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RESEARCH ARTICLE

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Minimizing Nonspecific Protein Adsorption in Liquid Crystal Immunoassays by Using Surfactants

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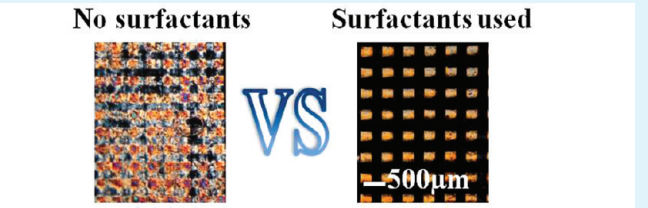
ABSTRACT: In this paper, we report the role of surfactants in minimizing nonspecific protein adsorption in liquid crystal (LC)-based immunoassays in which LC is used as a readout system. Among all surfactants tested, only nonionic surfactant such as Tween 20 can effectively reduce the nonspecific protein adsorption, while maintaining the selectivity of the LC-based immunoassay. We also show that to minimize nonspecific protein adsorption, Tween 20 can be added directly into the antibody solution to a final concentration of 0.8 mM. After the addition of Tween 20, better correlations between the antibody concentrations and the interference colors of LCs can therefore be obtained. For example, when Cy3 antibiotin was used, black, yellow, red, and green interference colors correspond to a concentration of 5, 25, 50, and 100 μg/mL, respectively. This feature gives LC immunoassay a unique advantage over the fluorescence-based immunoassay.



Effects of surfactants on liquid crystal immunoassays

KEYWORDS: nonspecific protein adsorption, Tween 20, immunoassay, liquid crystals, interference colors

INTRODUCTION

Immunoassays are techniques commonly used to detect antigens and antibodies by using immunological reactions. They have been widely used in fields such as medical diagnosis, drug discovery, and food testing. There are many different types of immunoassays including immunochromatographic lateral flow, enzyme linked immunosorbent assay (ELISA), immunomagnetic separation-electrochemiluminescence (IMS-ECL), time-resolved fluorescence immunoassay, and magnetic force immunoassay.[1−8] Among them, ELISA is very popular because of its high specificity, sensitivity, and high throughput. In a typical ELISA, antigens to be tested are first immobilized on the surface of microwells via physical adsorption.[9,10] Next, primary antibody which binds specifically to the test antigen is added. After the incubation, weakly bound primary antibody is washed off with buffer solutions. Then, a secondary antibody which can specifically bind to primary antibody is added. The secondary antibody is conjugated to an enzyme such horseradish peroxidase (HRP). Finally, a substrate is added and then a colorimetric response from the reaction between enzyme and substrate can be obtained.

However, one common issue in ELISA is the nonspecific adsorption of antibody onto the surface. The undesired nonspecific adsorption can decrease the sensitivity and specificity of the assay. To prevent the nonspecific adsorption of protein, several methods are readily available. The first method of reducing unwanted nonspecific adsorption is to block unoccupied binding sites of the surface of microtiter plate wells with different blocking agents.[11−17] These agents are classified into two categories: (1) Natural blocking reagents, such as milk and bovine serum albumin (BSA); (2) synthetic blocking reagents, for example, Tween 20, Triton X-100, sodium dodecyl sulfate (SDS), polyvinylalcohol (PVA), polyvinylpyrrolidone (PVP) and polyethyleneglycol (PEG).[18,19] In one example, after the adsorption of antigens, a blocking cocktail that contains (BSA) and Tween 20 is added to the microwells.[20] This blocking cocktail can adsorb on the surfaces to minimize the interactions between antibody and the surface. The incorporation of both BSA and Tween 20 offers an efficient strategy for minimizing nonspecific adsorption of proteins.

The second method of reducing nonspecific adsorption is by adding blocking agents into assay buffers during the incubation for antigen−antibody binding.[11,13,21−24] As reported by Brogan et al., to reduce the nonspecific adsorption, 0.005% (v/v) Tween 20 was mixed with hen egg ovalbumin (HOA) before binding to immobilized antibody on surfaces.[23] In another example, 1% (w/v) BSA and 0.5% Triton X-100 were added to the antigen and antibody solutions, respectively to minimize nonspecific adsorption.[11,13] The advantage of this method is that it avoids the blocking step and simplifies the experimental procedure. In the third method, surfactants such as Tween 20, Triton X-100 and SDS are added to the washing buffer solution to remove the nonspecific adsorption in immunoassays.[17,25−27] Xue et al. reported that the use of 1% SDS can effectively remove the nonspecific adsorption in the liquid crystal based immunoassay.[27] However, the disadvantage of using SDS is that it may denature the protein or antibody in the immunoassay.

