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Parallel, Open-Channel Lateral Flow (Immuno) Assay Substrate Based on Capillary-Channeled Polymer Films

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ABSTRACT

Presented here is a novel implementation of polypropylene capillary-channeled polymer (C-CP) films, functionalized for bioaffinity separations and implemented as a platform for lateral flow (immuno) assays. The parallel ~80 μm x 80 μm channels pass test solutions down the 30 mm film length via spontaneous wicking action, setting up the possibility for immobilizing different capture agents in the respective channels. The base-film modification process is divided into two steps: ultraviolet light treatment to improve hydrophillicity of the polypropylene substrate and the physical adsorption of a functionalized lipid tethered ligand (LTL) as a selective capture agent. The entire modification procedure is performed under ambient conditions in an aqueous solution without extreme pH conditions. In this demonstration, physical adsorption of a biotinylated-LTL onto the UV-treated PP surface selectively captures Texas Red-labeled streptavidin (SAv-TR) in the presence of enhanced green fluorescence protein (EGFP), which passes without retention in less than 5 s. In addition to the fluorescence imaging of the protein solutes, matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS) was used to confirm the formation of the LTL-SAv conjugates on the channel surface as well as to demonstrate an alternative means of probing the capture step. The present effort sets the groundwork for further development of C-CP films as a parallel, multi-analyte LFA platform; a format that to-date has not been described.

Keywords: Lateral flow assay, capillary-channeled polymer, film, lipid tethered ligand

INTRODUCTION

Lab-on-chip (LOC) devices focus on the miniaturization of laboratory-scale equipment to perform diagnosis on a small scale. One obvious point of development is towards low-cost, portable and disposable point of care diagnostic (POC) devices.[1-4] In virtually all POC implementations, there is a need for a multifunction matrix material onto which operations are affected. Many polymeric materials such as polyethylene, nitrocellulose (NC), Dacron, polyvinyl chloride (PVC), nylon, polyacrylonitrile (PAN) etc. have been studied as bioassay matrices, interfacing between materials and biological moieties.[5-8] A key component in the POC toolbox has been immunochromatographic assays, also known as lateral flow (immuno) assays (LFA). These devices use capillary action (wicking) to transport the analytes to detecting zones where the immunoreaction takes place,[9-19] greatly simplifying the operational overhead. The material of choice in the vast majority of LFA systems has historically been nitrocellulose,[12] however, there have been efforts towards introducing other material types into the market. Recently, thin layer materials with wicking power, such as paper, sponge rubber and glass fiber paper, have been employed to achieve bioseparation as a function of the analytes' physical and chemical properties.[20-27]

The primary advances in the development and use of LFAs has focused on strategies to affect greater selectivity and sensitivity,[14, 28] e.g. focusing on the detection event. In terms of the base fluidic matrices, paper-based assays have received a great deal of attention, though at this point no uniform format/approach has emerged.[25, 26, 29] The developments in this area have been more directed at alternatives to glass and plastic-based

microfluidics than simple LFAs. While perhaps a little dated, van Amerongen and co-workers have presented an interesting SWOT (strengths, weaknesses, challenges, and opportunities) analysis of the field lateral flow (immuno) assays.[30] In that review, they look to the various components of a typical LFA and project where challenges and opportunities exist. They note a few items that are consistent with other reviews, where aspects of greater sample-type versatility, greater stability, better sample utilization, and multi-component parallel assays are noted as key challenges. To solve these difficulties, new matrix/array materials will be needed.

Capillary-channeled polymer (C-CP) fibers have been explored in this laboratory for their use in protein chromatography and solid phase extraction.[31-36] C-CP fibers are melt-extruded from commodity polymers, the ones being focused on in this laboratory are polypropylene (PP), nylon 6, and poly(ethylene terephthalate) (PET). Structure-wise, the fibers have eight capillary channels extending the entire length of the fibers, which can self-align and results in a monolith-like structure with 1-5 μm capillary channels for fluidic flow when packed into a column. It has been shown that C-CP fiber packed columns exhibit excellent fluid transport properties suitable for macromolecular separations (i.e. achieving separation without suffering from van Deemter C-term broadening) therefore enhanced mass transfer efficiencies that allow very rapid separations with high recoveries.[34, 37, 38] C-CP materials can also be extruded in a ~5 mm wide film format, with parallel ~100 μm wide x ~100 μm deep channels running the length of the film. The use of individual channels versus a network of voids is very different from other thin layer materials currently being used as matrices for LFA. We have

previously demonstrated the ability to perform protein separations via wicking action, effecting doing thin layer chromatography (TLC) down the channels.[39] In that work, proteins were detected by matrix-assisted laser desorption mass spectrometry (MALDI-MS). However, during that study of the native C-CP film, the poor uniformity of the channels and the fact there were channels present on both sides of the films caused uneven solvent flow and poor reproducibility. Thus, a new PP film spinerette was designed by Specialty & Custom Fibers, LLC (Clemson, SC). This second-generation C-CP film has four uniform, individual channels on one side of the film, with a flat surface on the opposite side for easy mounting.

