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A pH-Sensing Optode for Mapping Spatiotemporal Gradients in 3D Paper-Based Cell Cultures

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Abstract

Paper-based cultures are an emerging platform for preparing 3D tissue-like structures. Chemical gradients can be imposed upon these cultures, generating microenvironments similar to those found in poorly vascularized tumors. There is increasing evidence that the tumor microenvironment is responsible for promoting drug resistance and increased invasiveness. Acidosis, or the acidification of the extracellular space, is particularly important in promoting these aggressive cancer phenotypes. To better understand how cells respond to acidosis there is a need for 3D culture platforms that not only model relevant disease states but also contain sensors capable of quantifying small molecules in the extracellular environment. In this work, we describe pH-sensing optodes that are capable of generating high spatial and temporal resolution maps of pH gradients in paper-based cultures. This sensor was fabricated by suspending microparticles containing pH-sensitive (fluorescein) and pH-insensitive (diphenylanthracene) dyes in a polyurethane hydrogel, which was then coated onto a transparent film. The pH-sensing films have a fast response time, are reversible, stable in long-term culture environments, have minimal photobleaching, and are not cytotoxic. These films have a pK_a of 7.61 ± 0.04 and are sensitive in the pH range corresponding to normal and tumorigenic tissues. With these optodes, we measured the spatiotemporal evolution of pH gradients in paper-based tumor models.

GRAPHICAL ABSTRACT

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b05015. SEM images of DPA particles used in the pH-sensing films; schematics for paper scaffolds, acrylic holders, and flow cell used to evaluate the pH-sensing film; fluorescence intensity profiles of M231-mCherry cells during prolonged incubation; equations describing fluorescence intensity normalization and Boltzmann fit of pH data (PDF)

FIJI macro used to process images from Metamorph at MSL on IX70 (PDF)

FIJI macro used to straighten and average across the channel culture to generate plot profiles of each fluorescence image in Excel (PDF)

Notes

The authors declare no competing financial interest.

In tumors, a combination of rapid cellular proliferation and aberrant vasculature leads to localized hypoxia and acidosis.¹ Hypoxic cells rely on anaerobic glucose metabolism (i.e., glycolysis) for ATP production.^{2,3} Glycolysis is energetically inefficient compared to aerobic glucose metabolism and requires increased glucose consumption to maintain normal cellular function. Glycolysis results in an increased production of acidic byproducts—such as lactate and hydronium ions—which are effluxed from the cell, and accumulate in the extracellular space. This acidification of the extracellular space results in a reverse pH differential across the cell membrane.⁴ In healthy breast tissue, the extracellular pH (pH_e) is ~ 7.4 , while intracellular pH (pH_i) is ~ 7.2 . In breast tumors, the pH_e can drop as low as ~ 6.5 , while the pH_i increases to ~ 7.4 .^{5,6} Cancer cells respond to acidosis by adopting aggressive phenotypes such as increased: radio and chemoresistance, proliferation rates, invasion into neighboring tissues and the eventual formation of metastatic sites, and rates of mutagenesis and clastogenesis.^{4,5,7} Gradients of pH have also been shown to direct cellular invasion.^{8,9}

Various in vitro culture platforms are available to quantify cellular responses in tumor-like environments.^{10–12} These platforms are preferred over traditional monolayer cultures for predicting in vivo responses as they contain the cell–cell contacts, extracellular matrices, and diffusion-dominated gradients found in poorly vascularized tumors.^{13–15} Paperbased cultures are an emerging platform in which 3D tumorlike structures can be assembled using equipment commonly available to any tissue culture laboratory.^{12,16} These cultures combine the ease of assembly and analysis associated with traditional monolayer cultures. They also provide a means of evaluating cellular responses in 3D structures with experimentally defined compositions and extracellular gradients.

Paper-based cultures, which were first described by Whitesides and colleagues,¹⁷ are prepared by seeding cell-laden hydrogels into wax-patterned sheets of paper. The hydrophobic wax borders isolate the cells into defined regions, allowing many cultures to be prepared in parallel on a single sheet of paper. We have used paper-based assays to quantify both cellular invasion^{18–21} and chemotherapy resistance²² in tumorlike environments. We have shown that cancer cells selectively invade regions of higher oxygen tension when placed in diffusion-limited environments where the cells are simultaneously exposed to overlapping gradients of oxygen, nutrients, signaling molecules, and waste products.