For liquid crystal (LC) based immunoassays, the nonspecific adsorption is also a problem,[27,28] but effective blocking strategies for LC-based immunoassays have not been reported before. To overcome the nonspecific adsorption problem, we study the effects of surfactants such as Tween 20 and Triton X-100 on

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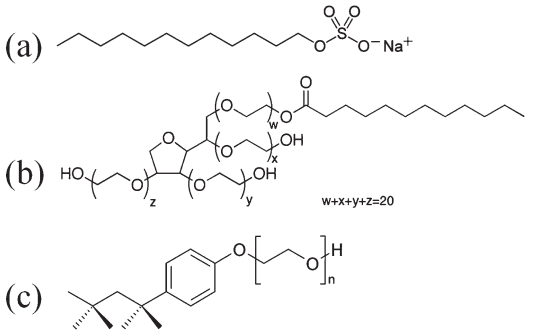


Figure 1. Molecular structures of surfactants used in the experiments. (a) SDS, (b) Tween 20, (c) Triton X-100.

nonspecific adsorption of protein and orientations of LC. By adding these surfactants to antibody solution, we demonstrate that the quantification of antibody concentration is far better than the one without any surfactants.

EXPERIMENTAL SECTION

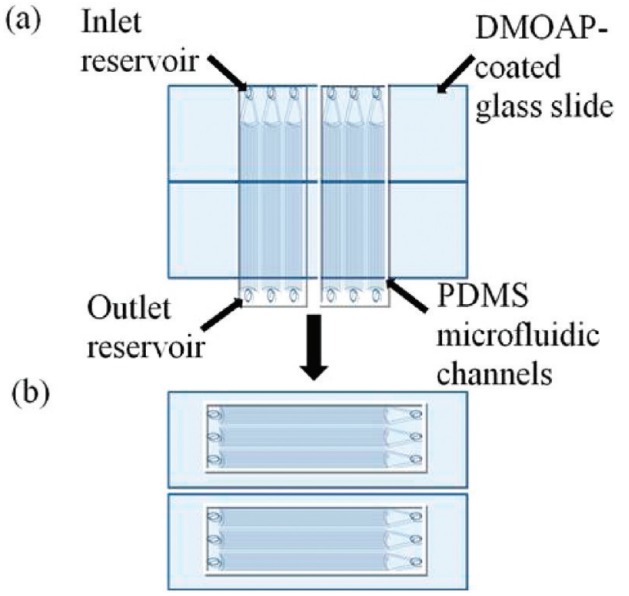
Materials. All glass slides were purchased from Marienfeld (Germany). Polysorbate 20 (Tween 20), Triton X-100 were purchased from Sigma-Aldrich (Singapore). Their molecular structures are shown in Figure 1. *N*,*N*-dimethyl-*N*-octadecyl-3-aminopropyltrimethoxysiyl chloride (DMOAP), human immunoglobulin G (human IgG), bovine serum albumin (BSA), biotin-labeled BSA (biotin-BSA), Cy3 antihuman IgG (produced in goat) and Cy3 antibiotin (produced in mouse) were purchased from Sigma-Aldrich (Singapore) and used without further purification. Sodium dodecyl sulfate (SDS), 10% (w/v) solution, and 10× phosphate buffer saline (PBS), were purchased from first BASE (Singapore). Poly(dimethylsiloxane) (PDMS) stamps were prepared from Sylgard 184 Dow Corning (U.S.A). Liquid crystal, 4-pentyl-4′-cyano-biphenyl (5CB), was purchased from Merck (Singapore). All solvents used in this paper were AR grade. Water was purified by using a Milli-Q system (Millipore, U.S.A).

