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Addressing the distribution of proteins spotted on μPADs

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Adsorption is the most common approach to immobilize biorecognition elements on the surface of paper-based devices. Adsorption is also the route selected to coat the substrate with albumin, therefore minimizing the interaction of other proteins. While similar in nature, the structure of the selected proteins as well as the conditions selected from the immobilization have a significant effect on the amount and distribution of the resulting composites. To illustrate these differences and provide general guidelines to efficiently prepare these devices, this article explores the interaction (adsorption and desorption) of BSA with 3MM chromatography paper. The experimental conditions investigated were the protein concentration, the interaction time, the number of times the protein was spotted, the pH of buffer solution, and the ionic strength of the buffer solution. The proposed approach mimics the steps involved in the fabrication (adsorption) and use (rinsing induced by the sample) of paper-based microfluidic devices. To identify the protein location following the rinsing step, the protein was fixed by dehydration in a convection oven and then stained using Coomassie Blue. The color intensity, which was found to be proportional to the amount of protein immobilized, was determined using a desktop scanner. To highlight the importance of understanding the adsorption process to the rational development of μPADs, results were complemented by experiments performed with lysozyme and immunoglobulin G.

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1. Introduction

One of the most active areas in analytical chemistry is the development and application of microfluidic paper-based analytical devices (μPADs). Since their introduction, about a decade ago, these devices have been extensively used in diverse clinical, environmental, and defense applications.[1] Their versatility, low-cost, portability, and simplicity offered for performing chemical tests are some of the most significant advantages of this technology.[2] Although different detection systems have been coupled with μPADs (*e.g.*, electrochemical, optical, chemiluminescence, and fluorescence), the most commonly used detection approach is based on the selective oxidation of the analyte using an enzyme followed by a peroxidase-based reaction that catalyzes the oxidation of a reagent, yielding a change in color. This strategy has been incredibly successful for the analysis of small molecules including biomarkers[3,4] and contaminants.[5] While it is expected that reagents and enzymes would remain in place and yield a uniform colorimetric response, the opposite is often reported,[6,7] rendering noticeable color gradients that affect the detection performance of these devices. To address this issue and strike a balance between the forces controlling the interaction (that can also induce conformational changes), a detailed understanding of the interaction of proteins with the cellulose substrate is clearly required.

The analysis of protein-based biomarkers is a second group of μPADs applications where understanding the interactions of proteins with the paper fibers can render significant improvements in performance. Although the number of reports in this category is significantly smaller than those concerning small molecules, μPADs have been successfully used to quantify hormones,[8] enzymes,[9,10] cancer biomarkers,[11–13] immunoglobulins,[14,15] and even viruses.[16,17] Because all of these applications require the quantitative transfer of the target molecules from the sample spot to the reaction chamber, 0.1–5% bovine serum albumin (BSA) is commonly added to the device in order to minimize non-specific adsorption of proteins and/or reagents.[6,18] BSA has also been used to optimize the immobilization of glucose oxidase to cellulose,[19] significantly increasing its storage stability.

Considering that nonspecific protein adsorption to cellulose has been identified as a major barrier to the use of paper as a platform for microfluidic bioassays,[20] this report aims to provide information related to how (and how fast) a model protein interacts with paper. As a first approach, BSA was chosen as the target molecule because it is a soft protein with well-known physico-chemical characteristics and wide-

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spread use in the μPAD community. BSA also tends to adsorb to a variety of surfaces, including those with hydrophilic characteristics.[21] While a variety of cellulose substrates have been reported in literature,[22] the herein described experiments were performed on 3MM chromatography paper because it provides a convenient balance between porosity and wicking speed.[23] The results obtained with BSA are also complemented by experiments performed with other proteins (lysozyme and Immunoglobulin G) with different physico-chemical characteristics.[24]

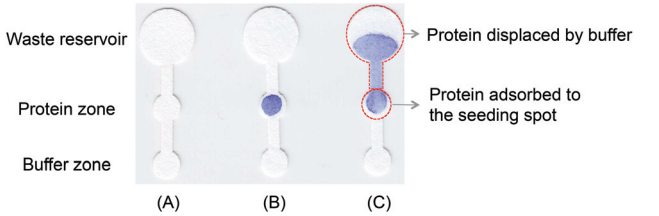

Fig. 1 Optical images showing the paper chips obtained after: the engraving (A), the protein spotting (B), and the introduction of running buffer solution (C). In all cases, the devices were thermally treated and then stained using Coomassie blue. The figure also shows the templates used to measure the average color intensity in each zone of the device.

