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.

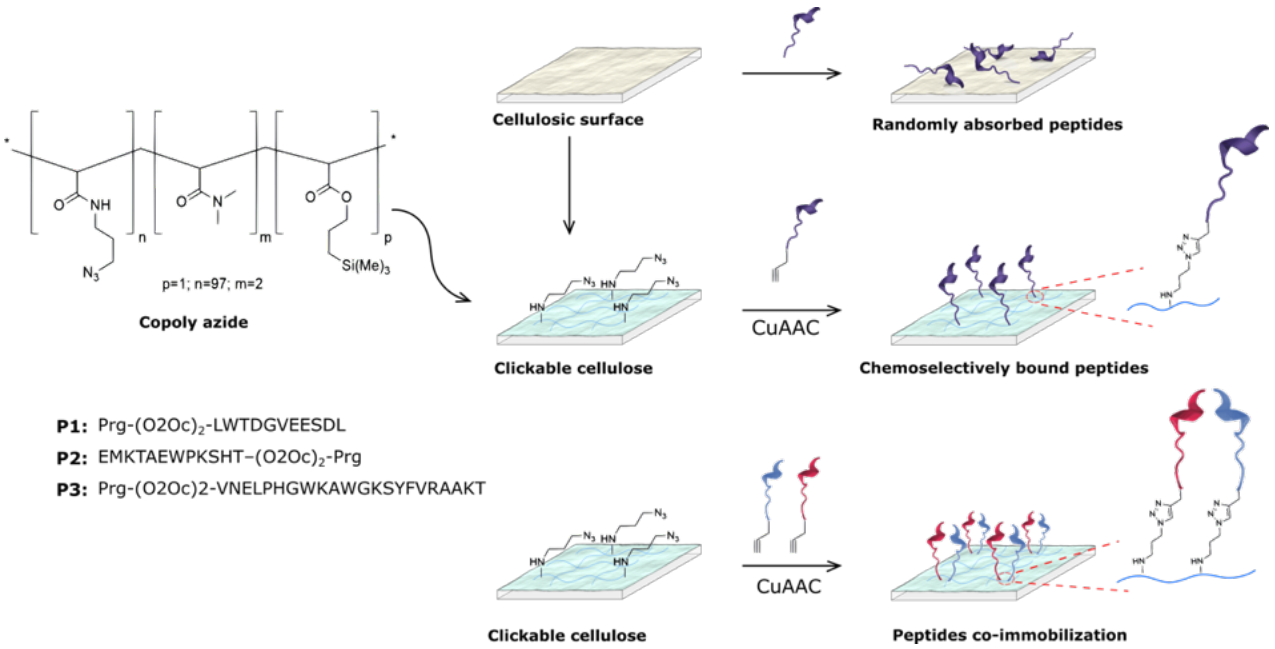
INTRODUCTION

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 immunodiagnostics, 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 bioconjugation [11-16]. 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-acryloyloxysuccinimide (NAS), and 3-(trimethoxysilyl)-propylmethacrylate (MAPS) copolymer [20, 21], using 3-azido-1-propanamine to introduce azido groups [22] (Scheme 1).

EXPERIMENTAL

Nitrocellullose was coated by immersion in Copoly Azide solution (1% w/v in 0.9M $(NH_4)_2 SO_4$) 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 (WCAhst).