Preparation of DMOAP-Coated Glass Slides. First, glass slides were immersed in 5% (v/v) of Decon-90 (a commercially available detergent) solution for 2 h. Then, they were rinsed with copious amounts of water and cleaned in an ultrasonic bath twice, each time for 15 min. Subsequently, the clean glass slides were immersed into an aqueous 0.1% (v/v) DMOAP solution for 5 min at room temperature. To remove the unreacted DMOAP from the surface, the DMOAP-coated slides were rinsed five times with water and dried under nitrogen gas. Finally, the DMOAP-coated slides were heated in a vacuum oven at 100 °C for 15 min to cross-link DMOAP.

Fabrication of PDMS Microfluidic Channels. The PDMS microfluidic channel was fabricated by using a conventional PDMS molding process. PDMS mixture was prepared by mixing elastomer Sylgard 184 with the curing agent at a ratio of 10:1. The prepared mixture of PDMS was poured onto a silicon master with microfluidic channel patterns (width × depth × length = 200 μm × 160 μm × 100 mm). The silicon masters were fabricated by defining the channel patterns via photolithography onto a negative photoresist (SU8−2050, Microchem, U.S.A) spin-coated onto a silicon wafer. The PDMS was then degassed in vacuum to remove bubbles and cured at 50 °C overnight. Inlet and outlet holes (3 mm in diameter) were punched by using a hole puncher. Subsequently, the PDMS microfluidic channels was peeled off and cleaned by a Soxhlet device with ethanol. The PDMS microfluidic channels were treated with oxygen plasma (100 W, 50 s) to facilitate the binding of microfluidic channels with DMOAP-coated glass slides.

Preparation of Microfluidic Immunoassays. Human IgG and biotin-BSA were first immobilized on a DMOAP-coated glass slide by flowing 15 μL of protein solutions (20 μg/mL) through microfluidic channels as shown in Scheme 1a. The two DMOAP-coated glass slides were placed side by side and secured by using binder clips. After 30 min of incubation, the microfluidic channels were removed, and the slides were rinsed with buffer solutions and dried under nitrogen gas. Subsequently, new microfluidic channels were placed on the DMOAP-coated glass slides, and 15 μL of Cy3 antihuman IgG (20 μg/mL), Cy3 antibiotin (20 μg/mL) were injected into two separate microfluidic channels as shown in Scheme 1b. After 30 min of incubation, the microfluidic channels were removed and the slides were rinsed and dried under nitrogen gas.

Scheme 1. Illustration of the Microfluidic Immunoassay[a]



[a] (a) Two DMOAP-coated glass slides were aligned side by side and secured using binder clips. PDMS microfluidic channels were treated with oxygen plasma and placed on top of the slide. To immobilize proteins on the surface, we injected 15 μL of protein solutions into the microfluidic channels and incubated for 30 min. (b) After the removal of "old" microfluidic channels, a new set of microfluidic channels were placed on the slide. Then, 15 μL of antibody solutions were injected into microfluidic channels.

Preparation of LC Cells. A LC optical cell can be fabricated by pairing a sample glass slide and a DMOAP-coated glass slide. The two slides were separated from each other with a fixed distance (~6 μm) by using two strips of Mylar films, and the optical cell was secured with two binder clips. To fill the empty cell, a drop of 5CB was dispensed onto the edge of the cell, allowing 5CB to fill the empty space between two glass slides by capillary force. Finally, the optical textures were observed under crossed polars with a polarized microscope (Nikon, Japan) in the transmission mode.

Fluorescence Detection. Fluorescence images of the slides were obtained using a fluorescence microarray scanner (GenePix 4100A, Molecular Devices, U.S.A.) equipped with a 532 nm laser. Calibration of the scanner was carried out by using a calibration slide and a hardware diagnostic tool in GenePix Pro 6.1 before each experiment. Fluorescence intensity profile was analyzed by using ImageJ (version 1.42).

RESULTS AND DISCUSSIONS

Blocking Effect of Surfactant on LC-Based Immunoassay. To investigate the blocking effects of surfactants, buffer solutions containing Cy3 antibiotin (25 μg/mL) mixed with one type of surfactants (Tween 20, Triton X-100 or SDS) were injected into microfluidic channels. These channels were supported on a

