSA and 0.05% (w/v) TWEEN 20 in PBS), and dried under ambient conditions for 10 min. Then, 2 μl of ALP-conjugated anti-rabbit IgG antibody (1:1000 dilution of the stock antibody produced in goat in 0.05% (w/v) TWEEN 20 in PBS) was added to the paper wells and incubated under ambient conditions for 1 min. Following the incubation, the test zones were washed twice by adding 10 μl of 1 × PBS each wash. Finally, 2 μl of ALP substrate (dissolved BCIP®/NBT tablet into 459 μM BCIP, 367 μM NBT, 5 mM $MgCl_2$ in 100 mM Tris buffer, pH 9.5) was added to each paper well for signal production. After signal amplification for 30 min, the paper wells were scanned by a CanoScan LiDE 210 scanner (set to color photo scanning at 600 dpi resolution) and analyzed using ImageJ.

3. Results and discussion

3.1. Surface characterization of the modified paper

Figure 2 shows SEM photographs of the surface morphologies of the cellulose paper modified by the five chemical routes. From the SEM photographs at 500×, one can observe that

Table 1. Relative element compositions of unmodified and modified cellulose paper characterized by XPS. The unmodified paper was used as benchmark control.

	Unmodified paper	CDI	DVS	PDITC	GA
C 1s	57.3 ± 0.2	56.4 ± 0.2	58.4 ± 0.3	54.0 ± 1.3	60.0 ± 0.2
O 1s	42.7 ± 0.2	42.9 ± 0.2	40.4 ± 0.2	43.7 ± 2.0	39.3 ± 0.2
N 1s		0.7 ± 0.3		1.1 ± 0.3	0.7 ± 0.2
S 2p			1.2 ± 0.1	1.2 ± 0.5	

Table 2. Comparison of the five modification methods.

	KIO_4 [27]	CDI [24]	DVS [25]	PDITC [23]	Chitosan/ GA [26]
Activation time (hours)	2	5	2	12	2 + 1
Reaction temperature (°C) [a]	65	90	RT	RT	RT
Modification efficiency (%)[b]	15	4	14	7	8
Immobilization strength index	22.4	6.5	22.2	14.9	16.1
Valid signal enhancement (%)	53.4	35.4	43	14.5	–14.1
Background noise reduction (%)	59.1	12.1	67	18	9.2
Bioactivity loss after storage (%)	13.9	32.1	24.4	-8	31

[a] RT: room temperature.
[b] The modification is considered successful if one glucose unit in the cellulose chain was modified completely.

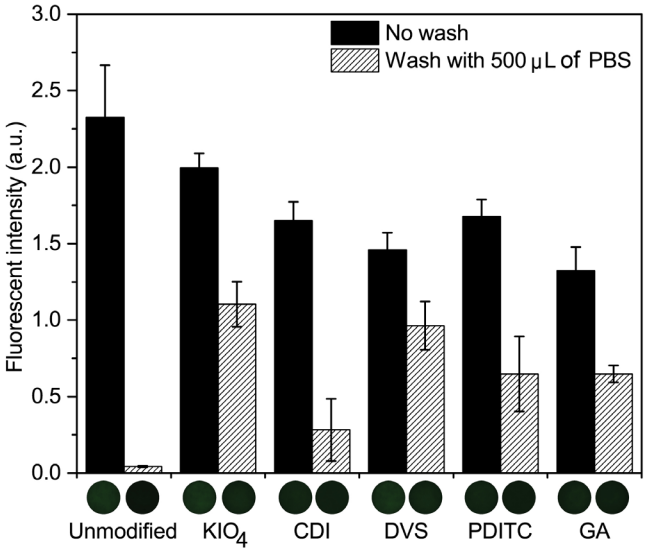

Figure 4. FITC-conjugated anti-rabbit IgG (50 μg ml^{-1}) was used to investigate the immobilization performance of unmodified and modified cellulose paper ($n = 7$). Fluorescence photographs of randomly selected paper spots on the multi-well paper plates are shown below the corresponding data columns. Error bars represent standard deviations.

the surface roughness of the cellulose microfiber increased slightly after the chemical modification processes. However, no obvious changes of the cellulose microfibers morphology and the porous structure were observed after the chemical modifications. In our ELISA experiments, we did not observe any change in the wicking speed of aqueous solutions in the modified paper wells. Thus, it was demonstrated that the capillarity of the modified cellulose paper keeps unchanged after the chemical modifications.