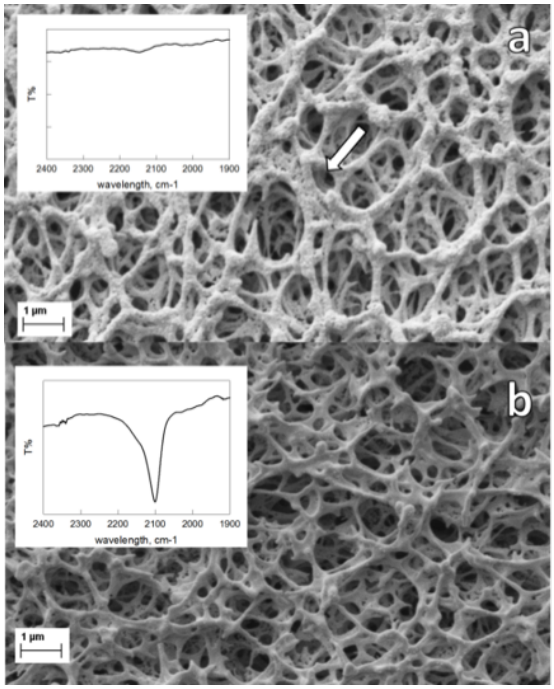


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Figure 1: SEM images and FT-IR spectra (inset) of the surfaces of (**a**) unmodified nitrocellulose and (**b**) copoly Azide coated nitrocellulose (scale bar 1 µm). The distinctive azide stretching band at 2100 cm−1 is clearly detectable on polymer 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 WCAhst 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 WCAhst 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 WCAhst measurements after prolonged exposure to water, which showed unaltered behaviour (WCAhst value of 16.4° after 4 days).

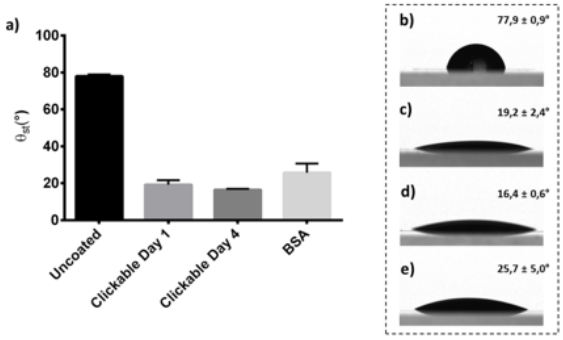

Figure 2 Water Contact Angle (WCA) measurements (**a**) of uncoated (**b**) and polymer coated nitrocellulose at day 1 (**c**) and after 4 days (**d**). Wettability after polymer coating is similar to that obtained after treatment with BSA (**e**).

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).

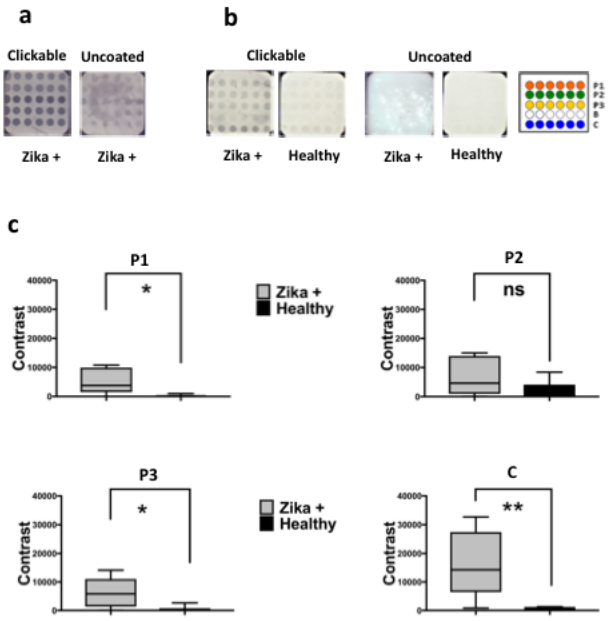

Figure 3. **a):** representative clickable and uncoated nitrocellulose pads spotted with peptide arrays after incubation with a Zika positive sample, dark-blue regular spots are detectable only on polymer coated pads. **b**): representative coated and uncoated nitrocellulose pads spotted with peptide arrays (see spotting scheme on the right) after incubation with a Zika positive sample and an 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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REFERENCES

(1) López-Marzo, A. M.; Merkoçi, A. Paper-Based Sensors and Assays: A Success of the Engineering Design and the Convergence of Knowledge Areas. Lab Chip 2016, 16 (17), 3150–3176. https://doi.org/10.1039/C6LC00737F.