DMOAP-coated glass slide with lines of immobilized 20 μg/mL of biotin-BSA. In the absence of surfactant, the fluorescence result in Figure 2a suggests that the antibiotin binds specifically to the biotin-BSA immobilized on the surface. However, the LC result implies that antibiotin binds to biotin-BSA, but it also binds nonspecifically to surrounding areas, and that causes a smearing LC image. This phenomenon suggests that LC is very sensitive to proteins adsorb on the surface, probably more sensitive than the fluorescence-based detection method on the basis of results shown in Figure 2a. In contrast, when 0.8 mM of Tween 20 is added to the antibiotin solution, Figure 2b shows that both fluorescence and LC images are well-resolved. This result suggests that Tween 20 can effectively reduce the nonspecific protein adsorption in the LC-based immunoassay. This is probably because Tween 20 can adsorb on the surface, preventing the protein to adsorb nonspecifically on the surface. Because Tween 20 is soluble in water, it can be removed from the surface during the rinsing procedure. We also note that Tween 20 is often used to remove the nonspecific protein adsorption in traditional immunoassays with a concentration range between 0.4 and 4 mM.[20,29−32] Because Tween 20 concentration used in our experiments is 0.8 mM, it is consistent with the past studies. For comparison, when surfactant SDS was used (3.5 mM), both fluorescence and LC results in Figure 2d show that SDS washes away antibody from the surface. Even though the SDS concentration is lower than its critical micelle concentration (8 mM),[33] it still have strong rising power and is difficult to control in this experiment. Another potential problem is that LC is very sensitive to ionic surfactant such as SDS. Past studies have shown that when SDS or other ionic surfactants adsorb at the water/LC interface, they tend to dictate the orientations of LC.[34−36] This result led us to propose that nonionic surfactant is probably better than anionic surfactant in reducing nonspecific protein adsorption in the LC-based immunoassay.

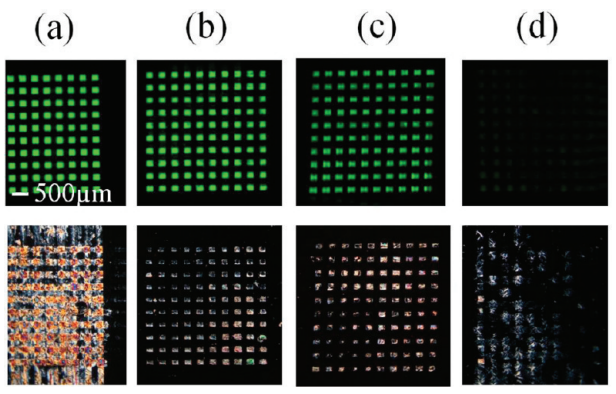


Figure 2. Comparison of the blocking effects of three surfactants by using fluorescence images (top row) and LC images (bottom row). Surfactants used are (a) no surfactant, PBS only, (b) Tween 20, (c) Triton X-100, and (d) SDS.

To test this hypothesis, another nonionic surfactant Triton X-100 was investigated. Figure 2c shows that when 1.6 mM of Triton X-100 was added, both fluorescence and LC-based immunoassays give clear and well-resolved images. Interestingly, for both Tween 20 and Triton X-100, the concentrations used were higher than their CMC (0.05 mM and 0.31 mM, respectively),[33] but they did not wash away antibody binds to the surface. Therefore, we can conclude that CMC is not a threshold concentration to wash away of antibody from the surface if nonionic surfactants are used. Although Tween 20 and Triton X-100 give similar results, in the following study, we chose Tween 20 as the blocking agent.

Optimizing Concentration of Tween 20. To investigate the optimal concentration of Tween 20 in the immunoassay, different concentrations of Tween 20 were added to the solutions containing 25 μg/mL of Cy3 antibiotin. Figures 3 shows that when the concentrations of Tween 20 is 0.4 mM or below, nonspecific binding of Cy3 antibiotin to the surface remains strong, as is evident by the smearing LC images. In contrast, when the concentration is 8 mM or above, both fluorescence and LC image suggest that the high concentration of Tween20 prevents the specific binding of Cy3 antibiotin to the surface. These results, when combined, suggest that the optimal Tween20 concentration is between 0.8 and 4 mM. In the following experiment, the Tween20 concentration is fixed at 0.8 mM.