C-CP films have several potential advantages as an LFA matrix; they are flexible, chemically stable, and optically transparent, thus easy to couple with various surface analyzing/detecting methods including absorbance spectroscopy, Raman/IR spectroscopy, florescence spectroscopy, and desorption/ionization mass spectrometry. A more subtle aspect is that the wicking action in the films is unidirectional, as opposed to the capillary action in a 3-dimensional matrices such as nitrocellulose or paper which effectively dilutes the sample into the depth of the matrix. As such, one might expect greater utilization of precious analyte as the movement is along a single surface as opposed to within a matrix that may not be accessed by the detection means. A final, very distinct advantage of the physical platform is that the films can be constructed with any number of parallel channels. As illustrated conceptually in Fig. 1, such a structure provides the possibility for parallel, multi-analyte determinations, as opposed to the single-analyte capabilities seen in common pregnancy or glucose tests. Thus, rapid, single-

sampling, multi-analyte determinations are envisioned on a support that offers ready chemical modification for specific applications. We are not aware of other parallel-format LFAs. While perhaps more expensive than nitrocellulose substrates, the support cost is minimal in comparison to capture ligands, while providing the above advantages.

While the previous protein separation on a C-CP film was performed on a native polypropylene (PP) surface to affect a reversed phase separation of proteins in Tris-HCl and phosphate buffered saline (PBS) matrices, there are a number of strategies which can be implemented to affect the selectivities desired in most LFA applications. Recently, this laboratory has focused on developing simple surface modification chemistries on C-CP fibers, which are transferable to the film format.[40-44] Of greatest relevance for the application of PP C-CP films has been the development of the lipid tethered ligand (LTL) methodology wherein the hydrophobic acyl chains actually intercalate into the polymer matrix to form an incredibly robust anchor.[45] Chemical selectivity is achieved by simple choice of the polar lipid head group. An in-house synthesized biotinylated-LTL has been used on PP C-CP fibers for capturing SAv-TR from EGFP-spiked *E. coli* cell lysate.[43]

Presented here is the proof of concept of the use of the synthesized biotinylated-LTL for the surface modification of UV-treated PP C-CP film and its use as an open-channel lateral flow (immuno) assay. As in the case of commercial LFAs, the open channel structure provides easy access in the chemical modification step, ready deposition of test samples, and visualization in the detection process as opposed to a closed structure such as meshes, tubes, and capillaries. Described are the simple methods for imparting

greater wicking activity as well as tethering of the capture ligand. It is believed that the present C-CP film format holds a great deal of promise towards multi-analyte LFAs.

EXPERIMENTAL

Chemicals and Reagents

Streptavidin-Texas Red (SAv-TR) was purchased through Southern Biotech (Birmingham, AL) and enhanced green fluorescence protein (EGFP) (found in p3051 retrovirus vector and amplified by PCR) was provided by Dr. G. Chumanov of the Chemistry Department of Clemson University (Clemson, SC). The biotiylated-LTL was synthesized in-house, as described previously[41]. A working solution containing 0.3 μg mL^{-1} each of (SAv-TR) and EGFP was prepared in 100 mM phosphate-buffered saline tween (PBST) buffer. PBST buffer was prepared by adding 1 mL of Tween-20 into 1 L of phosphate-buffered saline (PBS) buffer that was prepared by dissolving NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (1.44 g) and KH_2PO_4 (0.24 g) (all salts were purchased from Sigma-Aldrich, St. Louis, MO) in DI water (Milli-Q system, 18.2 MΩ cm^{-1}) to a final volume of 1 L and a pH of 7.4.

Protein separations on the C-CP films were achieved using a 40:60 acetonitrile:water (ACN:H_2O) eluent. MALDI matrix solutions consisting of 10 g L^{-1} of sinapinic acid, purchased from Fisher Scientific (Pittsburgh, PA, USA), were prepared in 0.1% trifluoroacetic acid (TFA), purchased from Sigma-Aldrich. ACN was purchased from VWR (West Chester, PA). Unless specified otherwise, all reagents and solvents were used without additional purification.

Preparation of PP C-CP films

The entirety of the film preparation, surface modification, and affinity capture is depicted in Fig. 2. Polypropylene (PP) C-CP films were melt-extruded at the Department of Materials Science and Engineering at Clemson University (Clemson, SC, USA). Films are available from a commercial source, Specialty & Custom Fibers, LLC (Clemson, SC, USA). They contain 4 channels ~80 μm in width and height, as shown in Fig. 3. It is important to note that the total channel volume of the 30 mm long C-CP film is only 0.77 μL. Film segments were washed sequentially with water, methanol, and acetonitrile, twice respectively, to remove any production residues. The films (30 mm in length) were attached via double-sided tape to a glass slide following the wash then treated in an ambient UV Irradiation chamber with a low pressure mercury grip lamp for 15 min (BioForce Nanosciences, Inc., Ames, IA). The intensity of the mercury grid lamp used was 19.4 mW cm^{-2} at a distance of 1.1 cm. The UV lamp had emission wavelengths from 185 to 450 nm (highest intensity located at 253.7 nm) and generated ozone from ambient air within the chamber.

