Clickable Cellulosic Surfaces for Peptide-Based Bioassays

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The use of peptides in paper-based analytics is a highly appealing field, yet it suffers from severe limitations. This is mostly due to the loss of effective target recognition properties of this relatively small bioprobes upon nonspecific adsorption onto cellulose substrates. Here, we address this issue by introducing a simple polymer-based strategy to obtain clickable cellulosic surfaces, that we exploited for the chemoselective bioconjugation of peptide bioprobes. Our method largely outperformed standard adsorption-based immobilization strategy in a challenging, real-case immunoassay, namely the diagnostic discrimination of Zika+ individuals from healthy controls. Of note, the clickable polymeric coating not only allows efficient peptides bioconjugation, but it provides favorable anti-fouling properties to the cellulosic support. We envisage our strategy to broaden the repertoire of cellulosic materials manipulation and promote a renewed interest in peptide-based paper bioassays.

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Clickable Cellulosic Surfaces for Peptide-based Bioassays

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ABSTRACT: The use of peptides in paper-based analytics is a highly appealing field, yet it suffers from severe limitations. This is mostly due to the loss of effective target recognition properties of this relatively small probes upon nonspecific adsorption onto cellulose substrates. Here, we address this issue by introducing a simple polymer-based strategy to obtain clickable cellulose surfaces, that we exploited for the chemoselective bioconjugation of peptide bioprobes. Our method largely outperformed standard adsorption-based immobilization strategy in a challenging, real case immunoassay, namely the diagnostic discrimination of Zika+ individuals from healthy controls. Of note, the clickable polymeric coating not only allows efficient peptides bioconjugation, but it provides favorable anti-fouling properties to the cellulosic support. We envisage our strategy to broaden the repertoire of cellulosic materials manipulation and promote a renewed interest in peptide-based paper bioassays.

INTRODUCTION

Cellulosic materials are widely used substrates in the fabrication of low-cost, de-centralized, point of care tests.¹⁻³ Paper-based assays are indeed routinely performed to diagnose infections, to quantify glucose in urine, to test pregnancy and for blood coagulation screening.^{4,9} Analytical bioprobes are typically immobilized on paper by noncovalent physical adsorption, which is mainly driven by hydrophobic interactions. Despite the high loading capacity provided by cellulose tridimensional structure, binding of relatively small-sized biomolecules such as peptides may suffer from remarkable variability and can undergo desorption during iterative assay steps. Also, nonspecific adsorption of peptide ligands can mask bio-reactive functionalities resulting in decreased target binding affinities and, overall, the analytical performance can be plagued both by low reproducibility and low signal-to-background ratio. This has made peptides still underused molecular probes for paper-based immunodiagnosics, as witnessed by the very few available commercial assays relying on peptide probes.¹⁰ On the other hand, peptides and celluloses may represent a powerful partnership for applications in low resource settings conditions (e.g. prolonged storage at noncontrolled temperature and humidity, no sophisticated lab infrastructures) given advantageous features in terms of ease of handling, costs, stability. In this direction, there is a high interest in developing new enabling strategies to expand the chemical space of celluloses and prime cellulosic surfaces for covalent and spatially resolved ligands bioconjugation¹¹⁻¹⁶. Of particular interest, photo-initiated thiol-yne click chemistry has been recently introduced by the Levkin group to develop micro-patterned

nanocellulosic substrates.¹⁷ Yet, click-bioconjugation on cellulose materials is still an unexplored venue for the design of paper-based analytical platforms. Of note, we previously demonstrated that peptides chemoselective immobilization on microarray surfaces can boost the diagnostic performances in real-case, complex bioassays^{18,19} and a similar effect could be expected to improve the analytical outcomes of paper-based devices

Here we report a straightforward procedure to obtain clickable cellulosic surfaces by means of a user-friendly, polymer-based strategy, that we applied to peptide-based microarray serodiagnostics. Our approach proved effective not only to enable the covalent and chemoselective binding of peptides, but also to tailor the wettability and anti-fouling properties of the cellulosic support. We showed that both features are highly beneficial for specific antibody capturing in challenging, analytical settings, filling the existing gap in peptide-based paper assays. To this aim, we took advantage of a recently developed clickable polymer obtained by post-polymerization modification of MCP-2, a commercially available N,N-dimethylacrylamide (DMA), N-acryloyloxy-succinimide (NAS), and 3-(trimethoxysilyl)-propylmethacrylate (MAPS) copolymer^{20, 21}, using 3-azido-1-propanamine to introduce azido groups²² (Scheme 1).