To verify the expected chemical modifications of the cellulose surface, FTIR and XPS analyses were performed. As the modification methods using CDI, DVS, PDITC, and GA all introduce chemical groups containing nitrogen (N) and/or sulfur (S) elements (figure 1), XPS analysis of the N and S elements was conducted on the cellulose surfaces modified by these methods. The KIO_4-based modification introduces aldehyde groups on the cellulose surface (figure 1); therefore, instead of using XPS, FTIR analysis was carried out on the KIO_4-modified cellulose surface to confirm the characteristic absorption band of the C=O double bond in the introduced aldehyde group. Figure 3 illustrates the FTIR spectra of the KIO_4-modified and unmodified cellulose surface. The characteristic adsorption band of the C=O double bond in the aldehyde group was detected at ~1726 cm^{-1}, confirming the successful modification of the cellulose surface with the KIO_4. Table 1 shows the XPS results of elemental compositions of cellulose surfaces modified by CDI, DVS, PDITC, and GA. Compared to the unmodified cellulose surface with only carbon and oxygen, the elemental signals of N and/or S were observed in the XPS spectra measured from the cellulose surfaces modified by CDI, DVS, PDITC, and GA. The results indicate the success of the chemical modifications of the cellulose paper by these four methods.

Cellulose consists of a linear chain of β (1 → 4) linked glucose units. To be consistent with previous studies [25], we counted the success of surface activation if a unit in the cellulose chain was modified completely. Thus, the modification efficiency becomes quantifiable for different surface chemistries based on the element composition change. On the basis of nitrogen-to-oxygen ratio, the modification efficiency was calculated to be 4% for CDI-modified cellulose paper. Similarly, the modification efficiencies are 14%, 7%, and 8% for DVS-, PDITC-, and GA-modified cellulose paper, respectively. For the KIO_4-based modification, the modification efficiency was previously reported to be 15% with the same modification protocol we used in this work [30]. The modification efficiencies of all the five surface chemistries are summarized in table 2. These values will be further validated by the data from the fluorometric testing of the protein immobilization strength and the colorimetric paper-based ELISA.

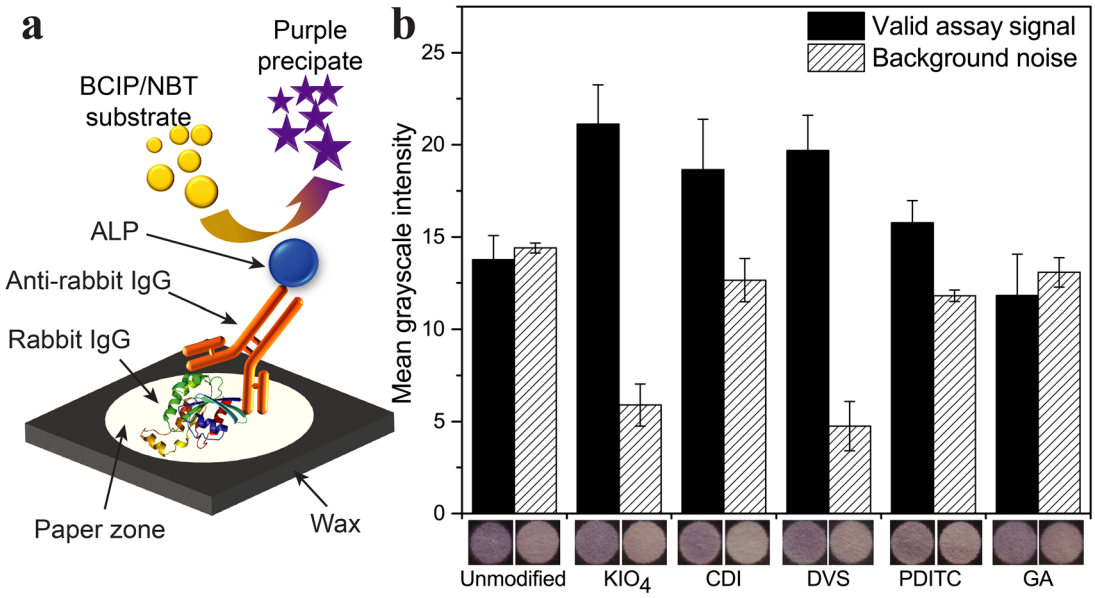

Figure 5. Colorimetric ELISA results on the unmodified and modified cellulose paper. (a) Schematic of colorimetric ELISA for detecting rabbit IgG. (b) Mean grayscale intensity values of control zone signals (background noise) and valid assay signals (test zone signal minus background noise) ($n = 7$). Colorimetric photographs of randomly selected paper spots on the multi-well paper plates are shown below the corresponding data columns. Error bars represent standard deviations.