2. Experimental design

Reagents and solutions

All aqueous solutions were prepared using 18 MΩ cm water (NANOpure Diamond, Barnstead; Dubuque, IA) and analytical reagent grade chemicals. Bovine serum albumin (BSA), sodium hydroxide, and sodium phosphate monobasic anhydrous were purchased from Fisher Scientific (Fair Lawn, NJ). Lysozyme (Lys, from chicken egg white) and Brilliant Blue R were obtained from Sigma-Aldrich (St Louis, MO). Immunoglobulin G (IgG) from human plasma was purchased from Lee BioSolutions, Inc. (St Louis, MO). Citric acid was acquired from Aldrich Chemical Co. (Milwaukee, WI). Glacial acetic acid and methanol were purchased from VWR International, LLC (Radnor, PA). Whatman 3 MM Chromatography Paper (189 g m^{-2}) was obtained from GE Healthcare (Pittsburgh, PA). The pH of different solutions was adjusted using 1 mol L^{-1} NaOH or HCl and measured using a glass electrode and a digital pH meter (Orion 420A+, Thermo; Waltham, MA). BSA and Lys solutions (0.4, 1.0, 1.5, 2.0, 4.0, 8.0, and 20.0 mg mL^{-1}) were prepared by dissolving a known amount of protein in 10 mmol L^{-1} buffer solution at a pH matching the corresponding IEP (isoelectric point), pH = IEP ± 1, or pH = 7.2. Solutions of IgG containing 0.4, 1.0, 1.5, 2.0, 4.0, 8.0, and 20.0 mg mL^{-1} of immunoglobulin were prepared in 10 mmol L^{-1} phosphate buffer at pH = IEP and pH = IEP ± 1. The staining solution was prepared by dissolving 10 mg of Brilliant Blue R in 10 mL of an aqueous solution containing 50% v/v methanol and 10% v/v acetic acid.

Substrate, staining procedure, and data processing

3MM chromatography paper was selected as substrate for the protein immobilization, following previous publications from our research group.[23,25] In order to pattern uniform paper chips, 3MM chromatography paper sheets were cut using a commercial CO_2 laser engraver (Mini24, Epilog Laser Systems; Golden, CO, USA) using the design shown in Fig. 1. In this case, the paper chips were composed of 3 circles interconnected by 2 channels (length = 5 mm and width = 2 mm). In order to mimic conditions leading to protein displacement, the selected protein (0.5 μL) was spotted in the central zone (d = 5 mm) and allowed to interact with the substrate under ambient conditions for 30 min (unless otherwise stated). At this point it is important to point out that this time was selected as a compromise between the time required to prepare the chip and that required for BSA to adsorb and undergo surface-induced structural rearrangements.[26] This consideration is also in line with results reported by our group,[27–30] specifically addressing the adsorption kinetics of BSA to various substrates. Then (and unless otherwise stated), 15 μL of buffer were spotted in the buffer zone (d = 5 mm) and allowed to wick through the device (through the protein-modified zone) towards the waste reservoir (d = 10 mm). This step was implemented to displace the unbound fraction of BSA.

Next, the paper chips modified with the selected protein were transferred to a convection oven (80 °C) for 10 min to fix the protein molecules to the paper before staining. Although it is unlikely that proteins would leach out to the staining solution (containing 50% v/v methanol and 10% v/v acetic acid), the thermal step was implemented to minimize that possibility. The following staining steps allowed for estimating what fraction of the protein remained in the central zone (where the protein was initially spotted) and what fraction was displaced by the buffer. For this purpose, the paper chips were then soaked in 0.1% w/v Coomassie blue for 10 min. Next, the excess dye was removed by placing the devices in a vial containing 10 mL of a mixture of 10% v/v acetic acid and 50% v/v methanol in water. This rinsing step was carried out under continuous agitation (80 rpm for 1 h), replacing the solution every 30 min. After drying at room temperature, an image of each paper chip was acquired using a flatbed scanner (Canon, CanonScan Lide700F) to be later analyzed using Adobe Photoshop CC 2015.5. For the analysis, the protein zone and the transfer zone were measured using a common template (see Fig. 1) and then the mean color intensity was calculated. In all cases, the reported data points and error bars correspond to the average and the standard deviation, respectively, of at least 5 devices. As a representative example, Fig. 1 contains images of the devices before the addition of BSA (A), after the addition of BSA (B, no buffer added), and after the addition of the buffer to the buffer zone (C).