To determine the role of acidosis in promoting invasion and drug resistance, there is a need for sensors capable of mapping the formation and maintenance of pH gradients in 3D cultures. When combined with the oxygen sensors previously developed in our laboratory,²³

these sensors will also determine if there are synergistic effects resulting from overlapping gradients of oxygen and pH.

Traditionally pH has been quantified with glass electrodes, which are rigid in design and limit measurements to large areas. Multianalyte microelectrode arrays provide higher spatial resolution without sacrificing sensitivity, and have been used to quantify extracellular analytes and pH in long-term cell cultures.^{24,25} Optical chemical sensors (optodes) are an attractive alternative for in situ pH measurements as they are easily fabricated, miniaturized, and are available in many forms.^{26–31} Thin film optodes are especially attractive as they are easily imaged and unmatched in their ability to spatially resolve and quantify analytes in real-time.^{9,32} Optodes have some disadvantages compared to electrochemical sensors, such as limited stability due to dye leaching from the optode matrix, photobleaching upon prolonged exposure to ultraviolet light, and sensitivity to changes in the composition of the medium being measured. Many of these disadvantages can be avoided by incorporating reference dyes or by selecting the appropriate sensing matrix.

In this work, we fabricated and characterized pH-sensing thin films. By placing these culture-compatible optodes in direct contact with a cell-containing paper scaffold, we were able to spatially and temporally map pH gradients over 48 h using fluorescence microscopy. The pH_e was determined from a ratio of two fluorescence intensities, which arise from a homogeneous mixture of pH-sensitive particles and pH-insensitive reference particles suspended in a polyurethane thin film. The pH-sensitive particles were short chitosan oligosaccharides labeled with fluorescein. The pH-insensitive reference particles were diphenylanthracene (DPA) encapsulated in polyacrylonitrile (PAN). The pH optode has a pK_a of 7.61 ± 0.04 ($I = 150$ mM) and is most sensitive from pH 6.6 to 8.6, spanning pH_e found in healthy breast tissue (~ 7.4) and breast tumors (~ 6.5).

EXPERIMENTAL SECTION

Reagents.

All reagents were used as received, unless otherwise noted. Fluorescein isothiocyanate and 9,10-diphenylanthracene were purchased from ACROS Organics, HydroMed D4 was purchased from AdvanSource Biomaterials, and Sylgard 184 Silicone Elastomer was purchased from Dow Corning. Acetone, dimethylformamide, ethanol (200 proof), hydrochloric acid, hydrogen peroxide (30%), methanol, sodium chloride, sodium hydroxide, and sodium phosphate dibasic (anhydrous) were purchased from Fisher Scientific. Chitosan (low MW) and polyacrylonitrile (150 000 MW) were purchased from Sigma-Aldrich. Dubelco phosphate-buffered saline (DPBS), HEPES, RPMI 1640 medium, and penicillin–streptomycin were purchased from ThermoFisher Scientific. Fetal Bovine Serum was purchased from VWR.

Particle Preparation and Functionalization.

pH-sensing particles were prepared using a protocol adapted from Lu et al.³³ Chitosan (2.5 g) was digested in an aqueous solution containing 15% H_2O_2 for 1 h at 85 °C. The solution was centrifuged at 7500g for 10 min, and the supernatant was filtered through Whatman 40

filter paper. Acetone (50 mL, 1 mL/s) was added to the filtrate, and the resulting precipitate was washed twice with methanol (40 mL). The precipitate was redissolved in pH 9 water (20 mL), fluorescein isothiocyanate (FITC, 20 mg) was added, and the solution was stirred for 3 h. To precipitate the fluorescein-labeled chitosan, methanol (50 mL, 1 mL/s) was added to the solution. The precipitated particles were washed with methanol (4×, 40 mL) and then stored in 1 mL of methanol in the dark at 25 °C.