Biotin-LTL functionalization, Sample Spotting, and Elution Process

The application of the biotinylated-LTL test strip is complicated by the high level of spontaneous wicking in the film channels. Simply, application of a droplet will cause the solution to spread across the entire film. To mitigate the spreading, two, 1 μL drops of DI-H_2O were placed 10 mm apart, and the LTL-containing ethanolic solution deposited in between. A 50 μg mL^{-1} biotin-LTL solution was prepared in 60% ethanol to deposit the capture strip. A 3 μL droplet of the solution was applied in the mid-film region between the water droplets. Due to the higher volatility of the ethanolic solution, that portion

evaporated more quickly than the water, effectively trapping the contents to an ~10 mm strip. This process was repeated five times. Following drying, a 10 µL aliquot of 60% ethanol was applied to one end of the film and wicked across the region to wash off any un-adsorbed biotin-LTL. For all on-film analysis, 2 µL of the protein test solution was spotted at one end, then washed by applying 10 µL DI-H_2O to the loaded protein. After 5 s, the wash solution was pipetted back off of the film. In both of these cases, the film's liquid capacity was overloaded, as such a small fraction wicks down the structure, with the majority left beaded on the end. The analytical elution was affected by applying 10 µL of 40:60 ACN:H_2O at the sample end of the film. In this case, a bundle of C-CP fibers loosely packed into 0.8 mm i.d. fluorinated ethylene propylene tubing (Cole Palmer, Vernon Hills, IL) placed at the far end acted as a sink to draw the entire volume across the film. In this way, the mixture was drawn across the biotinylated-LTL capture region.

Fluorescence Imaging of PP C-CP Films

Fluorescence imaging was achieved using a home-built spectrofluorometer system. A Xe arc lamp purchased from Chroma Technology Corporation (Bellows Falls, VT) with excitation filters set at 488 nm for EGFP, and 575 nm for SAv-TR. Fluorescence images were captured by an Ocra-ER (Hamamatsu) CCD camera, through an Olympus IX71, 2X /0.08 UPlanFI (infinity corrected) objective (Olympus, Center Valley, PA) with the emission filters set on 509 and 624 nm for EGFP and SAv-TR, respectively. The fluorescence data was processed using Micromanager 1.4 developed by Vale Lab (San Francisco, CA).

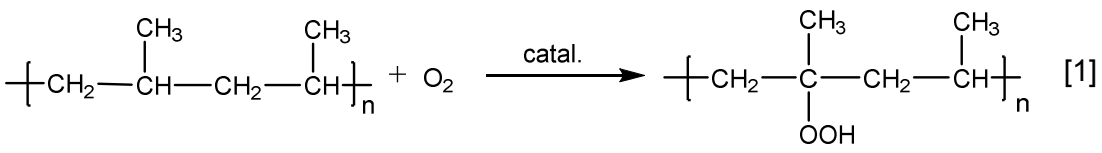
MALDI-MS Analysis

Films, after fluorescence imaging, were allowed to dry overnight in a dark environment and then double taped onto a MALDI target. The aforementioned MALDI matrix was sprayed onto the entire film at a low rate to limit analyte movement via wicking[39]. All analyte spectra were obtained on a Bruker Daltonics (Billerica, MA, USA) microflex LRF, a MALDI-TOF mass spectrometer, operating as linear, and positive ion detection mode. Instrument control and data processing were through Compass, a Bruker Daltonics software. The microflex is equipped with a nitrogen laser (337 nm) operating at a pulse rate of 60 Hz. Mass spectral acquisitions occurred using 100 laser shots at 35% laser power.

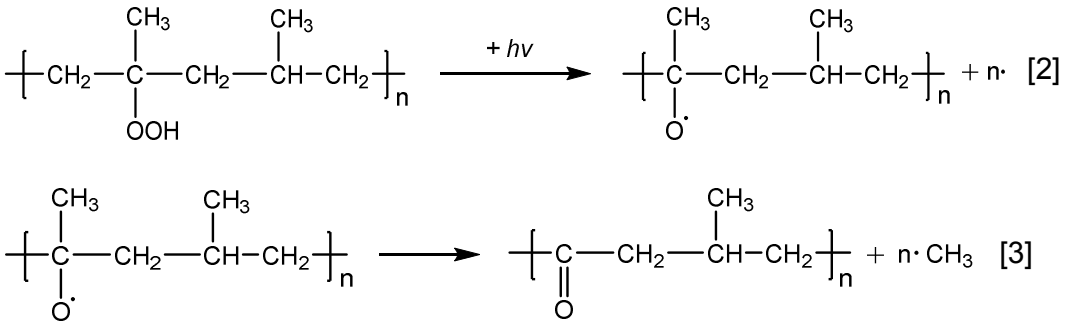
RESULT AND DISCUSSION

UV Surface treatment of the PP C-CP fiber

Ozone-generating UV light has been used for many years to clean organic contaminants from various surfaces.[46-49] It also has been used as one of the many surface treatment techniques for polymer films.[50] The purpose of the surface treatment in this study is to enhance the wicking propensity of the PP film by introducing more functional groups such as –OH, =O, –OOH through UV-induced oxidation, without inhibiting the LTL adsorption. Pure polypropylene does not absorb UV light at wavelengths < 200 nm. However, with the trace amounts of hydroperoxides and ketone chromophore/catalysts that exist in the PP films, the absorption range is extended below 200 nm through the processes depicted below.[50, 51]



