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Assessment of enhanced autofluorescence and impact on cell microscopy for microfabricated thermoplastic devices

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Abstract

Thermoplastics such as polystyrene (PS) and cyclo-olefin polymer (COP) have become common materials for fabrication of microfluidic cell-based systems because of a number of attractive properties. However, thermoplastics are also known to exhibit autofluorescence levels that may hinder their utility for cell-based and imaging applications. Here we identify and characterize a phenomenon causing an increase in the autofluorescence of polystyrene after thermal treatment. This effect is of particular importance for plastic microfluidic device fabrication because the ranges of pressures and temperatures causing this effect match the same range as those used for polystyrene bonding. Further, we find that the enhanced autofluorescence has significant impact on the image quality, accuracy, and ability to identify and quantify fluorescently labeled cells. We tested two alternative strategies – solvent bonding of PS or thermal bonding of COP – to alleviate the adverse effects of heterogeneous and enhanced autofluorescence on cell image analysis, and demonstrate that both strategies are viable options to thermal bonding of PS for specific applications where cellular imaging is of primary interest.

Keywords

polystyrene; cyclo-olefin polymer; microfluidics; fluorescence; cell imaging

Introduction

Polystyrene (PS) is the most widely used material in biology laboratory plasticware. The majority of Petri dishes, tissue culture flasks, and well plates used for cell culture are made from PS, largely because PS is inexpensive to manufacture in high volume, has excellent strength and durability, good optical clarity, and can be treated or modified to acquire desirable surface properties for a broad range of applications. Because of its popularity and ubiquity in biology laboratories, PS attracts significant interest as a material of choice for developing advanced cell culture platforms, including, most notably, microscale and microfluidic cell-based systems.¹ Recent advances in the microfabrication of polymeric materials, including PS, have demonstrated that rapid prototyping processes can help

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streamline plastic microdevice fabrication and thus increase the supply of plastic microdevices to biology laboratories.^{2,3}

While PS has many advantages, PS also exhibits notable drawbacks in certain applications, including its propensity to leach bioactive contaminants,⁴ and its need for more complicated microfabrication techniques to produce working PS devices.¹ In the context of epifluorescence microscopy, PS displays high levels of autofluorescence particularly between the 300- to 400-nm wavelength range, which coincides with the excitation and emission spectra of common fluorophores used in cell-based biological assays. Although some basic studies of PS autofluorescence have been previously reported,^{5–9} and a number of reports have also discussed autofluorescence of other common materials for microfluidic applications, such as polycarbonate (PC), poly(methyl methacrylate) (PMMA), poly(dimethylsiloxane) (PDMS), cyclo-olefin copolymer (COC), and others,^{9–13} the effects of PS autofluorescence have not been described in the specific context of cell microscopy and image analysis for cell-based assays, particularly in PS-based microfluidic devices. Furthermore, the impact of different microfabrication techniques on autofluorescence has not been assessed. Given the growing interest in this research area and in the development of microfluidic cell-based assays in general, there is a need to bring awareness to the issue of autofluorescence, and assess its potential impact on microfluidic assay development.

In this brief article, we discuss the autofluorescence of polymeric materials, specifically in the context of microfluidic systems where different thermoplastics are exposed to treatments associated with microdevice fabrication. Specifically, we identify a phenomenon where heat-treated PS exhibits a significant increase in autofluorescence levels for wavelengths above 400 nm through an increase in overall autofluorescence as well as the generation of heterogeneous spots comparable in size and brightness to fluorescently labeled cells. We analyzed autofluorescence images of heat- and pressure-treated PS sheets, and characterized the impact of the resulting autofluorescence on a typical cell-based experiment involving fluorescence microscopy in microfabricated thermoplastic devices. The results indicated that the “enhanced autofluorescence” phenomenon significantly impedes the ability to accurately perform cell image analysis. We tested two alternative fabrication strategies for minimizing enhanced autofluorescence conceptually close to the original fabrication approach: (1) use of cyclo-olefin polymer (COP) instead of PS, based a recent report that demonstrated the use of COP for microfluidic cell-based screening¹⁴ and (2) use of solvent bonding instead of thermal bonding (Figure 1). Our goal here is to bring awareness to this issue, identify the conditions for which it may be a significant limitation for the development of cell-based assays, and provide simple alternatives that can circumvent the problem.