It is also important to note that when the buffer was added to the device after the thermal treatment, no significant differences were observed, yielding to devices that resemble the one shown in Fig. 1B.

3. Results and discussion

As previously stated, the described experiments were performed by adsorbing BSA under various experimental conditions, including the protein concentration, the time between when the protein was spotted and washed with the buffer solution (0–90 min), the number of times the protein was spotted (1–5) before washing with buffer solution, the pH of buffer solution (IEP ± 1, and physiological pH = 7.2), and the ionic strength of the buffer solution (adding 0.1–100 mg mL^{-1} NaCl). Additional experiments were performed with two other proteins (Lys and IgG) to identify the role of protein structure on the adsorption process. Although the results presented in this manuscript were obtained using 3MM chromatography paper, preliminary experiments indicated that the overall conclusions could be extended to other paper types; once the differences in thickness/porosity[23] are accounted for (data not shown).

In order to evaluate the effect of protein concentration on the adsorption of BSA on 3MM chromatography paper, solutions containing 0.4–20.0 mg mL^{-1} were prepared in 10 mmol L^{-1} citrate buffer at pH = 4.7, which is BSA's isoelectric point (IEP). The solutions were first used to confirm the proportionality between the color intensity and the amount of protein in the paper. For these experiments, the protein solution was spotted on the protein zone and allowed to interact with the substrate at room temperature for 30 min. Then, the paper chips were placed in the oven, stained, and the color intensity measured as described in the Experimental section. Fig. 2A presents a summary of the results obtained, where the color intensity (measured in the protein zone or the adjacent channel), was analyzed as a function of the protein concentration used. Because these results were collected by fixing the spotted protein (no buffer rinsing step, equivalent to the device shown in Fig. 1B), they are equivalent to a calibration curve. In this case, it can be observed that a negligible color intensity was detected in the adjacent channel, therefore confirming that all the protein spotted remained in the seeding spot. Fig. 2A also shows that under these conditions, color intensities of up to 80 A.U. can be considered proportional to the amount of protein present in the spot. This value corresponds to 4 μg of BSA (0.5 μL of a solution containing 8 mg mL^{-1}) distributed in the seeding spot (d = 5 mm). Although a sensitivity of 10.6 ± 0.6 A.U. mg^{-1} mL can be calculated from these results, it is important to mention that the color intensity would be affected by the spatial distribution of the protein and that very likely, not all of the protein present in the 3D structure of the paper is detected by the scanner. For these reasons, the color intensity will be considered to provide only semi-quantitative information about the amount of protein in each area.

The subsequent set of experiments were designed to determine how much of the spotted protein remained in the protein zone and how much would be displaced by the buffer. This experiment mimics the typical steps involved in the preparation and use of a device, respectively. As it can be observed in Fig. 2B, the results resemble those obtained in a classic adsorption isotherm and show a rapid increase in the adsorbed amount (color intensity) leading to a plateau when the [BSA] in solution reached approximately 8 mg mL^{-1}. While it is possible that the color intensity of the plateau could be influenced by the staining/reading procedure, these results are analyzed also considering the color development in the adjacent channel/reservoir. Although the saturation value herein calculated is significantly higher than those reported by Jeyachandran[21] (saturation reached at ~1 mg mL^{-1}) or Norde[26] (saturation reached at ~4 mg mL^{-1}), the difference can be reasonably attributed to the fact that the exposed area in paper is significantly larger than the corresponding geometric area. It is critically important to state that because these experiments are very different from traditional adsorption experiments (solvent evaporation, no equilibrium, *etc.*), estimating accurate thermodynamic parameters from these results would be extremely challenging.[31] Fig. 2B also shows that when the BSA concentration was greater than 4 mg mL^{-1}, a fraction of the protein spotted was transferred upon the addition of buffer. This behavior is especially important at higher protein concentrations (*e.g.* 20 mg mL^{-1} BSA), where similar color intensities were observed in both the seeding spot and the