The labeled oligosaccharides were analyzed with gel permeation chromatography, on a Waters liquid chromatograph equipped with Waters Ultrahydrogel 250 and 120 columns (in series), a Waters 141 refractive index detector, and a Wyatt miniDAWN TREOS MALS detector. The chromatographic parameters include: a mobile phase consisting of 0.1 M acetate buffer (pH = 4.60) and 0.5 M sodium nitrate, a flow rate of 0.6 mL/min, a column temperature of 25 °C, and a sample volume of 100 μ L. The labeled oligosaccharides have an average molecular weight of 3464 Da and a polydispersity of 1.848.

pH-insensitive reference particles were prepared by coprecipitating 9,10-diphenylanthracene (DPA) and polyacrylonitrile (PAN), according to a protocol first described by Wang et al.³² PAN (0.3 g) was dissolved in 30 mL of DMF containing 3 mM DPA. To precipitate the particles, 70 mL of water was added to the solution (1 mL/s) followed by 20 mL of brine (5.5 M NaCl, 1 mL/s). The mixture was then stirred for 1 h. Particles were washed with ethanol (5×, 100 mL), followed by water (4×, 100 mL), and then stored in 5 mL of water in the dark at 4 °C.

The morphology and size of the PAN particles were characterized using scanning electron microscopy (SEM, Hitachi S-4700) at an accelerating voltage of 2.0 kV, a working distance of 4.5 mm, and a magnification of 50 000×. For sample preparation, a single droplet of particles suspended in EtOH was deposited on a silicon support, dried, and then sputter coated with 13 nm of a AuPd alloy. The particles are 3.3 ± 0.6 nm in diameter (Figure S1).

Film Preparation.

The sensing matrix was prepared by dissolving 1.2 g of HydroMed D4 in 14 mL of 95% (v/v) ethanol. A 1 mL suspension of the fluorescein-labeled chitosan was sonicated for 30 min, centrifuged, and then resuspended in 4 mL of HydroMed D4. A 1 mL suspension of the DPA reference particles were sonicated for 30 min, centrifuged, and resuspended in 0.33 mL of water before being added to the HydroMed D4. The sensing matrix (0.5 mL) was spin coated onto 18 mm round PET films (overhead transparency, Staples) for 10 s at 450 rpm. The freshly prepared films were placed in a clean Petri dish and dried overnight under ambient conditions. Prior to use, the films were rehydrated in DPBS for 48 h to remove any unreacted dye and then sterilized under UV light for 30 min. Film thickness was evaluated using a stylus profilometer (KLA Tencor P-6). Films are approximately 100 μ m thick with variances of ± 2 μ m across a single film.

Cell Culture.

MDA-MB-231 breast adenocarcinoma cells (ATCC) were transduced with lentiviral particles (LPP-MCHR-LV105-025, GeneCopoeia) according to the manufacturer's protocol. Single clones of the mCherry-expressing cells (M231-mCherry) were selected. Cells were

maintained as monolayer cultures at 5% CO₂ and 37 °C in 1640 RPMI medium with L-glutamine, supplemented with penicillin–streptomycin (1%) and fetal bovine serum (10%). Cells were passed when the culture was 70–80% confluent and medium exchanged every 48 h.

Scaffold and Holder Preparation.

Paper scaffolds were prepared as detailed previously.¹⁸ Three channels (11 × 2 mm) were wax-patterned onto both sides of Whatman 105 paper using a ColorQube 8570 printer. Scaffolds were baked for 15 min at 150 °C and then sterilized under UV light overnight. PET films were cut using a Silver Bullet cutting machine, cleaned with ethanol, and autoclaved prior to use. Holders were prepared by laser cutting (Universal Laser Systems, ILS9.75) sheets of clear cast acrylic (McMaster-Carr). Both the top (6.25 mm thick acrylic) and the bottom (3.125 mm thick acrylic) pieces were laser cut with settings of: 100% power, 2% speed, and 1000 ppi. Holders were cleaned with ethanol prior to use. Detailed schematics of the paper scaffolds (Figure S2) and acrylic holders (Figure S3) are available in the Supporting Information (SI).

Flow Cell Device Preparation.

The flow cell used in this work consisted of a 6.35 μ m thick polydimethylsiloxane (PDMS) flow guide sandwiched between two 3.175 mm thick acrylic blocks (Figure 1a). All components were laser cut, using the settings described above. The top acrylic block included three ports—two inlets and a single outlet—which were interfaced with 0.79 mm Tygon tubing via a wall-plug technique described by Capretto et al.³⁴ Prior to device assembly, a pH-sensing film was placed between the bottom acrylic piece and PDMS flow guide. Detailed schematics of each component of the flow cell (Figure S4) are available in the SI.