Materials and Methods

Plastic Materials

PS sheets (1.2 mm thickness; #ST313120) and PS film (190 μm thickness; #ST311190; no additives) were purchased from Goodfellow (Cambridge, MA, USA), and Zeonor® cyclo-olefin polymer (COP) film (130 μm thickness; ZF14-188, 1420R grade) was acquired from Zeon Chemicals. PS sheets were patterned with features and cut to size using a personal CNC micromilling machine (Tormach pCNC 770, Waunakee, WI, USA). PS and COP films

were cut to size with scissors. COP sheets were purchased from Ajedum Films (Ajedum, Solvay Solexis Inc., Newark, DE, USA) as 610- μ m thick sheets made from Zeonor COP (1420R grade).

Thermal and Solvent Treatments

For thermal treatment tests on PS and COP film, films were cut into $\sim 10 \times 20$ mm rectangular pieces, and placed onto a programmable 15-ton hydraulic press with heated platens (Model #3889, Carver Press, Wabash, IN, USA) for 30 min at 90°C (T90) or 125°C (T125), with or without pressure (P) (simulates optimal bonding conditions for PS to achieve high bond strength and high quality replication of microfeatures, as presented in ref. [2]). Pressure was applied at 3000 lb (or 9.7 ksi), while for samples without pressure, a 160-g aluminum block was placed on top of the sample to ensure contact of the sample piece with the heated platen surface. For solvent tests (S) on PS film only, acetonitrile (ACN; A998-4, Fisher Chemicals) was applied to the surface of the film for 10 seconds using a polyethylene transfer pipet (Samco, Thermo Scientific). Excess ACN was drained on a Kimwipe, and any remaining traces of ACN were evaporated by placing the film on a hot plate for 10 min at 65°C. To prepare the sample pieces for imaging, each rectangular sample piece was secured to an underlying glass cover slip (#3335, Thomas Scientific) with scotch tape.

Bonding of Micromilled Devices

For micromilled devices, PS or COP sheets were milled with microwell features (1.5 mm $\times$ 1.5 mm $\times$ 1.2 mm deep), and then bonded to PS or COP films, respectively, using procedures similar to the thermal and solvent treatment steps described above. Briefly, thermal bonding of PS was achieved with 3000 lb at 90°C for 30 min, while thermal bonding of COP was achieved with 3000 lb at 125°C for 30 min. Solvent bonding of PS was achieved by applying ACN to the surface of the film for 10 seconds using a transfer pipet, draining excess ACN on a Kimwipe, and then mating the ACN-treated film surface to the untreated surface of the PS sheet that had been pre-warmed to 65°C. Thumb pressure was simply applied uniformly across the device for ~ 1 min to allow the solvent to react with the polymer at the interface between the mated surfaces. The device was left at 65°C for an additional 10 min after force application.

Cell Culture and Labeling

RPMI8226 cells (human-derived multiple myeloma cell line of B-cell origin) were obtained from ATCC. RPMI8226 cells were routinely cultured at 37°C with 5% CO₂ in high-glucose DMEM containing 10% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin (1% P/S), and 10 mM HEPES buffer at 1.0 to 1.5 $\times 10^5$ cells/mL, and passaged every 2–3 days. Ramos cells (human-derived Burkitt's lymphoma cell line of B-lymphocyte origin) were obtained from ATCC. Ramos cells were routinely cultured at 37°C with 5% CO₂ in RPMI1640 containing 10% FBS, and 1% P/S at 2.0 to 2.5 $\times 10^5$ cells/mL, and passaged every 2–3 days.