Quantification of Antibody Concentrations. An important requirement for an immunoassay is its ability to quantify different concentrations of antibodies or antigens. For example, in ELISA, the protein concentration is proportional to the absorbance of the solution. To investigate whether this LC-based immunoassay can be used to determine protein concentrations, we analyzed 4 different samples, which contain 5, 25, 50, and 100 μg/mL of Cy3 antibiotin, respectively, by using fluorescence. Figure 4a−d shows that the changes in the fluorescence intensity is easily visible with the naked eye when the Cy3 antibiotin concentration is increased from 5 μg/m to 25 μg/mL, but when the Cy3 antibiotin concentration is further

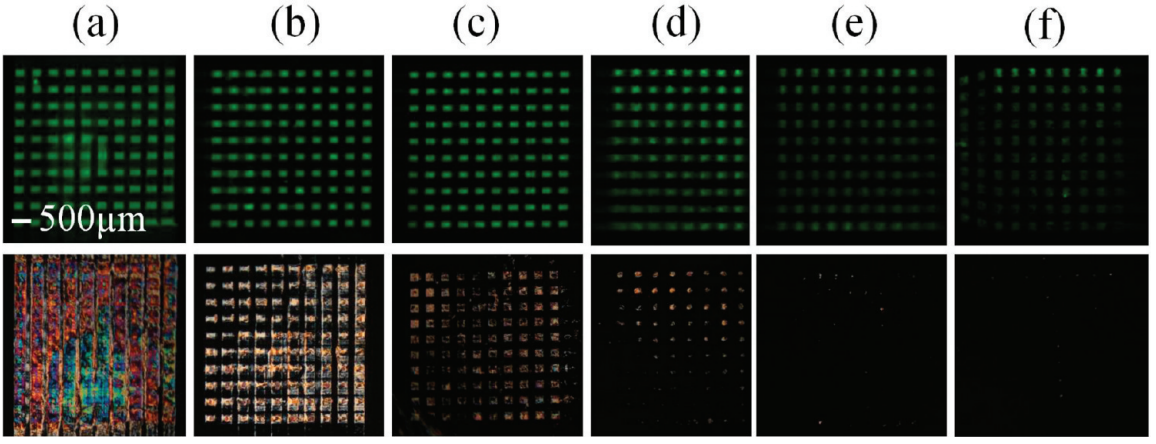


Figure 3. Blocking effects of Tween 20 on fluorescence images (top row) and LC images (bottom row) at the following Tween 20 concentrations: (a) 0.08, (b) 0.4, (c) 0.8, (d) 4, (e) 8, and (f) 40 mM.

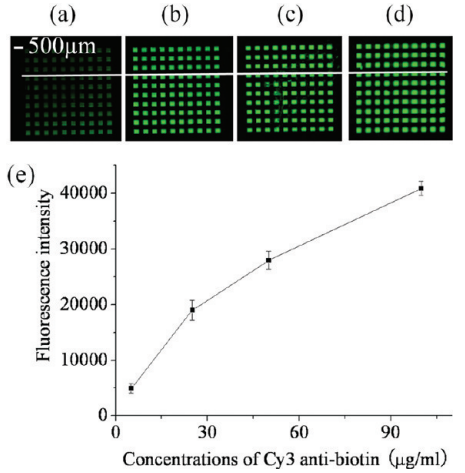


Figure 4. Fluorescent images of the immunoassay at different antibody concentrations. Concentrations of Cy3 antibiotin are (a) 5, (b) 25, (c) 50, and (d) 100 μg/mL. (e) Fluorescence intensity values across the line in part a−d.

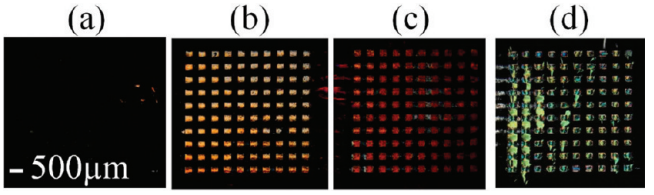


Figure 5. Optical images of LC showing the effect of antibody concentration on the colors of LCs. Concentrations of Cy3 antibiotin are (a) 5, (b) 25, (c) 50, and (d) 100 μg/mL. Different concentrations of antibiotin can be differentiated by using the colors of LCs.

increased to 50 μg/m or 100 μg/mL, the difference in the fluorescence intensity is not distinguishable with the naked eye. However, when we analyze the fluorescence intensities by using Image J, the fluorescence intensities indeed increases with the increasing Cy3 antibiotin concentration as shown in Figure 4e. For comparison, the LC result in Figure 5 shows that different Cy3 antibiotin concentrations lead to distinct LC colors, which follow the order of black, yellow, red and finally green. Therefore, we can easily differentiate the different concentrations of Cy3 antibiotin concentration by using the LC colors with the naked eye, and that gives LC based immunoassay a unique advantage over the fluorescence based immunoassay. Because human eyes can detect approximately 10 million different colors, this principle will be very useful in the development of portable immunoassay in which different antibody/antigen concentrations result in different visible colors.