[1]



The effects of treatment time on the ozone level generated over time and the oxygen uptake on PP were reported in detail by Walzak and co-workers.[52] In that work, the O:C ratio on the PP surface was found to increase linearly with the treatment time for up to 15 min, however no significant change occurred between 15-30 min of treatment. The contact angle was tested in air and results showed a decrease in the contact angle with increasing oxygen uptake, as anticipated. This treatment method was reproduced closely for the PP C-CP films, yielding appreciably higher wicking rates for both aqueous and mixed solvents, though not adding so much hydrophilic nature so as to inhibit the LTL adsorption.[42]

Biotin-LTL-functionalized PP C-CP films for the affinity capture of streptavidin-Texas Red

While the present protocol for depositing both the capture ligand and analytical sample are relatively crude, the concept is easily demonstrated. Figure 4 presents the fluorescent images obtained for SAv-TR and EGFP at three locations along the C-CP film. Figure 4a consists of images of the two proteins as-applied to the film prior to elution. As seen, both proteins are concentrated at the end, spreading down-film over distance of ~4 mm. Note that application of the relatively large droplet causes some protein buildup on the tops of the walls between the channels. Application of the eluting solvent

causes transport of the proteins along the solvent front with post-elution imaging clearly showing selective capture of the SAv-TR target. Figure 4b shows that the SAv-TR is indeed located within the channels, where expected, with no indication of the much more strongly fluorescing EGFP, which would reflect non-specific binding. While no assumption of the quantitative correctness of the fluorescent images is made here (though they were taken under the same conditions), the images taken at the end of the films (Fig. 4c) reflect a depletion of the SAv-TR and a loss in EGFP response, likely due to dilution.

As in any sort of selective bioassay, the question of non-specific binding is crucial. Previous studies have clearly shown that proteins have a very high affinity for PP fiber surfaces. The initial driving force for the UV oxidation was to increase the hydrophilicity of the PP surface, and thus the propensity for wicking of aqueous phases. Perhaps an equally important effect should be a reduced tendency for non-specific binding of proteins, which would occur through hydrophobic surface interactions. Previous studies with PP C-CP fibers dealt with the issue of non-specific binding by effecting the separations in the presence of the detergent Tween. As shown in Fig. 5 for the case of a film not subjected to the biotin-LTL deposition, the UV modification of the PP C-CP films results in a surface that appears to be immune to non-specific binding. As was seen in Fig. 4a, the image of the initial deposition zone (Fig. 5a) shows the proteins concentrated at the film head. Images taken post-elution at the mid-film region (Fig. 5b) reflect no accumulation of either protein along the elution channels, with the film-end images (Fig. 5c) showing accumulation of both. Thus, at this preliminary

stage, it appears that the UV-treatment does indeed impart sufficient hydrophilicity to inhibit non-specific protein binding to a large extent.

Using the C-CP PP film as MALDI Target for Direct Analysis of Analyte on Film

In the case that the target of an immunoassay does not have a fluorescence tag, MALDI-MS is an opportunity for identification and perhaps quantification of the captured molecule. Certainly this capability is more relevant in the case of class-specific captures, such as glycoproteins on a lectin capture surface. As demonstrated previously, C-CP films can be used as separation media with subsequent detection using the imaging capabilities of most MALDI-MS systems. Figure 6 demonstrates sequential use of MALDI imaging for the capture/separation of the SAv-TR/EGPF mixture. As described in the experimental section, the C-CP film was sprayed with MALDI matrix solution, allowed to dry again, then taped onto a MALDI target. Figs. 6a and b are the MALDI-MS spectra taken from the head (starting end) of the film, before and after the elusion step. In the first case (Fig. 6a), the proteins show very strong responses. Following elution with the 40:60 ACN:H_2O solvent, the region is depleted of the SAv, with a small amount of EGFP appearing to remain (Fig. 6b). MALDI-MS imaging of the central portion of the film (Fig. 6c), in the region of the biotin-LTL capture zone, reflects the capture of the SAv-TR as was demonstrated in the fluorescent images, with no indication of non-specifically bound EGFP. Finally, the MALDI spectrum acquired at the film end (Fig. 6d) reflects the accumulation of the non-retained proteins. In this case, it is clear from the relative spectral responses that the SAv-TR was selectively captured in preference to the EGFP. The presence of

the SAv at the end reflective of an overload situation versus the amount of adsorbed capture ligand. Utilizing the PP film as a MALDI substrate would appear to be an exemplary benefit in comparison to other thin layer film/membranes.[53, 54] In this case, the fact that the solute exists on the film surface and not within a permeable structure should provide greater analytical sensitivity and less spectral background.