EXPERIMENTAL

Nitrocellulose was coated by immersion in Copoly Azide solution (1% w/v in 0.9M (NH₄)₂ SO₄) for 30 minutes, rinsed with water, dried under nitrogen and cured for 15 min under vacuum at 80°C. Full experimental details in the Supporting Information

Scheme 1: cellulose modification by copoly Azide allows immobilization of yne-modified peptides via copper-catalyzed-azide-alkyne-cycloaddition (CuAAC). In contrast to random adsorption to unmodified cellulose, the clickable cellulose allows chemoselective binding of peptides and enhanced immunoreactivity of probes by cooperative interaction resulting upon proximal peptide co-immobilization

RESULTS AND DISCUSSION

Nitrocellulose blotting membranes, a common cellulosic support for bio-analysis, was coated using a mild dip-and-rinse protocol by immersion in a polymer aqueous solution (Supporting Information). The polymer readily self-adsorbed onto the cellulosic substrate (30 minutes), without the need of surface pre-treatments. Efficient polymer adsorption was verified by FT-IR spectroscopy, showing the peculiar azide stretching band at 2100 cm^{-1} (Figure 1 inset). Scanning electron microscopy (SEM) was used to compare the morphology and structure of the cellulosic surfaces. The uncoated nitrocellulose membrane is constituted by a weaving of fibers, forming elliptic micrometric cavities superimposing on different parallel planes and providing a porous structure (Figure 1-a). Moreover, sub-micron small pores can be observed along nitrocellulose fibers (indicated by the arrow). Such sub-micron porosity is instead lost upon polymeric coating, as fibers appear as covered by a layer with a melt morphology (Figure 1-b).

Interestingly, these results were duplicated using Whatman brand chromatography 1 paper (see Supplementary Information, Figure S1) suggesting applicability of the method to any cellulosic materials; however given our envisaged use of clickable paper supports for microarray immunoassays, we concentrated on the use of ready-to-use, 16-pads nitrocellulose layered on glass slides for microarrays. The wettability properties of untreated and clickable nitrocellulose were compared by static water contact angle measurements (WCA_{hst}).

Figure 1: SEM images and FT-IR spectra (inset) of the surfaces of (a) unmodified nitrocellulose and (b) copoly Azide coated nitrocellulose (scale bar 1 μm). The distinctive azide stretching band at 2100 cm^{-1} is clearly detectable on polymer treated nitrocellulose. Full FT-IR spectra of the functionalized nitrocellulose/polymer containing nitrocellulose are provided in Supplementary information. However, no major morphological alterations are visible and the high porosity of the nitrocellulose 3D structure is preserved.

Figure 2 shows a remarkably different wettability for the unmodified and polymer-coated paper, with WCA_{Hst} values of 77.9° and 19.2°, respectively. It is evident that the thin polymeric coating sensibly increased the surface hydrophilicity. This is in accordance with our previous findings on MCP-2 coated surface of silicon and glass. We anticipated that the observed coating-induced hydrophilization could have an impact on the anti-fouling properties of the cellulosic surface, as the WCA_{Hst} was comparable to that of cellulose treated with BSA, commonly used to suppress the background noise. We verified the stability of the polymeric coating by repeating WCA_{Hst} measurements after prolonged exposure to water, which showed unaltered behaviour (WCA_{Hst} value of 16.4° after 4 days).

Figure 2 Water Contact Angle (WCA) measurements (a) of uncoated (b) and polymer coated nitrocellulose at day 1 (c) and after 4 days (d). Wettability after polymer coating is similar to that obtained after treatment with BSA (e).

The analytical performances of clickable and uncoated nitrocellulose were then compared in a real-case microarray immunoassay to reveal arbovirus infections by detection of antibodies directed against the Zika viral antigen NS1. To validate our platform, we selected a small panel of known NS1-antigen-derived immunoreactive peptides (Scheme 1). While peptide P1 is reactive as single molecular entity, we previously demonstrated that the effective discrimination of Zika positive individuals by peptides P2 and P3 relies on their cooperative interaction resulting upon spatially proximal co-immobilization (Scheme 1).²³ In this latter and more demanding case, a fine control of peptidic probes display inducing a cooperative binding mechanism is crucial to efficiently capture Zika virus antibodies. To enable click bioconjugation onto azido-modified nitrocellulose, P1-P3 peptides were modified with a short-chain PEG spacer (O2Oc) including a terminal alkyne moiety. Peptides were arrayed by a piezoelectric spotter, either dissolved in PBS to allow spontaneous adsorption on uncoated nitrocellulose or reacted via CuACC to provide chemoselective covalent binding (See Supporting Information). To suppress nonspecific interactions during the assays, untreated nitrocelluloses were preliminary incubated with a 2% w/v BSA solution, whereas azido-modified surfaces, due to the expected favorable anti-fouling properties provided by the polymeric coating, were simply washed in a 2 mM EDTA aqueous solution. The two 16-pads nitrocellulose slides were incubated with Zika positive human sera and captured antibodies were detected using an alkaline phosphatase (AP) labelled secondary antibody to provide colorimetric quantification of peptide immune-recognition, using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). We could immediately assess a striking difference