3.2. Quantification of protein immobilization performance

To evaluate the protein immobilization performance of the modified cellulose surfaces and verify its relationship with the measured modification efficiencies, we employed the FITC-conjugated anti-IgG as a representative protein for fluorometric testing of the protein immobilization strength. After the addition of FITC-conjugated anti-IgG to the paper wells, the antibodies were immobilized via covalent binding on the chemically modified cellulose paper and physical adsorption on the unmodified cellulose paper. After washing with 500 μl of washing buffer, the quantity of remaining antibodies immobilized on the paper well indicates the protein immobilization strength. Figure 4 shows the average fluorescence intensities of the modified and unmodified paper wells before and after washing. One can see that, on the unmodified paper wells, most of the immobilized antibodies were washed off because of the weak physical adsorption (figure 4). This result is in a good agreement with the previously reported result [14]. In contrast, more immobilized antibodies remain on the chemically modified paper wells, proving the higher immobilization strength achieved by covalent binding. We define an index of the immobilization strength as the ratio of the after-washing fluorescence signal of the modified paper well to the one of the unmodified well, and the calculated indices are shown in table 2. Specifically, KIO_4- and DVS-modified cellulose paper exhibited the two highest immobilization strength indices (22.4 for KIO_4 and 22.2 for DVS). PDITC- and GA-modified cellulose paper showed moderate levels of immobilization strength, while CDI-modified cellulose paper revealed the lowest level of immobilization strength. From table 2, we can also observe that the modification efficiency and immobilization strength index data well correlate with each other. The results in figure 4 confirms that the covalent protein immobilization greatly reduces protein loss during the washing step. We further envision that the chemical modifications will promote the assay signal and reduce the background noise of colorimetric ELISA on the modified paper wells.

We then conducted colorimetric direct ELISA of rabbit IgG on the modified and unmodified paper wells (figure 5(a)) to further evaluate the efficacy of the modification methods for improving the assay signal and reducing the background noise. The valid output signal of an assay on a specific type of paper well was calculated as the total colorimetric signal of the experimental group minus the one of the negative control group (pure PBS as the sample). The background noise on a specific type of paper well was calculated as the difference of the total colorimetric signals measured from a paper well before and after a negative control assay. For unmodified cellulose paper, the analyte proteins (rabbit IgG) were immobilized to the cellulose surface via physical adsorption. However, the physical adsorption caused high background noise (as shown by the 'unmodified' data in figure 5(b)) because of its high level of nonspecific binding [8, 31]. In contrast, the chemical modification activated more sites on the cellulose surface with functional groups that can form covalent binding with proteins. This enhanced the immobilization efficiencies of both the analyte protein (rabbit IgG) and the inert blocking proteins, thus improved the output signal of the assay and reduced the background noise. As shown in figure 5(b), the valid output signals of assays on paper wells modified by KIO_4, CDI, and PDITC are all higher than the one on the unmodified paper. Table 2 shows the valid signal enhancement and the background noise reduction levels (both in percentage) of assays on different types of the modified paper wells. The KIO_4- and DVS-modified paper provided the two highest improvement percentages of the valid output signal (53.4% for KIO_4 and 43% for DVS). The only exception is the GA-modified paper that exhibited a valid signal

reduction comparing to the unmodified cellulose paper. This may be attributed to a structural distortion of the label enzyme (ALP in our assay) caused by its extensive crosslinking with GA, which reduces the bioactivity of the enzyme and thus the valid assay signal [32].

The background noise of an ELISA is mainly caused by the nonspecific binding of the enzyme-conjugated antibodies on the cellulose surface, and the blocking step of the ELISA is designed to reduce this nonspecific binding. In the blocking step, blocking buffer containing inert proteins is added to block unoccupied binding sites on the cellulose surface and sterically prevent the enzyme-conjugated antibodies from absorption to the surface. Although we followed this blocking step in our ELISA experiments, high background noise was still observed from assays on the unmodified paper well (figure 5). The CDI-, PDITC-, and GA-modified cellulose paper also exhibited slightly high background noise levels possibly due to the low modification efficiencies. In contrast, KIO_4- and DVS-modified cellulose paper wells have larger numbers of chemically modified cites for covalent immobilization of the rabbit IgG. Thus, more enzyme-conjugated antibodies were covalently bound to the immobilized IgG rather than being physically adsorbed to the cellulose surface; after the washing step, more enzyme-conjugated antibodies remained on the cellulose paper because of the strong covalent bonds. In summary, the KIO_4- and DVS-based modification methods provide the highest modification efficiency, the strongest immobilization strength, and the best biosensing performance (highest valid signal and lowest background noise).