CONCLUSIONS

In the present study, we developed a surface modification method for polypropylene C-CP films to be used as an open-channel lateral flow (immuno) assay substrate. The entire process, including the UV-light surface treatment to enhance wicking and the physical adsorption of the biotin-LTL, was achieved under ambient conditions. The general methodology was illustrated for the cases of optical (fluorescent) and MALDI-MS detection. The ability of protein separation on film opens a wealth of opportunities in the realm of protein solution processing for MALDI-MS, including simply reducing matrix effects (desalting) for the bio-molecules by using an on-film wash. Such concepts open many opportunities for bio-recognition and affinity separation when appropriate functional LTLs are attached utilizing the proposed method. The proposed method is predicted to be effective not only for the polypropylene films, but also for other types of film materials including poly(ethylene terephthalate), and polyamide (nylon 6). Certainly, use of different detection modalities, specifically visual/colorimetric observations as are common in the point-of-care market, would expand the utility of the method.

A couple of issues need to be addressed in future work to reduce this concept to practice. First, the method of application of the capture ligand must be improved. In future studies, a molecular printing system should be utilized as the application technique, to insure a uniformed surface on the desired regions. A potential extension is that different affinity functional groups could be applied onto separate channels, generating a multi-analyte, parallel LFA. Second, the method of applying the initial sample in a more uniform way would ultimately help the quantification. In this case, not performing the UV treatment at the film head would restrict the movement of the initial sample, concentrating the solute species to a narrower region. This could be used in concert with much lower sample volumes. Finally, the versatility for a variety of C-CP film compounds will be investigated in the near future along with the detailed study of role UV light treatment conditions on both the wetting and non-specific binding.

The presented study also demonstrated the principle of using C-CP films as processing platforms and MALDI targets for proteins analysis/identification. We have encountered problems and there are challenges remaining, but the overall simplicity, flexibility, and practicality of the approach showed its worthy of further development. In conclusion, The C-CP films as biosensor platforms, have a high potential path for point-of-care/lab-on-chip applications.

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FIGURE CAPTION

Figure 1. Conceptual illustration of the use of a capillary-channeled polymer (C-CP) film as a parallel, multi-analye lateral flow (immuno) assay. Sample solution wicking would flow left-to-right, with the analyte-specific capture ligands (e.g., the biotinylated-LTL here) immobilized within adjacent channels, and the terminus likely consisting of a sorbent pad (sink) to maintain capillary flow.

Figure 2. Schematic illustration of sequential surface modification and analyte capture processes for affinity targeting of streptavidin on PP C-CP films.

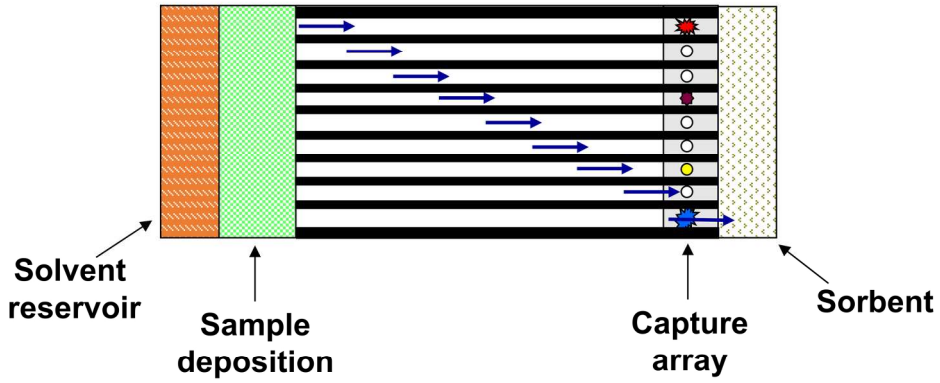
Figure 3. Microscope image (4x) of the CC-P films: a) cross section, and b) the surface.

Figure 4. Fluorescence image of the PP C-CP films after surface treatment of UV light, and modification with biotin-LTL in the middle of the film, and application of the protein mixture of SAv-TR and EGFP. a) before applying the 40:60 ACN:H_2O eluting solution at initial sample deposition zone, b) after elution at biotinylated region and c) after elution at the end of the film. Presence of SAv-TR indicated in red and EGFP in green.

Figure 5. Fluorescence image of the PP C-CP films following exposure to UV light and application of the protein mixture of SAv-TR and EGFP, a) before applying the 40:60 ACN:H_2O eluting solution at initial sample deposition zone, b) after elution at the body region and c) after elution at the end of the film. Presence of SAv-TR indicated in red and EGFP in green.

Figure 6. MALDI-TOF mass spectra of the PP C-CP films after surface treatment by UV light and modification with biotin-LTL and application of the protein mixture of SAv-TR and EGFP. a) before applying the 40:60 ACN:H_2O

eluting solution at initial sample deposition zone, b) after elution at the initial sample deposition zone, c) after elution, in the biotinylated region, and d) after elution, at the end of the film.