For fluorescence labeling, CD138⁺ / CD20⁻ RPMI8226 cells and CD138⁻ / CD20⁺ Ramos cells were mixed in a 1:1 ratio to achieve a final cell density of 0.5 $\times 10^6$ cells/mL. The mixed cell suspension was stained for 1 h in a 4°C cold room by adding a 1:10 dilution of mouse anti-human CD138 antibody conjugated with AlexaFluor 647 (Clone B-A38,

MCA2459A647T, AbD Serotec), a 1:5 dilution of mouse anti-human CD20 antibody conjugated with AlexaFluor 488 (Clone 2H7, MCA1710A488T, AbD Serotec), and a 1:1000 dilution of Hoechst dye (Invitrogen, USA). The mixture of cells and antibodies mimicked common procedure for dealing with mixed cell samples for flow cytometry analysis. Cells were centrifuged at $200\times g$ for 5 min, resuspended at ~ 200 cells/ μL , and loaded directly into microfabricated well arrays by dispensing 4 μL per well. Arrays were incubated for 3 h at 37°C in 5% CO_2 , and representative images for each well were acquired for phase contrast, 350-nm, 488-nm, and 647-nm imaging channels.

Microscopy and Image Analysis

All fluorescent images were taken at room temperature using a Nikon Eclipse Ti inverted fluorescent microscope (Nikon Instruments) with a PlanFluor 10 $\times$ objective (NA= 0.30, through air, Nikon Instruments) coupled to a Nikon DS-Qi1Mc CCD camera (Nikon Instruments). Four filters for four different excitation/emission wavelengths were available: ex-350 nm/em-400 nm; ex-492 nm/em-517 nm; ex-572 nm/em-600 nm; ex-647 nm/em-700 nm. Images were acquired with NIS-Elements Version D 3.10 software (Nikon Instruments). Image analysis for autofluorescence tests on bare samples after thermal and solvent treatments were performed in ImageJ with custom macros and plugins.

For the cell imaging experiment, cells were identified and counted using an in-house program (Je'Xperiment; source code available upon request). The cell identification algorithm included background subtraction, auto-thresholding via the "Li" algorithm (Fiji imaging software) with a lower threshold level of 12 U.I., erosion, dilation, watershedding steps, and execution of the particle analyzer procedure from the ImageJ API. The algorithm was performed on each of three channels (350 nm = 'blue', 488 nm = 'green', and 647 = 'red'). Blue particles were considered as valid cells. Each blue particle center of mass was used to determine colocalization with green and red regions of interest (ROIs). If the center of mass of the blue particle was colocated inside a green or red ROI, the cell was counted as a 'true positive' green or 'true positive' red cell. If a blue particle was not colocated with green or red ROIs, it was deemed as an 'unlabeled' or negative cell. Finally, if a green or red ROI did not contain a blue ROI, the cell was considered a 'false positive' green or 'false positive' red cell.

Statistical Analysis

For thermal and solvent treatment tests, one-way analysis of variance (ANOVA) for each wavelength was performed, followed by Tukey's procedure for multiple comparisons. Results were considered statistically significant for $P < 0.05$. Sample size $n = 5$ for all tests. For the cellular counting tests, a two-tailed Wilcoxon rank-sum test was performed on a sample size of $n = 4$.

Results and Discussion

We first aimed to quantify the average level of autofluorescence in both PS and COP after various treatments associated with microdevice fabrication. PS and COP sample pieces were treated at either 90°C or 125°C , approximately 10°C below their respective glass transition

temperatures,¹⁵ and either with or without pressure, or with brief exposure to acetonitrile in the case of solvent treatment. The polymer sample was secured to an underlying glass coverslip, and imaged at four different fluorescence excitation wavelengths. Images were taken at the vertical edge of the sample piece such that the left half of the image showed the surface of the plastic, while the right half of the image showed the surface of the glass (Figure 2A). Two equally sized regions of interest (ROI) were sampled (avoiding the bright edge), one from each half of the image. Background levels from the glass were subtracted from the plastic.