(2) Parolo, C.; Merkoçi, A. Paper-Based Nanobiosensors for Diagnostics. Chemical Society Reviews. 2013, pp 450–457. https://doi.org/10.1039/c2cs35255a.

(3) Klemm, D.; Heublein, B.; Fink, H.-P.; Bohn, A. Cellulose: Fascinating Biopolymer and Sustainable Raw Material. Angew. Chemie Int. Ed. 2005, 44 (22), 3358–3393. https://doi.org/10.1002/anie.200460587.

(4) Martinez, A. W.; Phillips, S. T.; Whitesides, G. M.; Carrilho, E. Diagnostics for the Developing World: Microfluidic Paper-Based Analytical Devices. Anal. Chem. 2010, 82 (1), 3–10. https://doi.org/10.1021/ac9013989.

(5) Quesada-González, D.; Merkoçi, A. Nanoparticle-Based Lateral Flow Biosensors. Biosens. Bioelectron. 2015, 73, 47–63. https://doi.org/10.1016/j.bios.2015.05.050.

(6) Singh, A. T.; Lantigua, D.; Meka, A.; Taing, S.; Pandher, M.; Camci-Unal, G. Paper-Based Sensors: Emerging Themes and Applications. Sensors (Switzerland). 2018. https://doi.org/10.3390/s18092838.

(7) Hu, J.; Zhang, Z.-L.; Wen, C.-Y.; Tang, M.; Wu, L.-L.; Liu, C.; Zhu, L.; Pang, D.-W. Sensitive and Quantitative Detection of C-Reaction Protein Based on Immunofluorescent Nanospheres Coupled with Lateral Flow Test Strip. Anal. Chem. 2016, 88 (12), 6577–6584. https://doi.org/10.1021/acs.analchem.6b01427.

(8) Morales-Narváez, E.; Naghdi, T.; Zor, E.; Merkoçi, A. Photoluminescent Lateral-Flow Immunoassay Revealed by Graphene Oxide: Highly Sensitive Paper-Based Pathogen Detection. Anal. Chem. 2015, 87 (16), 8573–8577. https://doi.org/10.1021/acs.analchem.5b02383.

(9) Li, H.; Steckl, A. J. Paper Microfluidics for Point-of-Care Blood-Based Analysis and Diagnostics. Anal. Chem. 2019, 91 (1), 352–371. https://doi.org/10.1021/acs.analchem.8b03636.

(10) de Puig, H.; Bosch, I.; Gehrke, L.; Hamad-Schifferli, K. Challenges of the Nano–Bio Interface in Lateral Flow and Dipstick Immunoassays. Trends in Biotechnology. 2017, pp 1169–1180. https://doi.org/10.1016/j.tibtech.2017.09.001.

(11) Kong, F.; Hu, Y. F. Biomolecule Immobilization Techniques for Bioactive Paper Fabrication. Anal. Bioanal. Chem. 2012, 403 (1), 7–13. https://doi.org/10.1007/s00216-012-5821-1.

(12) Riccardi, C.; McCormick, S.; Kasi, R.; Kumar, C. A Modular Approach for Interlocking Enzymes in Whatman Paper. Angew. Chemie 2018, 130 (32), 10315–10319. https://doi.org/10.1002/ange.201805074.

(13) Deraney, R. N.; Mace, C. R.; Rolland, J. P.; Schonhorn, J. E. Multiplexed, Patterned-Paper Immunoassay for Detection of Malaria and Dengue Fever. Anal. Chem. 2016, 88 (12), 6161–6165. https://doi.org/10.1021/acs.analchem.6b00854.

(14) Große, S.; Wilke, P.; Börner, H. G. Easy Access to Functional Patterns on Cellulose Paper by Combining Laser Printing and Material-Specific Peptide Adsorption. Angew. Chemie Int. Ed. 2016, 55 (37), 11266–11270. https://doi.org/10.1002/anie.201601603.