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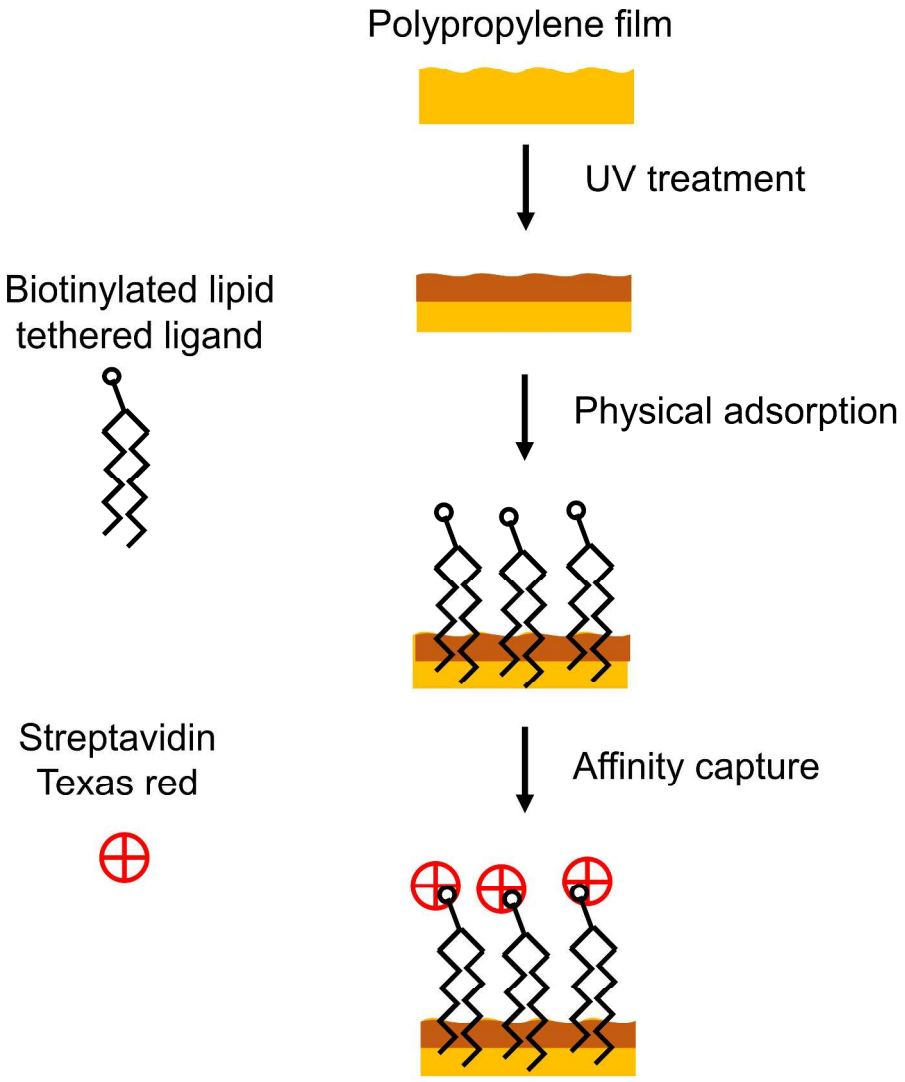

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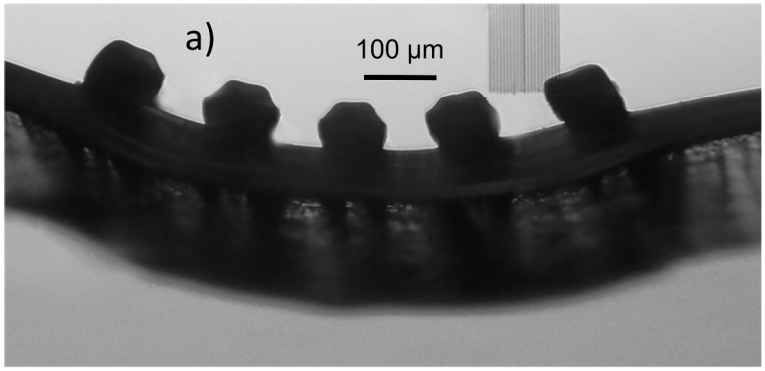