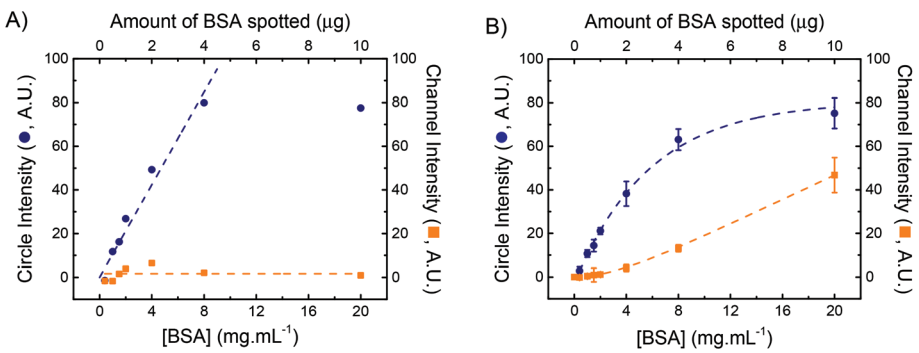

Fig. 2 A. Intensity of the stained BSA at the protein zone and the adjacent channel/reservoir as a function of the BSA concentration used for spotting. Lines included to guide the eye. B. Intensity of the stained BSA at the protein zone and the adjacent channel/reservoir as a function of the BSA concentration used for spotting, upon the running buffer solution was introduced. Lines included to guide the eye.

adjacent channel (see Fig. 1C). Because these experimental results show that only a fraction of the BSA would adsorb to the cellulose, researchers interested in using BSA as a blocking agent should carefully balance the concentration required to saturate the surface of the paper (approximately 8 mg mL^{-1}) with the possibility of BSA leaching to downstream structures (more significant when [BSA] > 4 mg mL^{-1}). While accurate information about surface coverage is currently not available, this information is critical for the rational design of μPAD because (as a strong ligand) BSA could displace other proteins from the cellulose by competition and therefore affect the analytical performance of the device.

While the formation of multilayer arrangements of BSA (during a single adsorption event) is unlikely, a common practice during the development of μPADs is the deposition of proteins using multiple spotting steps. In order to follow this practice and possibly increase the amount of BSA attached to the surface, 0.5 μL of either 1 mg mL^{-1} or 2 mg mL^{-1} BSA in 10 mmol L^{-1} citrate buffer solution at pH = 4.7 were spotted multiple times on different paper chips. During each experiment, the BSA solution spotted on the paper chip was allowed to dry (at room temperature) for 30 min before re-spotting. The experimental results are shown in Fig. 3, where the color intensity in both the spotting zone and the adjacent channel/reservoir was measured.

As can be observed in Fig. 3A and B, this strategy led to significant increases in the color intensity of both the protein zone and the adjacent channel, showing a direct relationship between the color intensity and the number of times the protein was spotted on the paper chip. Because this strategy allowed the newly spotted protein to interact with the modified surface, these results suggest that this may be the most convenient option to increase the amount of protein in the seeding spot (Fig. 3A). As expected, the color intensity was significantly higher when a solution containing 2 mg mL^{-1} BSA was used. However, it is important to note that the latter conditions (Fig. 3B) led to the displacement of a larger fraction of the BSA spotted, clearly visible when BSA was spotted 5 times. In agreement with the previous experimental data described in Fig. 2, the greater the amount of BSA dispensed (higher concentration or spotting multiple times), the greater the adsorbed amount. Again, for the selected spot dimension (d = 5 mm), 2–4 μg of BSA seem to saturate the surface without significantly leaching to downstream elements.

The aforementioned experiments were performed considering (based on literature reports) that 30 min represents a reasonable balance between the time required to fabricate the devices and time required for the interaction between the proteins and the cellulose fibers to reach an equilibrium. To determine if this assumption can be considered valid when adsorbing BSA to cellulose fibers, the effect of the time elapsed between the moment when the protein was spotted on the protein zone and the time when the buffer solution was dispensed on the device was investigated. In this case, 0.5 μL of 2 mg mL^{-1} BSA in 10 mmol L^{-1} citrate buffer at pH = 4.7 was spotted on the chip and allowed to dry at room temperature for a selected period of time, ranging between 0 and 90 min. Next, the paper chips were processed and measured as previously described. Fig. 4 includes a summary of the results obtained, showing the distribution of BSA on either the central circle or the adjacent channel, as a function of the interaction time (note the difference in scale with respect to previous figures).