3.3. Stability of modified cellulose paper

We have demonstrated that the modified cellulose paper enhanced the protein immobilization strength and the biosensing performance. We also verified the stability of the modified cellulose paper over storage. Colorimetric ELISA was performed on the modified cellulose paper after 30 d storage in a dry and dark container at room temperature to mimic the packaging condition of a commercial biosensor. Figure 6 shows the valid output signals of assays on fresh modified paper wells ('Day 1' data) and the ones after 30 d storage ('Day 30' data). Overall, all five types of modified cellulose paper retained $\geqslant$70% of its original output signal level on Day 1. One can see that the PDITC-modified paper revealed no decrease of the output signal after 30 d storage, and the KIO_4-modified paper has a slight signal decrease after 30 d storage. These results could be attributed to the high stability of the functional groups introduced by the PDITC- and KIO_4-based methods under the storage environment. Although the DVS-modified cellulose paper showed high protein immobilization strength and good biosensing performance, it turned into light yellow after the storage. This caused higher background noise and reduced valid signals. Considering all the quantified characteristics (modification efficiency, protein immobilization strength, biosensing performance, and storage stability) of the surface modification methods, the KIO_4-based method provides the best overall performance for protein functionalization on μPADs.

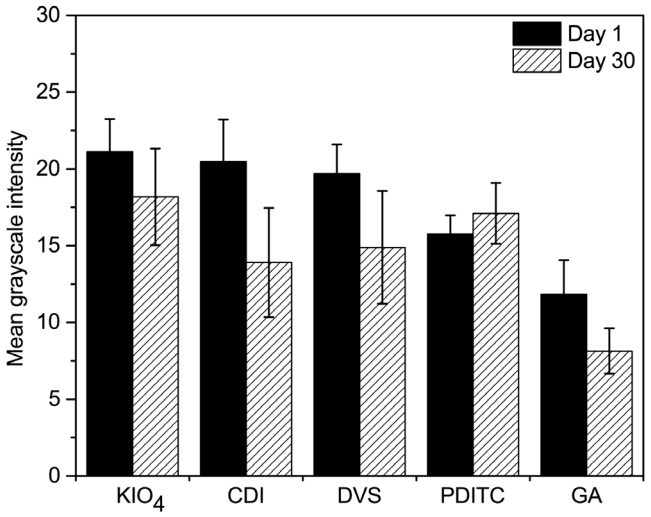

Figure 6. Colorimetric ELISA results on the modified paper after 30 d storage ($n = 7$). Error bars represent standard deviations.

4. Conclusion

On the front of the biofunctionalization of μPADs, several groups have investigated different modification methods on cellulose paper [33–35]. The modification methods can be utilized for surface functionalization of cellulose substrates for detection of nucleic acids [36, 37], proteins [38] and cells [39, 40]. However, no systematic study were performed for comparing the efficiency of these methods for covalent protein immobilization on μPADs. In this article, we compared the five cellulose modification methods for enhancement of protein immobilization. The surface characterization was performed first to evaluate the modification processes and calculate the modification efficiencies of different methods. All the modification methods were capable of activating the cellulose with functional groups to immobilize proteins via covalent binding. The immobilization strength was quantified through fluorometric testing of immobilized proteins after washing. It was demonstrated that the KIO_4- and DVS-based modification methods provided the highest modification efficiencies and the highest immobilization strength levels. Colorimetric ELISAs were also performed on the modified paper to evaluate their biosensing performance. The KIO_4- and DVS-based modification methods revealed highest improvement levels of the valid assay signal, and the largest reductions of the background noise. Finally, the modified cellulose paper was tested over 30 d storage to investigate their stability. The PDITC- and KIO_4-based methods exhibited the best stability levels after the 30 d storage. Considering all the tested characteristics of the modification methods, it was concluded that the KIO_4-based modification method has the best overall performance for protein immobilization on cellulose paper.

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