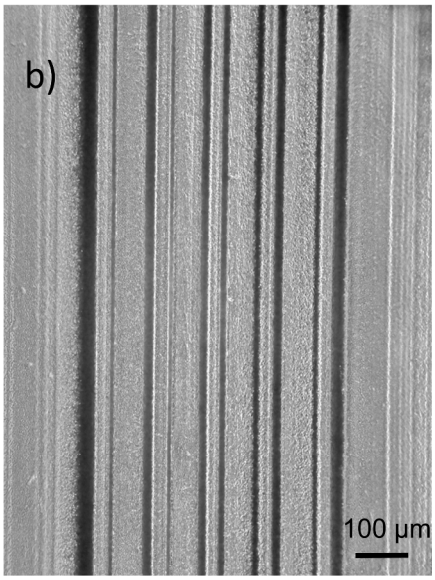

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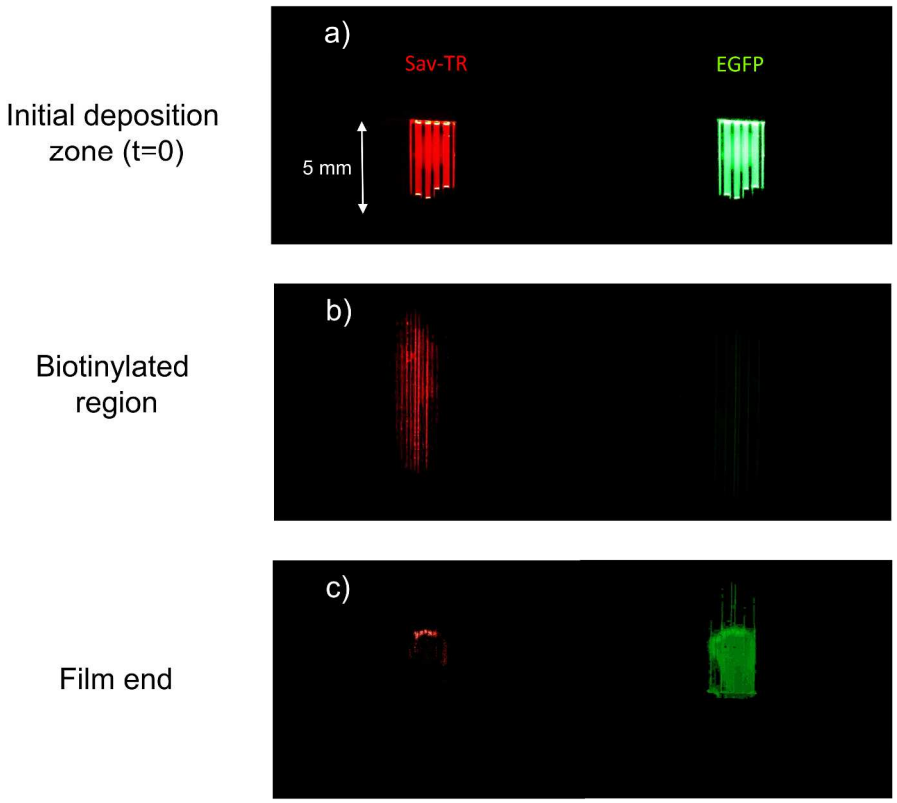

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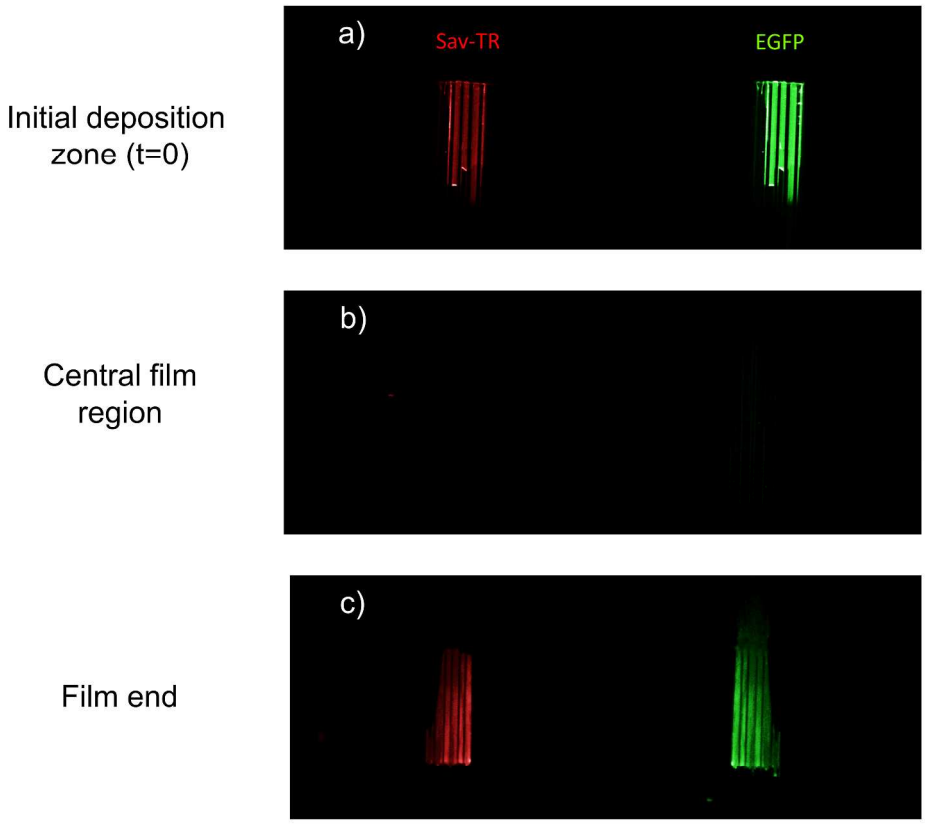