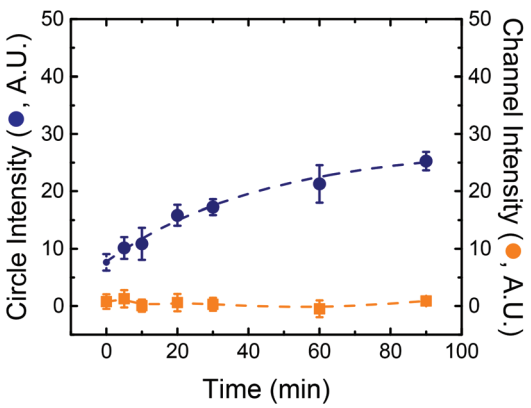

Fig. 4 Intensity of the stained BSA at the protein zone and the adjacent channel/reservoir as a function of the time allowed to the BSA solution to dry before the introduction of the buffer solution. Lines included to guide the eye.

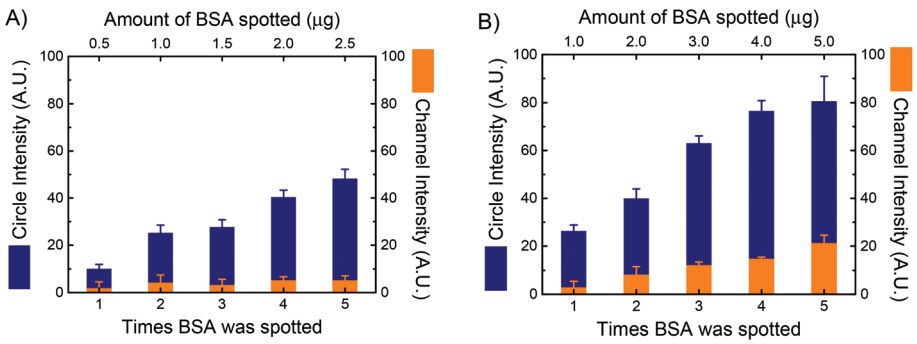

Fig. 3 Intensity of the stained BSA at the protein zone and the adjacent channel/reservoir as a function of the number of spots performed on the paper chip upon the running buffer solution was introduced. The BSA concentrations used were 1 mg mL^{-1} (A) and 2 mg mL^{-1} (B).

As it can be observed, increasing the interaction time yielded moderate but systematic changes in the distribution of BSA in the paper device. In general, the longer the interaction time, the higher the amount of BSA immobilized on the seeding spot. In agreement with adsorption data reported for other hydrophilic substrates, BSA displays high affinity for the cellulose fibers, resulting in its quick attachment to the substrate. In one extreme, it can be observed that when the protein was rinsed immediately after spotting, about 40% of the dispensed BSA was displaced to the adjacent channel/reservoir. In the other extreme, almost all the protein can be confined to the seeding spot (no protein detected in the adjacent channel) if allowed to interact for 90 min before introducing the buffer (see Fig. 4). Based on these results it can be concluded that the minimum time required to quantitatively attach BSA in μPADs is 30 min. However, if the development process can afford it, the longer the wait, the higher the adsorbed amount. It is important to highlight that homogeneous blue spots were always obtained, indicating that a uniform distribution of the protein molecules on the protein zone was accomplished at all selected times (no coffee ring effect).

Because they are often reported as key variables affecting both the kinetics and final outcome of the adsorption process, the effects of the pH and ionic strength of the buffer solution were also evaluated on the distribution of BSA. In these experiments, the paper chips were spotted with 0.5 μL of 2 mg mL^{-1} BSA contained in 10 mmol L^{-1} citrate buffer at pH = 4.7. After 30 min, either citrate or phosphate buffer at the selected pH or ionic strength was introduced to wash the protein zone. The pH of the buffer solution was investigated at the IEP = 4.7 of BSA, IEP ± 1, and pH = 7.2 (physiological pH). The ionic strength was studied at the IEP of BSA by adding 0.1, 1, 10, and 100 mg mL^{-1} NaCl in 10 mmol L^{-1} citrate buffer (pH = 4.7). The experimental results of pH and ionic strength are shown in Fig. 5A and B, respectively.