Cell Culture Assembly and Analysis.

Cells were suspended in Matrigel at 150 000 cells/ μ L and stored on ice to prevent gelation. A 2 × 2 mm section of each channel was filled with 1 μ L of the cell suspension as depicted in Figure 2a. The remainder of the channel was filled with 2 μ L of Matrigel. The cell-seeded scaffolds were incubated in culture medium for 4 h prior to being sandwiched between a pH-sensing film and a PET film. The PET films were configured to either expose the entire culture to medium (an “open configuration”) or limit exposure to one end of the channel (a “closed configuration”). The entire stack was enclosed in an acrylic holder, placed in a six-well plate, covered with culture medium, and incubated.

Assembled devices were imaged with an Olympus IX70 inverted wide field fluorescence microscope. The assembled devices were housed in an incubator, which maintained the cultures at 37 °C and 5% CO₂. Images were captured with a Hamamatsu Flash 4.0 V2+ sCMOS camera with a 4×/0.13 PhL UPlanFL objective. M231-mCherry cells were imaged with a 555/25 nm excitation and a 605/52 nm emission filter, with 400 ms exposure. pH-sensitive particles were imaged with a 490/20 nm excitation and a 525/36 nm emission filter, with 400 ms exposure. DPA reference particles were imaged with a 376/30 nm excitation and a 455/50 nm emission filter, with 100 ms exposure.

Statistical Analysis.

All values are reported as the average and standard deviation, unless stated otherwise. Data sets were analyzed with GraphPad Prism. A two-way ANOVA with Tukey's multiple comparison post-test was used to compare different data sets. A p value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Sensor Design and Fabrication.

We prepared a pH-sensing optode, which contained a mixture of pH-sensitive and pH-insensitive particles (Figure 2c). The pH-insensitive particles served as a reference and eliminated any variations arising from inhomogeneity in film thickness, composition, or dye distribution. We selected the polyurethane hydrogel HydroMed D4 as our sensing matrix. This particular hydrogel is attractive for our applications as it is commercially available, soluble in 95% ethanol, easily prepared as a film with spin or knife coating, cell culture compatible, and able to facilitate rapid proton transfer.^{35–40} It also is ideal for cell-culture-based measurements as polyurethane films have low sensitivity to ionic strength,^{41–43} ensuring the observed changes in fluorescence intensity were due to changes in the pH of the extracellular environment. This insensitivity to ionic strength can be attributed to the film's neutral charge and high water content, which eliminates a discrete interface between the optode and culture medium.

A number of cell culture appropriate pH-sensing optode formats have been described in the following review articles.^{26,44} Some examples include a dual-dye system similar to those reported in this work. Others used self-referencing dyes, which eliminate the need for a reference particle but can suffer from leaching or be costly.^{26,37,45} We chose fluorescein as our pH-sensitive dye because it is inexpensive, has a relatively good photostability, and has a number of commercially available variants that allow for covalent attachment to a substrate.⁴⁶ In this work, we used fluorescein isothiocyanate (FITC) to covalently attach the dye molecules to amine-containing substrates. The pH-insensitive reference particles contained diphenylanthracene (DPA) molecules encapsulated in polyacrylonitrile (PAN).

The first-generation films contained fluorescein-modified silica or polystyrene microparticles. Films containing the fluorescein-labeled silica particles were prone to extensive leaching of the dye despite thorough washing before use. Films containing the fluorescein-labeled polystyrene particles did not suffer from leaching but were difficult to reproducibly fabricate. We found that small changes in the labeling time resulted in significant shifts in pK_a of the particle-immobilized fluorescein molecules, ranging from 6.90 to 7.95.

The second-generation films, which were ultimately used in this work, contained fluorescein-modified chitosan oligosaccharides. These oligosaccharides were generated by the oxidative degradation of low molecular weight chitosan polymers using a previously reported method.³³ These films had a pK_a of 7.61 ± 0.04 at a physiologically relevant ionic strength ($I = 150$ mM), could be reproducibly prepared, and were not prone to leaching. The

remainder of the experimental details presented in this work pertains to the second-generation films, which were spin coated onto circular PET films.

Sensor Characterization.