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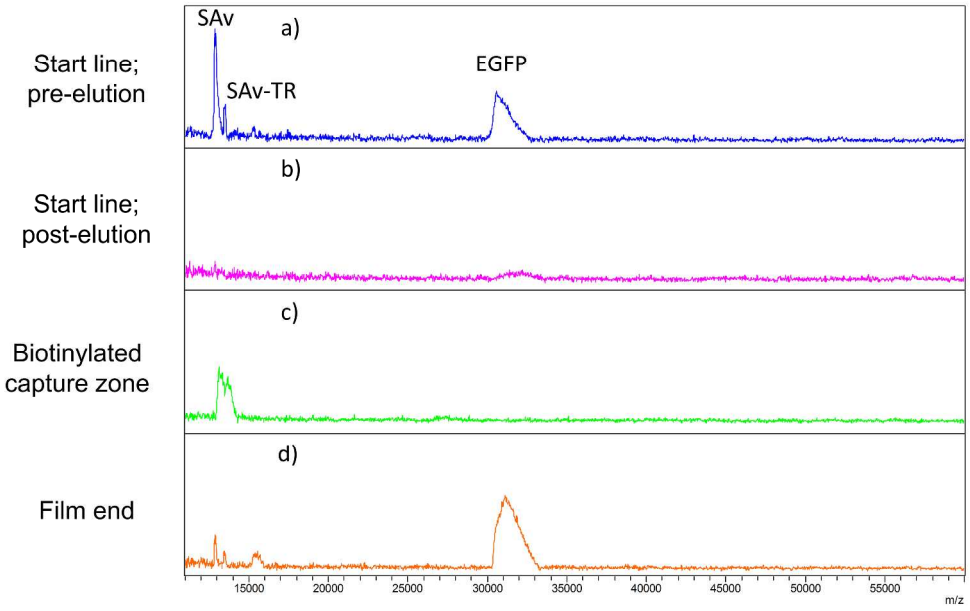

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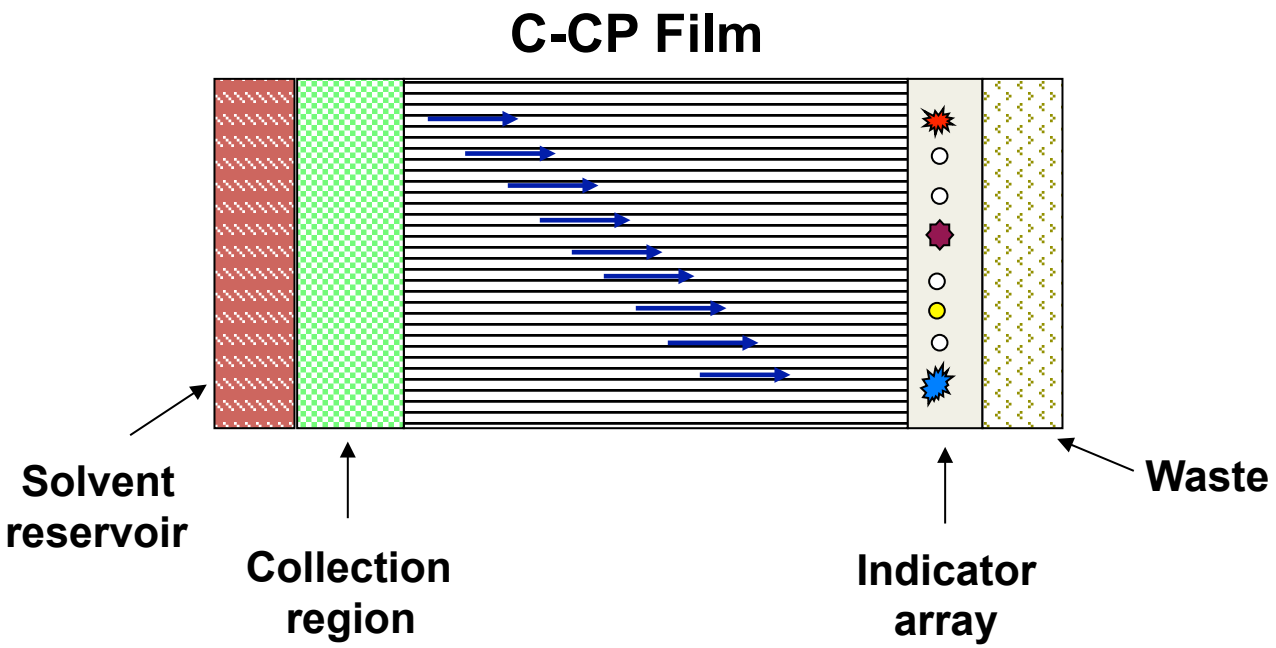
C-CP Film
Solvent reservoir
Collection region
Indicator array
Waste