It can be observed in Fig. 5A that the maximum color intensity developed on the protein zone was obtained at the IEP of the protein. Only slightly lower intensities were obtained under conditions leading to electrostatic repulsion between proteins. Because cellulose is neutral in the studied pH range, these results indicate that the interaction with the substrate is mostly driven by structural rearrangement of the protein leading to the exposure of its hydrophobic groups to the cellulose fibers. It is important to note that pH = 7.2 led not only to the lowest color intensity on the protein zone but also the highest color intensity in the adjacent channel. These findings suggest that mild (but noticeable) protein–protein electrostatic interactions can also affect the adsorption/desorption process and that pH values close to the corresponding IEP could lead to maximizing the amount of protein adsorbed. In line with these results, the color intensity in the protein zone was only marginally affected when the rinsing buffer contained increasing amounts of NaCl (Fig. 5B). These results not only highlight the relevance of these results to the rational design of paper-based devices, but also demonstrate how careful researchers should be when selecting a substrate, as the presence of functional groups could have significant effects on the adsorption.[32]

Thus far, the experiments were focused on the adsorption of BSA to the paper substrate and provide guidelines to use this strategy when BSA is used to block the surface and subsequently minimize the non-specific adsorption of other proteins. Based on a general literature survey, it is evident that it is often assumed that proteins (including BSA) will interact with the substrate and remain in the seeding spot. Taken at face value, adsorption studies could then be considered redundant and not required for the rational design of analytical devices. However, the presented results demonstrate that several experimental variables can influence the amount and location of proteins.

To illustrate the effect of protein structure on their adsorption/desorption behavior, two additional proteins (Lys and IgG), with different structural characteristics were selected. Lys is a hard protein with low molecular weight (14 kDa). On the other extreme, IgG is a soft protein almost three times larger than BSA. As it can be observed in Fig. 6, the differences between these proteins are striking. In the first case (Fig. 6A), it can be observed that the amount of Lys adsorbed to the cellulose (at the seeding spot) is smaller than that obtained with

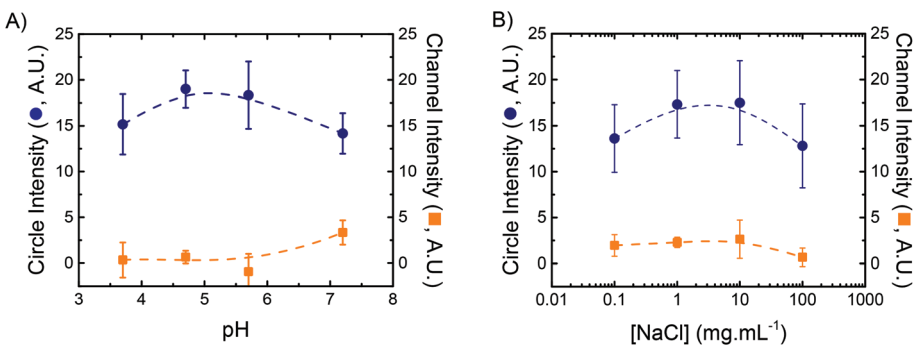

Fig. 5 Intensity of the stained BSA obtained at the protein zone and the adjacent channel/reservoir as a function of the pH (A) and ionic strength (B) of the protein solution. Lines included to guide the eye.

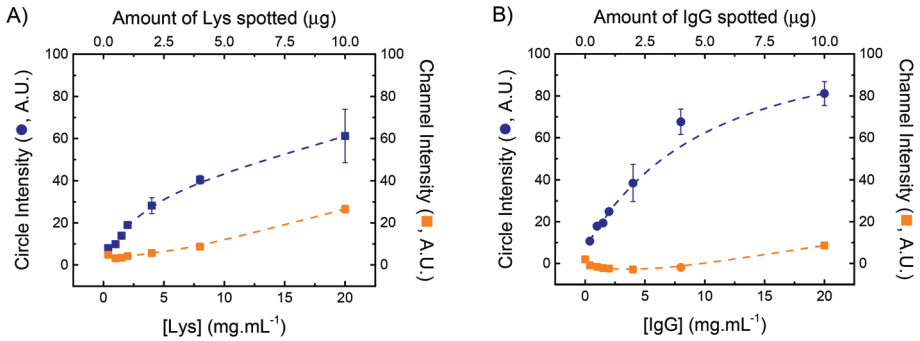

Fig. 6 A: Intensity of the stained Lys at the protein zone and the adjacent channel/reservoir as a function of the Lys concentration used for spotting and upon the rinsing step. Lines included to guide the eye. B: Intensity of the stained IgG at the protein zone and the adjacent channel/reservoir as a function of the IgG concentration used for spotting and upon the rinsing step. Lines included to guide the eye.