(15) Draz, M. S.; Moazeni, M.; Venkataramani, M.; Lakshminarayanan, H.; Saygili, E.; Lakshminaraasimulu, N. K.; Kochehbyoki, K. M.; Kanakasabapathy, M. K.; Shabahang, S.; Vasan, A.; et al. Hybrid Paper-Plastic Microchip for Flexible and High-Performance Point-of-Care Diagnostics. Adv. Funct. Mater. 2018, 28 (26), 1707161. https://doi.org/10.1002/adfm.201707161.

(16) Dong, Y.; Chen, H.; Zhang, S.; Wang, P.; Lu, X.; Liu, W.; Wu, Z. "Click-Chemical" Modification of Cellulose Acetate Nanofibers: A Versatile Platform for Biofunctionalization. J. Mater. Chem. B 2018, 6 (28), 4579–4582. https://doi.org/10.1039/c8tb01401a.

(17) Guo, J.; Filpponen, I.; Johansson, L.-S.; Heiβler, S.; Li, L.; Levkin, P.; Rojas, O. J. Micro-Patterns on Nanocellulose Films and Paper by Photo-Induced Thiol–yne Click Coupling: A Facile Method toward Wetting with Spatial Resolution. Cellulose 2018, 25 (1), 367–375. https://doi.org/10.1007/s10570-017-1593-2.

(18) Gori, A.; Sola, L.; Gagni, P.; Bruni, G.; Liprino, M.; Peri, C.; Colombo, G.; Cretich, M.; Chiari, M. Screening Complex Biological Samples with Peptide Microarrays: The Favorable Impact of Probe Orientation via Chemoselective Immobilization Strategies on Clickable Polymeric Coatings. Bioconjug. Chem. 2016, 27 (11), 2669–2677. https://doi.org/10.1021/acs.bioconjchem.6b00426.

(19) Gori, A.; Cretich, M.; Vanna, R.; Sola, L.; Gagni, P.; Bruni, G.; Liprino, M.; Gramatica, F.; Burastero, S.; Chiari, M. Multiple Epitope Presentation and Surface Density Control Enabled by Chemoselective Immobilization Lead to Enhanced Performance in IgE-Binding

Fingerprinting on Peptide Microarrays. Anal. Chim. Acta 2017. https://doi.org/10.1016/j.aca.2017.06.027.

(20) Pirri, G.; Damin, F.; Chiari, M.; Bontempi, E.; Depero, L. E. Characterization of A Polymeric Adsorbed Coating for DNA Microarray Glass Slides. Anal. Chem. 2004, 76 (5), 1352–1358. https://doi.org/10.1021/ac0352629.

(21) Cretich, M.; Di Carlo, G.; Longhi, R.; Gotti, C.; Spinella, N.; Coffa, S.; Galati, C.; Renna, L.; Chiari, M. High Sensitivity Protein Assays on Microarray Silicon Slides. Anal. Chem. 2009, 81 (13). https://doi.org/10.1021/ac900658c.

(22) Sola, L.; Damin, F.; Gagni, P.; Consonni, R.; Chiari, M. Synthesis of Clickable Coating Polymers by Postpolymerization Modification: Applications in Microarray Technology. Langmuir 2016, 32 (40), 10284–10295. https://doi.org/10.1021/acs.langmuir.6b02816.

(23) Sola, L.; Gagni, P.; D'Annessa, I.; Capelli, R.; Bertino, C.; Romanato, A.; Damin, F.; Bergamaschi, G.; Marchisio, E.; Cuzzocrea, A.; et al. Enhancing Antibody Serodiagnosis Using a Controlled Peptide Coimmobilization Strategy. ACS Infect. Dis. 2018, 4 (6), 998–1006. https://doi.org/10.1021/acsinfecdis.8b00014.