on the extent of captured antibodies between clickable and uncoated nitrocellulose. While for untreated nitrocellulose colored spots were indeed barely detectable and inhomogeneous, i.e. highlighting poor immune recognition, the chemoselectively bound probes were efficiently recognized giving rise to dark-blue colored regular spots (Figure 3-a). This is mostly indicative that peptides affinity to their target antibodies is strongly compromised by a nonspecific strategy of immobilization, where immune-reactive regions are sacrificed upon surface adsorption. This may be particularly relevant in all of those demanding assays that do not rely on an exquisite affinity of the immobilized bioprobe for the target antibody. We then confirmed that the preserved immunoreactivity of P1, P2, P3 probes on the clickable nitrocellulose support could be translated into efficient discrimination between Zika positive individuals and healthy controls (Figure 3-b). As we previously observed, the combined presentation of peptides P2 and P3 (probe C) proved the most effective in providing statistically relevant discrimination between the two groups with $p < 0.01$ (Figure 3-c).

Figure 3. a): representative clickable and uncoated nitrocellulose pads spotted with peptide arrays after incubation with a Zika positive sample, dark-blue regular spots are detectable only on polymer coated pads. b): representative coated and uncoated nitrocellulose pads spotted with peptide arrays (see spotting scheme on the right) after incubation with a Zika positive sample and a healthy control, discrimination between infected and healthy serum is only possible on clickable nitrocellulose. c): Scatter plots reporting mean immunoreactivity with SD of Zika positive and healthy control individuals and results of the unpaired t Test Zika infection diagnosis probe P1, P2, P3 and C (combination of peptides P2 and P3). ns = not significant. Significant: $p < 0.05$; * = $p < 0.05$; ** = $p < 0.01$. Photographs of whole 16-pad microarray slides available in the Supporting Information (Figure S3 and S4)

This underlines the possibility to finely control peptidic probes surface display even on cellulosic supports, which is, to the best of our knowledge, an unprecedented result in paper-based bioassays. It is also worth underlining that, in the same assay, an alternative oriented bioconjugation strategy based on biotinylated peptides and streptavidin functionalized

nitrocellulose resulted in poor immunoreactivity and inconsistent discrimination between healthy and infected samples (Supporting Information, Figure S5). These results are worth of particular consideration as celluloses are rather complex substrates, mostly due to peculiar porosity properties and related high surface-to-volume ratio that can amplify interface effects such as nonspecific interactions and the formation of interfering multiple bioprobe layers.

CONCLUSIONS

Here, we move beyond current limitations of paper-based bioassays by introducing an easy and user-friendly method for peptide chemoselective bioconjugation onto nitrocellulose supports based on a clickable polymeric coating. We demonstrate that our strategy is suitable for peptide probes stable binding while avoiding to compromise their immunoreactivity to target antibodies, since nonspecific interactions with the nitrocellulose support are abolished. The structural properties of nitrocellulose are maintained, but the wettability and anti-fouling properties provided by the polymeric coating are effective in suppressing background noise signal even when complex biological samples, such as sera (see Supplementary Information Figure S6). We demonstrate that these favorable features can be exploited to perform sophisticated molecular bioassays, in this case the serological discrimination between Zika positive individuals and healthy controls, using a peptide-based assay. Overall, our results enrich the toolbox for the controlled manipulation of cellulosic material interfacial properties, broadening their compatibility with small-sized peptide molecular probes.