BSA or IgG and that a small amount of the enzyme can always be displaced by the rinsing step with buffer. According to these results, a solution containing at least 8 mg mL^{-1} Lys would be required to saturate the surface. The second case (Fig. 6B) shows that IgG can be readily adsorbed to the paper displaying a behavior that is similar to that of BSA. However, the results show that much smaller concentrations of IgG are required to yield to significant color changes and that unless saturation is reached (>10 mg mL^{-1}), most of the protein will remain in the seeding spot.

These differences could be attributed to a number of factors. Following the mechanism proposed for the adsorption of BSA (driven by structural rearrangements to enhance the interaction of hydrophobic amino acids with the surface),[33,34] it is reasonable to consider that stiffer proteins, like Lys, would be more resistant to undergo conformational transitions and to adsorb to the cellulose fibers (neutral and hydrophilic). Albeit being more stable, a fraction of these proteins can be desorbed during rinsing steps or displaced by the sample introduction, originating the color gradients often reported in literature. The second factor to be considered is that the evaporation and rehydration in the presence of salts has been reported to induce changes in the solubility of proteins like BSA.[35] Considering that these changes in solubility are accompanied by significant structural rearrangements, these results provide additional evidence about the importance of closely monitoring to preserve their functionality.

4. Conclusions

The experiments discussed describe the experimental conditions to favor the interaction of BSA with cellulose fibers. The protein was spotted and then rinsed, following the steps usually involved in the preparation and use of paper-based microfluidic devices. The subsequent staining step (performed with Coomassie Blue) provided a simple and selective avenue to obtain semi-quantitative information and determine the distribution of the protein on the device. The results obtained with BSA can be used to implement rational strategies to immobilize and control the location of proteins in µPADs. Experiments performed with Lys and IgG not only highlighted the differences in adsorption behavior of these proteins but also suggest that the adsorption behavior could be related to the tendency of the proteins to undergo surface-induced structural rearrangements. Considering the obvious differences in the experiments, these results also support the hypothesis that the main issue related to color gradients generated by enzymes can be related to an excess of enzymes (with respect to the amount dictated by the surface) added during the preparation of the device and that remains loosely-bound in the device.

Conflicts of interest

Authors have declared no conflict of interests.

Acknowledgements

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References

1 D. M. Cate, J. A. Adkins, J. Mettakoonpitak and C. S. Henry, *Anal. Chem.*, 2015, **87**, 19–41.
2 Y. Yang, E. Noviana, M. P. Nguyen, B. J. Geiss, D. S. Dandy and C. S. Henry, *Anal. Chem.*, 2017, **89**, 71–91.
3 A. K. Yetisen, M. S. Akram and C. R. Lowe, *Lab Chip*, 2013, **13**, 2210–2251.
4 Y. Xia, J. Si and Z. Li, *Biosens. Bioelectron.*, 2016, **77**, 774–789.
5 N. A. Meredith, C. Quinn, D. M. Cate, T. H. Reilly, J. Volckens and C. S. Henry, *Analyst*, 2016, **141**, 1874–1887.