At 350-nm wavelength excitation, there was significant autofluorescence across all materials and conditions tested, with all COP sheets (treated or untreated) significantly lower than untreated or solvent-treated PS, and all thermal treated PS significantly higher than untreated or solvent-treated PS (Figure 2). At 647-nm wavelength excitation, there was negligible autofluorescence across all materials. For 492-nm and 572-nm wavelength excitations, however, autofluorescence levels depended critically on material and treatment conditions. Specifically, all COP sheets exhibited negligible autofluorescence at both wavelengths, similar to autofluorescence at 647 nm. In contrast, untreated and solvent-treated PS were both significantly higher in autofluorescence than COP, while heat-treated PS with and without pressure were both significantly higher in autofluorescence than untreated and solvent-treated PS. In particular, high-pressure thermal treatment of PS further enhanced autofluorescence at 488- and 572-nm wavelengths. A possible explanation for the increased levels of autofluorescence is the formation of excimers resulting from polymer chain reorganization.⁵ This explanation is consistent with the observation that the excimer emission peak has a longer wavelength than the monomer emission peak, so increased excimer formation would likely shift or broaden the peak of the fluorescence spectrum to even longer wavelengths. While an in-depth analysis and discussion of polymer chemistry is not the main purpose of this report, we note that the observed effect of enhanced autofluorescence at >400 nm wavelengths is relevant and important because these are wavelengths that are most commonly used for cell-based assays and applications. Thus, care must be taken during experimental design in cell biology to account for this increase in autofluorescence.

Closer examination of the autofluorescence at both 350-nm and 488-nm wavelengths at higher magnification revealed that the average autofluorescence across the image was not only higher, but that the autofluorescence was characterized by relatively large, distinct circular patches or spots in a heterogeneous pattern (Figure 2B). Importantly, these spots were similar in size to typical biological cells, and thus the physical phenomenon identified here has important and direct implications in cell biology research. The enhanced and heterogeneous autofluorescence of PS above 350-nm wavelength makes it more difficult to choose appropriate fluorophores for fluorescence-based applications. Typically, this problem can be circumvented either by choosing strong fluorophores to render the autofluorescence negligible, or by avoiding the highly autofluorescent channels altogether. However, the use of thermal bonding procedures as described above would significantly increase autofluorescence levels across a broad range of commonly used fluorescence wavelengths.

To assess the actual impact of the enhanced and heterogeneous autofluorescence for cell-based applications, we performed a simple experiment involving cell imaging and analysis of a mixed culture of two immunolabeled suspension cell lines. The effects of three different fabrication methods (thermal bonding with PS, solvent bonding with PS, and thermal bonding with COP) on enhanced autofluorescence, and its impact on cell identification and image analysis, were evaluated and compared (Figure 3A). Ramos cells ($CD138^- / CD20^+$) were labeled with a directly conjugated AlexaFluor488-CD138 antibody, and were thus expected to coincide with an emission wavelength prone to the autofluorescence phenomenon. In contrast, RPMI8226 cells ($CD138^+ / CD20^-$) were labeled with a directly conjugated AlexaFluor647-CD138 antibody, and were thus expected to coincide with an emission wavelength not prone to autofluorescence. This experimental design also simulated a low-fluorescence intensity situation typical of flow cytometry analyses that employ directly conjugated antibodies. To aid cell counting, Hoechst stain was applied to all cells and in all conditions, and was expected to stain cell nuclei with an intensity significantly higher than the autofluorescence at 350 nm, thus being clearly identifiable. Cells co-stained with Hoechst and either CD138 (red) or CD20 (green) were considered ‘true positive’ cells. Cells stained with only Hoechst were considered ‘unlabeled’ cells, or cells that likely expressed the specific surface antigen in such low numbers that they could not be identified with the image algorithms used. In contrast, identified ‘red’ or ‘green’ cells without colocalized Hoechst stain were considered “false positives”. These cells were considered as miscounted cells resulting from inaccuracies of the algorithm or from spurious signals generated by autofluorescence of the device material. We note here that the number of true and false positives detected inherently depends on thresholding and other image analysis parameters, but that varying these parameters increased the “true positive” and “false positive” ratios accordingly.

In the case of thermal bonding with PS, the low levels of autofluorescence at 647 nm resulted in only 8% detection of false positive $CD138^+$ RPMI8226 cells with only red signal (and no nuclei). We considered this error to be inherent to the image analysis algorithms employed. In contrast, the enhanced and heterogeneous autofluorescence at 492 nm led to 54% detection of false positive $CD20^+$ Ramos cells with only green signal (and no nuclei). The heterogeneous autofluorescence is particularly difficult to remove by image filtering because it displays as bright spots with a size range similar to that of typical mammalian cells. Furthermore, intensity of these spots was in the same range as the cells of interest, increasing the detection of false positives significantly.