ASSOCIATED CONTENT

Supporting Information

Full experimental details and additional Figures are available in the Supporting Information file

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Supplementary Information

Clickable cellulosic surfaces for peptide-based bioassays

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Materials and Methods

Reagents

Reagents for peptide synthesis were from Iris Biotech (Marktredwitz, Germany). Other chemicals were from Sigma-Aldrich (St. Louis, MO, USA) if not stated otherwise. All solvents for solid-phase peptide synthesis (SPPS) were used without further purification. HPLC grade acetonitrile (ACN) and ultrapure 18.2 Ω water (Millipore-MilliQ) were used for the preparation of all buffers for liquid chromatography. The chromatographic columns were from Phenomenex (Torrance CA, USA). HPLC eluent A: 97.5% H₂O, 2.5% ACN, 0.7%TFA; HPLC eluent B: 30% H₂O, 70% ACN, 0.7%TFA. Alkaline Phosphatase-conjugated AffiniPure F(ab')₂ Fragment Rabbit Anti-Human IgG was obtained from Jackson ImmunoResearch (West Grove, PA, USA). LowCross-Buffer was from Candor (Wangen, Germany).

Copoly Azide was obtained from MCP-2 (Lucidant Polymers, Sunnyvale CA) by post-polymerization modification reaction with 3-azido-1-propanamine as reported in [L. Sola, F. Damin, P. Gagni, R. Consonni, M. Chiari, Synthesis of Clickable Coating Polymers by Postpolymerization Modification: Applications in Microarray Technology, Langmuir. 32 (2016)]

Peptide Synthesis

Peptides p1-p3 were prepared and characterized as previously reported [L. Sola, P. Gagni, I. D'Annessa, R. Capelli, C. Bertino, A. Romanato, F. Damin, G. Bergamaschi, E. Marchisio, A. Cuzzocrea, et al., *ACS Infect. Dis.* **2018**, *4*, 998–1006]. Briefly, all peptides were synthesized by stepwise microwave-assisted Fmoc-SPPS on a Biotage ALSTRA Initiator+ peptide synthesizer according to Fmoc-based protocols. Sequences were assembled on a 2-CTC resin and chain elongation was performed by iterative cycles of amino acids coupling (1 x 20min, 50 C; Oxyma/DIC as activators) and Fmoc-deprotection using a 20% piperidine solution in DMF (1x10min). Upon complete chain assembly, peptides were cleaved from the resin using a 2.5% TIS, 2.5% thioanisole, 2.5% water, 92.5% TFA mixture. Crude peptides were then purified by preparative RP-HPLC. MS analysis was performed separately on purified material.

Samples

A panel of positive ZIKV serum samples was purchased from Boca Biolistics (Pompano Beach, FL). Samples were proved positive by MAC Elisa test performed by the purchaser and confirmed positive for anti-Zika IgG by an NS1 based ELISA test (ZIKV IgG from Dia.Pro. (Sesto San Giovanni, Italy) according to manufacturer instructions. Human sera from healthy donors used in the study were obtained from voluntaries, which donated blood for transfusion purposes with the

collaboration of Transfusion Centre (IRCCS Ca' Granda Policlinico Ospedale Maggiore in Milan, Italy) and their use was permitted for research purposes only. Both donor and recipient's identities are protected by the privacy laws governing the Italian Transfusion System. The purpose of production of blood products is only of the donor's free will and with the latter's consent.

Nitrocellulose and Whatman paper coating

Nitrocellulose slides (Fast Slide 16-Pad, Whatman®), nitrocellulose blotting membranes (BioTrace NT Blotting membranes from PALL), Whatman filter paper 1, were coated by Copoly Azide by immersion in a polymer solution (1% w/v in 0.9M $(\text{NH}_4)_2 \text{SO}_4$) for 30 minutes, then rinsed with water, dried under nitrogen and cured for 15 min under vacuum at 80°C. Blotting membranes were used for the FT-IR measurements and for the SEM microscopy experiments. 16-Pad Slides were used for the contact angle measurements and the bioassays

Peptide microarrays

Peptides were first dissolved in DMSO to 1 mM stock solution and then diluted to the final spotting concentration (100 μM) into the printing buffer for uncoated slides (PBS) or for CuACC conjugation on Copoly Azide coated surfaces (25 mM Na/Acetate pH 4.8, 15 mM Trehalose, 100 μM CuSO_4 , 400 μM THPTA and 6.25 mM Ascorbic Acid). Microarrays were arrayed using a non-contact S12 Spotter (Scienion Co., Berlin, Germany), depositing 5 drops for each spot. All the peptides samples were printed in replicates. Up to sixteen arrays were spotted on the slides, corresponding to the sixteen compartments created by NEXTERION* IC-16 sixteen well Incubation Chamber from Schott (Jena, Germany). The same protocol was applied to nitrocellulose uncoated slides.