To characterize the pH-sensing films and determine their suitability for prolonged cell culture applications, we measured their: (i) pH working range and sensitivity; (ii) temporal responsiveness to changes in pH; (iii) relative photostability; (iv) stability in the presence of cell culture conditions; and (v) cytotoxicity. When possible, we compared these values to other pH-sensing films that used HydroMedD4 as a sensing matrix (Table 1). To ensure the films could be reproducibly fabricated, we measured the variability across each film as well as between independently prepared films. To determine the variability across a single film, we divided the sensor into subsections and measured the average response within those regions. All reported values are from at least three independently prepared films.

pH Working Range.—We calibrated the pH-sensing films with a series of high ionic strength ($I = 150$ mM) phosphatebuffered solutions ranging from pH 5.50 to 8.50. To best mimic the setup used in our cell cultures, we placed the films in a custom built acrylic holder (Figure 2a) adjacent to a waxpatterned paper scaffold. We measured the fluorescence intensity across the entire film with an inverted fluorescence microscope and used a Boltzmann function (Figure 3a eq S2) to relate the fluorescence intensity ratio of the fluorescein and DPA particles and pH. The average pK_a of the chitosanimmobilized fluorescein molecules was 7.61 ± 0.04 , which is similar to the values others have found for fluorescein encapsulated in the HydroMed D4 gels.^{35–37}

The uniformity and reproducibility of the films' ability to measure pH is quite good, with an RSD of 0.52% across a single film and an RSD of 0.53% across three films. Using the slope of the calibration curve, we determined the films have a maximum sensitivity of 0.92 and can reliably resolve changes as small as 0.13 pH units within ± 1 pH unit of the pK_a . This pH range is appropriate for our paper-based tumor models, based on values reported in healthy breast tissue and in breast tumors (pH = 6.5–7.5).

Responsiveness to Changes in pH.—To characterize the responsiveness of the pH-sensing films, we constructed a flow cell (Figure 1a) that allowed us to quickly alternate the delivery of two phosphate-buffered solutions (20 mM, $I = 150$ mM) with pH values of either 6.00 or 8.00. Using an inverted fluorescence microscope, we measured the fluorescence intensity of both dye molecules at 1 s intervals. The average response time (t_{90}) across three independently prepared films was 20.7 ± 4.3 s (Figure 1b). The films were also reversible over at least five cycles (Figure 1c). The response time and reversibility values are comparable to polyurethane-based pH sensors used in other works.^{38–40}

Film Photostability.—To evaluate sensor photostability, we measured the fluorescence intensity of both dye molecules across the entire sensor under constant illumination for 1 h at pH 6.00 and 8.00. While we observed a decrease in signal for both DPA and FITC (Figure 1d), we are able to normalize the signal intensity to a film subjected to the same exposure conditions and account for any changes due to photobleaching (eq S1). We also note that for

our particular application, illumination was limited to a short period of time: 4 s over a 48 h period.

Film Cytotoxicity.—To ensure the pH-sensing films were compatible with our cell culture setup, we seeded M231mCherry cells in paper scaffolds and cultured them for up to 48 h in contact with either: a bare PET film, a PET film coated with HydroMed D4, or a PET film coated with a HydroMed D4 containing the fluorescein and DPA particles. After 48 h, the cultures were disassembled, and the paper scaffolds were imaged on a Typhoon 9400 scanner as described in previous publications.^{19,22} The average fluorescence intensity of each cell-containing region (Figure 3b) is statistically insignificant for all of the culture configurations, suggesting that our pH-sensing films are not cytotoxic over the duration of our experiments. Previous setups in our lab have utilized unmodified PET films to limit the exchange of oxygen and nutrients between the culture and fresh medium. We have shown that this material is not cytotoxic.

Film Stability.—To confirm that the films were stable in culture conditions, we measured their fluorescein/DPA intensity ratio before and after a 5 day incubation period in spent culture medium collected from M231-mCherry cells. We imaged the sensors in a pH 7.00 phosphate-buffered solution and found there was no statistical significance in the measured pH value after prolonged exposure to culture medium (Figure 3c).

Mapping pH Gradients Formed in Paper-Based Channel Cultures.