Specificity of LC-Based Immunoassay with Tween 20. Finally, to study the role of Tween 20 in the specificity of LC-based microfluidic immunoassay, we immobilized parallel lines of human IgG and biotin-BSA on the same DMOAP-coated slide, and then flew antibody solutions containing 0.8 mM of Tween 20 through horizontal microfluidic channels covered on the slide. A LC image of this microfluidic immunoassay is shown in Figure 6a. The results demonstrate that LC only appears bright at intersections where antibodies meet their specific target proteins. From these results, we conclude that the proteins immobilized by microfluidic channels can still be recognized by their respective antibodies with high specificity in this LC-based immunoassay.

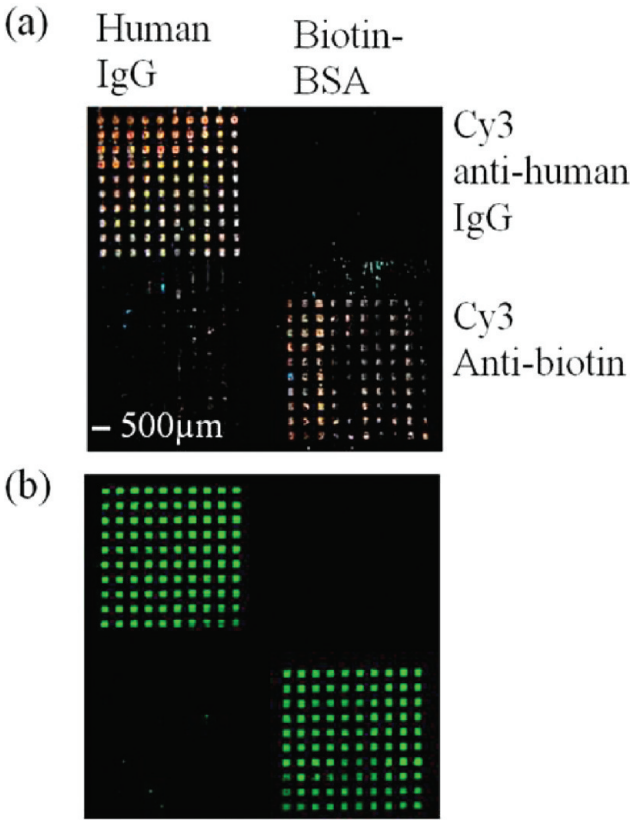


Figure 6. Specificity of LC-based microfluidic immunoassay after the addition of Tween 20. (a) Fluorescence images. (b) LC images.

Furthermore, the result of this LC-based immunoassay was confirmed by the typical fluorescence-based immunoassay as shown in Figure 6b. We point out that Xue et al.[27] used 1% SDS solution as washing buffer to remove the nonspecifically adsorbed proteins in the LC-based microfluidic immunoassay. However, in the current study, Tween 20 was added into antibody solution and used as a blocking agent to minimize the nonspecific protein adsorption. Comparing these two methods, this method is superior because it is difficult to control the final rinsing step.

CONCLUSIONS

We have studied the blocking effects of surfactants such as Tween 20, Triton X-100 and SDS on LC-based immunoassay. We showed that Tween 20 is superior to SDS on alleviating the nonspecific protein adsorption. The optimum concentration of Tween 20 used in LC-based immunoassay is 0.8 mM, which can effectively reduce the nonspecific adsorption of proteins and improve test results. In this LC-based immunoassay, when the Cy3 antibiotin concentrations increase from 5 to 100 μg/mL the LC results show the black, yellow, red, and green interference color, respectively. On this basis, we can easily differentiate the different concentrations of Cy3 antibiotin with the naked eye, and that gives LC based immunoassay a unique advantage over the fluorescence-based immunoassay. We also show that the proteins immobilized by microfluidic channels can still be recognized by their respective antibodies with high specificity in this LC-based immunoassay. These findings show the importance of adding Tween 20 to the antibody solution in the LC-based immunoassay.

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