Printed slides were placed in a humid chamber and incubated overnight at room temperature. The following day the slides coated with Copoly Azide were immersed in 2 mM EDTA water solution for 1h, while uncoated slides were immersed in a solution of 1% BSA in PBS. Finally, the slides were washed with water and dried under a stream of nitrogen.

Bioassay

Sera were diluted 3:50 v/v in LowCross-Buffer and 50 μL of sample were added into each microarray well, incubated for 30 minutes on a shaker (70 rpm, 22°C). ZIKV positive and healthy sera were incubated in parallel app to 16 samples per slide. Negative controls included blank arrays incubated only with the incubation buffer. The microarray slides were then rinsed for 15 minutes with washing buffer (0.05 M Tris/ HCl pH 9, 0.25 M NaCl, 0.05% v/v Tween 20) and incubated statically with 50 μL of Alkaline Phosphatase-conjugated AffiniPure F(ab')₂ Fragment Rabbit Anti-Human IgG 1mg/mL diluted 1:300 in LowCross-Buffer for 1 hour. The slides were

washed with PBS for 5 minutes and then with washing buffer for further 5 minutes. For colorimetric development, the slides were incubated with BCIP/NBT (Blue Liquid Substrate System for Membranes) from Sigma for 10 minutes in static manner, then washed with water and dried under nitrogen.

Detection

The results were visible by naked eye. Pictures of the arrays were taken using a stereo-microscope equipped with a Nikon SMZ 1500 camera and the Infinity Capture software from Lumenera Corporation. ImageJ software was used to analyse the images as follows: luminosities of spots and background were evaluated through the “Measure” function designing circular areas whose colour intensity was calculated by subtracting the background around the spots. The results were expressed as the absolute values of the negative contrast.

Material characterization

Infrared spectra of paper strips were recorded using a Nicolet Nexus FTIR spectrometer equipment with U-ATR device from 400 to 4,000 cm^{-1} . For each sample, spectra were collected (128 scans) using solid FT-IR support. The morphology of the gold sputtered materials was fully characterized using a EvoMA10 (Zeiss, Oberkochen, Germany) Scanning Electron Microscope (SEM) equipped with a LaB6 filament. The wettability properties of untreated and clickable nitrocellulose were evaluated by static water contact angle measurements using a KSV Instruments Contact Angle equipped with a CAM 200 camera.

Supplementary Figures

Figure S1: SEM images of the surfaces (a) unmodified b) polymer-coated Whatman filter paper (scale bar 1 μm).

Figure S2: FT-IR spectra of the functionalized nitrocellulose/polymer containing nitrocellulose (black dashed line) and the uncoated nitrocellulose (grey solid line). The band at 2100 cm⁻¹ is assigned to azide group stretching.

Figure S3: photographs of 16 pad nitrocellulose slides incubated with 14 Zika positive serum samples on copoly Azide modified surface (left) and on uncoated support (right). Immunoreactivity of peptide spots is much higher on the clickable surface. Peptide spots on the uncoated slide are barely visible . Colorimetric reaction was prolonged on both slides to enhance the visibility of peptide spots on the uncoated nitrocellulose.

Figure S4: photographs of 16 pad nitrocellulose slides incubated with 6 healthy control sera (columns H) and 6 Zika positive serum samples (column Z+) on copoly Azide modified surface (left slide) and on uncoated support (right slide). Slides were used in parallel using the same bioassay conditions. Colorimetric reaction with enzyme substrates BCIP/NBT was standardized at 5 minutes for all surfaces and samples. On clickable surface, for all analysed samples, the Zika positive sera provided distinguishable coloured spots of much higher intensity in comparison to that provided by the healthy controls. On the contrary, on uncoated nitrocellulose, in the same conditions of assay and colorimetric reaction, no spots were distinguishable from background.

Figure S5: uncoated 16 pads nitrocellulose slide co-spotted with 3 droplets streptavidin (1 mg/mL) and 3 droplets of 50 μ M biotinylated peptides. Spots are barely visible for some of the samples and no discrimination between healthy and positive sera is feasible

Figure S6: comparison of background signal nitrocellulose pads after incubation with serum and Alkaline Phosphatase-conjugated AffiniPure F(ab')₂ Fragment Rabbit Anti-Human IgG. Colorimetric reaction with enzyme substrates BCIP/NBT was standardized at 5 minutes for all surfaces revealing differences in background among the tested surfaces. The uncoated nitrocellulose provided the highest background, even when surface was blocked by BSA. The clickable (copoly Azide modified) nitrocellulose showed the lowest background. Notably, background is not affected by blocking or washing, demonstrating the inherent anti-fouling properties of the copoly Azide coating.

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