To image the evolution of pH gradients in the paper channels, we sandwiched a cell-laden scaffold between a pH-sensing film and a PET film and enclosed the entire structure in an acrylic holder (Figure 2a). The PET films determined the level of exchange between the cells and culture medium. In the open configuration (Figure 4a), the entire culture was exposed to culture medium. In the closed configuration (Figure 4b), exchange with the culture medium was limited to one end of the channel. By limiting this exchange, we are able to generate a diffusion-limited environment similar to that found in poorly vascularized tumors. We have used these different culture configurations previously to quantify cellular invasion in real-time.¹⁸ To simplify data analysis and focus on characterizing the pH-sensing film's ability to map gradients in cell cultures, we sequestered the cells in the center of each paper channel with thin wax-patterned lines that prevented invasion (Figure 2b).

We imaged both the M231-mCherry cells and the pHsensing film in situ, over the course of a 48 h experiment, with an inverted fluorescence microscope (Figure 4). Each assembled channel image is a composite of five individual images, which were stitched together at a 10% overlap using a custom FIJI macro (FIJI Macro S1). To enable quantitative image analysis, each individual image was subtracted using the camera offset and normalized to the uneven illumination in the field of view prior to stitching. The assembled images allowed us to generate a spatial distribution of pH across the channel, while the different time points allowed us to visualize temporal changes. The resolution of the pH-sensing films is limited by the microscope objective and able to spatially resolve a single pixel ($2.62 \mu\text{m}^2$). Figure 4 contains representative images of the open and closed culture configurations after 1 h of incubation. Figures 4c,e illustrate the position of the sequestered M231mCherry cells.

Figures 4d,f are the heat maps, which correspond to the spatial distribution of pH values, generated from the ratio of fluorescein and DPA fluorescence intensities. To better visualize changes in pH along the channel, we developed a macro to reduce the two-dimensional image to a single dimensional profile by averaging every y -pixel for a given x -pixel across a 5×1.5 mm section (FIJI Macro S2). To demonstrate the film's ability to map pH over an extended culture period, we generate representative profiles after 1, 6, 12, 24, and 48 h of incubation. Figure 4g is the profile for the open configuration culture. Figure 4h is the profile for the closed configuration culture. These profiles illustrate how the pH gradients evolve over 48 h in our 3D tumor model.

The pH profiles across the length of the closed configuration culture (Figure 4h) resemble those predicted for spheroids and solid tumor masses.⁴⁷ The pH steadily decreases from the region of free exchange with culture medium ($\text{pH} = 7.26 \pm 0.07$) to the edge of the cell mass and continues to drop until the center of the cell-seeded region ($\text{pH} = 6.54 \pm 0.03$). Over time, the slope of this pH gradient slowly decreases as pH_c steadily increases in the center of the cell-seeded region. These changes in pH at the center of the culture are statistically significant ($p < 0.50$) between 1 and 6 h, 6 and 24 h, and 12 and 48 h. Images of the cell-containing region show a decrease in fluorescence intensity across the entire population with increasing culture time (Figure S5). These data suggest the increase in pH is due to decreased metabolic activity and that the buffering capacity of the culture medium is able to mitigate the reduced production of the acidic species.

We note that the observed changes in the closed configuration are due to the culture conditions and not to pH-sensing films, as a starkly different result is observed for the open culture configuration. Here, the pH gradient is spatially consistent across the entire channel and temporally constant after the first hour (Figure 4g). We attribute the initial drop in pH to the acclimation of the culture medium to 5% CO_2 conditions of the incubator. These results confirm that we are able to discern spatiotemporal differences in pH across paperbased cultures.

CONCLUSION

This work describes the preparation and characterization of a pH-sensing thin film that can be directly incorporated into monolayer and 3D cell cultures. These sensors are prepared by suspending two different particles in a polyurethane hydrogel, which was then coated onto a PET film. The pH-sensitive particles are fluorescein-modified chitosan oligosaccharides. The pH-insensitive particles were DPA molecules encapsulated in polyacrylonitrile. These pH-sensing films are the first, which we are aware of, to utilize chitosan as a support for the attachment of pH-sensitive molecules. The chitosan supports are ideal for film fabrication because, unlike other amineterminated microparticles, they were not susceptible to leaching upon prolonged incubation in aqueous environments. The regular spacing of the amine groups on the chitosan oligomers also ensured reproducible labeling, resulting in independently prepared films with consistent pK_a values and sensitivities.

The pH-sensing films have a fast response time, are reversible and stable in long-term cultures, have minimal photobleaching, and are not cytotoxic. Using fluorescence

microscopy, we were able to generate spatially resolved maps of pH across 3D cultures of cell-containing paper scaffolds. These films also allowed us to map the temporal evolution of the pH gradients across a 48 h period.