To address this issue of heterogeneous autofluorescence in annealed PS sheets, we evaluated two other fabrication processes, including solvent bonding of PS and thermal bonding of COP, which are commonly used materials and procedures in microfabrication. Both alternatives displayed low levels of false positives in the 647-nm wavelength, as expected, and thus allowed reliable identification of the $CD138^+$ RPMI8226 cells. Importantly, both alternatives performed significantly better in cell identification at the 488-nm wavelength, and only detected 23% and 28% false positive $CD20^+$ Ramos cells for PS solvent bonding and COP thermal bonding, respectively, which are both significantly lower than the 54% detection for PS thermal bonding. These results suggest that both alternatives will provide

more accurate cell identification in applications involving fluorescence microscopy, specifically low fluorescence intensity applications.

It is important to note that this phenomenon is particularly critical when imaging low-level fluorescence signal, such as that from directly conjugated antibodies or endogenously expressed fluorescent proteins. High fluorescence signals, such as those from cytoplasmic tracker dyes or cells with highly expressed proteins, can be imaged without requiring special attention toward autofluorescence issues. It is possible that the source or grade of PS and COP used for fabrication affects this enhanced autofluorescence phenomenon. Thus, while we have used bi-axially oriented PS sheets and 1420R grade COP in our experiments, it remains to be determined whether other forms of these raw material (e.g., injection molded, pellets, etc.) display the same phenomenon at the same levels. Ultimately, the design engineer of the platform must choose a material and fabrication process that is compatible with the application, and in the process give consideration to the autofluorescence issues described above.

Conclusions

In summary, we report a phenomenon by which the autofluorescence level of PS sheets is enhanced particularly after thermal treatment. The autofluorescence observed occurs both in average background intensity, but also in heterogeneous bright spots with size similar to that of biological cells. The autofluorescence phenomenon described has inherent implications in cell-based microfluidic applications because thermal diffusion bonding of PS is commonly employed for the microdevice fabrication. Applications specifically sensitive to this phenomenon are applications involving low fluorescence intensity microscopy, such as the imaging of directly immunostained cells, or imaging of fluorescent reporter cell lines for proteins with low-level endogenous expression. We have identified alternative fabrication methods for these cases, using either solvent bonding of PS or thermal bonding of COP, which were both found to mitigate the autofluorescence issues described.

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THERMAL BONDING OF POLYSTYRENE

ALTERNATIVE MATERIAL: COP

ALTERNATIVE METHOD: SOLVENT BONDING

Figure 1.

Autofluorescence of polystyrene (PS) is well documented, but this issue is exacerbated after thermal treatments that are common during bonding procedures of microfabricated polymeric devices. Two alternative strategies for addressing this issue are to choose a different material such as cyclo-olefin polymer (COP), or to choose a different bonding method, such as solvent bonding.

Figure 2.

Autofluorescence intensity of PS and COP with various treatments. (A) Phase contrast and fluorescence microscopy images from four different excitation wavelengths. Scalebars = 200 μ m. (B) Magnified images of thermally treated PS, solvent treated PS, and thermally treated COP. Scalebar = 50 μ m. (C) Background-subtracted average fluorescence intensity measurements. PS – polystyrene; COP – cyclo-olefin polymer; (T90+P) – with 90°C and pressure; (T90) – with 90°C only; (S) – with solvent; (NT) – no treatment. $n = 5$ for all conditions. Error bars = SE. Lines above bars indicate statistical significance at $P < 0.05$.

Figure 3.

(A) Microscope images of cells in microwell arrays fabricated using different methods including PS thermal bonding (left column), PS solvent bonding (middle column), and COP thermal bonding (right column). The images represent a 1:1 mix of AlexaFluor488-CD20⁺ and AlexaFluor647-CD138⁺ labeled cells. Top row - overlay of microscope images; bottom row - overlay of software-generated masks. (B) Cell counts for the three different methods of device fabrication. Light blue – Hoechst only cells; Green/blue stripes – Hoechst⁺/CD20⁺ cells; Red/blue stripes – Hoechst⁺/CD138⁺ cells; Green - CD20⁺ only cells (false positive green); Red – CD138⁺ only (false positive red). * indicates statistical significance at $P < 0.05$.