6 K. Yamada, H. Shibata, K. Suzuki and D. Citterio, *Lab Chip*, 2017, **17**, 1206–1249.
7 G. G. Morbioli, T. Mazzu-Nascimento, A. M. Stockton and E. Carrilho, *Anal. Chim. Acta*, 2017, **970**, 1–22.
8 L. Cao, C. Fang, R. Zeng, X. Zhao, Y. Jiang and Z. Chen, *Biosens. Bioelectron.*, 2017, **92**, 87–94.
9 R. Cao, L. Guan, M. Li, J. Tian and W. Shen, *Sens. BioSens. Res.*, 2015, **6**, 13–18.
10 Y. Zhang and D. Rochefort, *Anal. Chim. Acta*, 2013, **800**, 87–94.
11 Y. Wang, H. Liu, P. Wang, J. Yu, S. Ge and M. Yan, *Sens. Actuators, B*, 2015, **208**, 546–553.
12 C. Ma, W. Li, Q. Kong, H. Yang, Z. Bian, X. Song, J. Yu and M. Yan, *Biosens. Bioelectron.*, 2015, **63**, 7–13.
13 M. Zhao, H. Li, W. Liu, Y. Guo and W. Chu, *Biosens. Bioelectron.*, 2016, **79**, 581–588.
14 K. Abe, K. Kotera, K. Suzuki and D. Citterio, *Anal. Bioanal. Chem.*, 2010, **398**, 885–893.
15 A. C. Glavan, D. C. Christodouleas, B. Mosadegh, H. D. Yu, B. S. Smith, J. Lessing, M. T. Fernández-Abedul and G. M. Whitesides, *Anal. Chem.*, 2014, **86**, 11999–12007.
16 K. F. Lei, C.-H. Huang, R.-L. Kuo, C.-K. Chang, K.-F. Chen, K.-C. Tsao and N.-M. Tsang, *Anal. Chim. Acta*, 2015, **883**, 37–44.
17 M. S. Khan, T. Pande and T. G. M. van de Ven, *Colloids Surf., B*, 2015, **132**, 264–270.
18 K. Scida, B. Li, A. D. Ellington and R. M. Crooks, *Anal. Chem.*, 2013, **85**, 9713–9720.
19 E. W. Nery and L. T. Kubota, *J. Pharm. Biomed. Anal.*, 2016, **117**, 551–559.
20 X. Deng, N. M. B. Smeets, C. Sicard, J. Wang, J. D. Brennan, C. D. M. Filipe and T. Hoare, *J. Am. Chem. Soc.*, 2014, **136**, 12852–12855.
21 Y. L. Jeyachandran, J. A. Mielczarski, E. Mielczarski and B. Rai, *J. Colloid Interface Sci.*, 2010, **341**, 136–142.
22 E. Kontturi, T. Tammelin and M. Osterberg, *Chem. Soc. Rev.*, 2006, **35**, 1287–1304.
23 E. Evans, E. F. M. Gabriel, W. K. T. Coltro and C. D. Garcia, *Analyst*, 2014, **139**, 2127–2132.
24 T. E. Benavidez, D. Torrente, M. Marucho and C. D. Garcia, *Langmuir*, 2015, **31**, 2455–2462.
25 E. Evans, E. F. M. Gabriel, T. E. Benavidez, W. K. T. Coltro and C. D. Garcia, *Analyst*, 2014, **139**, 5560–5567.
26 W. Norde and C. E. Giacomelli, *J. Biotechnol.*, 2000, **79**, 259–268.
27 L. E. Valenti, P. A. Fiorito, C. D. García and C. E. Giacomelli, *J. Colloid Interface Sci.*, 2007, **307**, 349–356.
28 J. L. Wehmeyer, R. Synowicki, R. Bizios and C. D. García, *Mater. Sci. Eng., C*, 2010, **30**, 277–282.
29 M. R. Nejadnik and C. D. Garcia, *Colloids Surf., B*, 2011, **82**, 253–257.
30 K. Y. Chumbimuni-Torres, R. E. Coronado, A. M. Mfuh, C. Castro-Guerrero, M. F. Silva, G. R. Negrete, R. Bizios and C. D. Garcia, *RSC Adv.*, 2011, **1**, 706–714.
31 R. A. Latour, *J. Biomed. Mater. Res., Part A*, 2015, **103**, 949–958.
32 K. S. Ha, H. Hinago, A. Sakoda and M. Suzuki, *Stud. Surf. Sci. Catal.*, 1993, **80**, 251–258.
33 W. Norde, *Colloids Surf., B*, 2008, **61**, 1–9.
34 S. A. Bhakta, E. Evans, T. E. Benavidez and C. D. Garcia, *Anal. Chim. Acta*, 2015, **872**, 7–25.
35 W. R. Liu, R. Langer and A. M. Klibanov, *Biotechnol. Bioeng.*, 1991, **37**, 177–184.