These pH-sensing films will enable studies of extracellular gradients on cellular movement, viability, and drug resistance. We will continue to use these films to spatially resolve pH gradients in our 3D cultures and determine how small changes in culture environment (e.g., cellular density, cell type, different conditions of limited exchange) manifest changes in gradient formation as well as cellular responses to those gradients. The ease of preparation and compatibility of these films with human cancer lines suggests that they can be incorporated into other devices as well as monitor pH gradient formation and maintenance in bacterial, eukaryotic, and other tissue-like cultures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. pH sensor characterization. (a) Diagram of the flow cell used to exchange buffered solutions in contact with the pH-sensing film. (b) Fluorescein fluorescence intensity values as the buffered solution was exchanged from pH 6.00 to 8.00. The average response time (t_{90}) of two subsections from three pH sensors was 20.73 ± 4.27 s. (c) Fluorescence intensity of fluorescein as pH 6.00 (low signal) and 8.00 (high signal) buffered solutions were delivered. Two subsections of three independently prepared films were monitored and demonstrated that the pH sensor was reversible. (d) Fluorescence intensity of both the fluorescein- (top) and DPA-containing (bottom) particles subjected to constant illumination (490 or 376 nm). Reported values are the average of five subsections from three independently prepared films in pH 6.00 and 8.00 phosphate-buffered solutions. Error bars correspond to standard deviation.

Figure 2.

(a) Diagram of the pH-sensing film, which was integrated into the paper-based culture platform. A single sheet of paper, wax-patterned with three identical channels, was seeded with cells, sandwiched between a PET film and pH sensor, and placed in a custom-made acrylic holder. (b) Representative micrographs of a (top) single channel seeded with mCherry-expressing MDA-MB-231 cells, (middle) the pH-sensitive fluorescein particles, and (bottom) the pH-insensitive DPA reference particles. The wax lines were used to limit cellular movement during the experiment and are visible through the sensing film. (c) Schematic of the pH sensor (not to scale).

Figure 3. pH sensor characterization. (a) Fluorescence intensity of the pH-sensing film when exposed to buffered solutions of different pH at 37 °C. The average pK_a value of 15 subsections from 3 independently prepared films was 7.61 ± 0.04 ($I = 150$ mM). (b) Fluorescence intensity of MDA-MB-231-mCherry cells seeded in paper-based scaffolds and cultured adjacent to the pH-sensing films for either 24 or 48 h. Fluorescence intensity values were normalized to the absolute fluorescence intensity measured on day zero and are the average of 10 biological replicates. (c) Fluorescence intensity of films, which were incubated in spent culture medium for 5 days and placed in a pH 7.00 buffered solution. Values are from 10 subsections of 3 separate films. Error bars in each figure correspond to the standard deviation.

Figure 4.

pH-sensing in paper-based cell cultures. (a) Diagram of the open culture configuration, in which culture medium freely exchanges with the entire channel. (b) Diagram of the closed culture configuration, in which medium exchange is limited to one side of the channel. Representative fluorescence micrographs of M231-mCherry cells in the (c) open and (d) closed culture configuration. Representative heat maps of the pH values across the cell-containing channels in the (e) open and (f) closed culture configuration. The vertical lines in images (e) and (f) are the wax lines, which were printed on the adjacent paper scaffold and used to isolate cell populations throughout the duration of the experiment. All scale bars shown are 250 μm . Average pH profiles across the cell culture over a 48 h period in the (g) open and (h) closed culture configuration. Each plot is the average across three cell cultures. We note that the cell-containing regions represented by the dotted lines are also the average of three different cultures and that differences in the shapes of these profiles are a result of manually seeding the cells in each paper channel. The error bars indicate the standard error of the mean.

Table 1.

pH-Sensing Thin Films Prepared in HydroMed Polyurethane Matrices

dye	incorporation into matrix	t_{90} (s)	pK_a	ref.
fluorescein	immobilized on chitosan	20	7.61	this work
5-hexadecanoylamino fluorescein	encapsulated	16	7.15	36
OHButoxy-aza-BODIPY	encapsulated	20	7.96	35
SNARF-DE	encapsulated	12	7.70	37