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Comparative Study of Poly (ϵ -Caprolactone) and Poly(Lactic-co-Glycolic Acid) -Based Nanofiber Scaffolds for pH-Sensing

Wenjun Di¹, Ryan S. Czarny², Nathan A. Fletcher², Melissa D. Krebs², and Heather A. Clark^{1,*}

¹Department of Pharmaceutical Sciences, Northeastern University, Boston, MA 02115

²Chemical and Biological Engineering, Colorado School of Mines, Golden, CO, 80401

Abstract

Purpose—This study aims to develop biodegradable and biocompatible polymer-based nanofibers that continuously monitor pH within microenvironments of cultured cells in real-time. In the future, these fibers will provide a scaffold for tissue growth while simultaneously monitoring the extracellular environment.

Methods—Sensors to monitor pH were created by directly electrospinning the sensor components within a polymeric matrix. Specifically, the entire fiber structure is composed of the optical equivalent of an electrode, a pH-sensitive fluorophore, an ionic additive, plasticizer, and a polymer to impart mechanical stability. The resulting poly(ϵ -caprolactone) (PCL) and poly(lactic-co-glycolic acid) (PLGA) based sensors were characterized by morphology, dynamic range, reversibility and stability. Since PCL-based nanofibers delivered the most desirable analytical response, this matrix was used for cellular studies.

Results—Electrospun nanofiber scaffolds (NFSs) were created directly out of optode material. The resulting NFS sensors respond to pH changes with a dynamic range centered at 7.8 ± 0.1 and 9.6 ± 0.2 , for PCL and PLGA respectively. NFSs exhibited multiple cycles of reversibility with a lifetime of at least 15 days with preservation of response characteristics. By comparing the two NFSs, we found PCL-NFSs are more suitable for pH sensing due to their dynamic range and superior reversibility.

Conclusion—The proposed sensing platform successfully exhibits a response to pH and compatibility with cultured cells. NFSs will be a useful tool for creating 3D cellular scaffolds that can monitor the cellular environment with applications in fields such as drug discovery and tissue engineering.

Keywords

pH detection; electrospinning; nanofibers; poly(ϵ -caprolactone); poly(lactic-co-glycolic acid)

*Corresponding author: Heather A. Clark, h.clark@neu.edu, Phone: (617) 373-3091.

SUPPLEMENTARY INFORMATION

Supplementary information is available in the online version of the paper.

INTRODUCTION

Two-dimensional cell culture studies have played a significant role in furthering our understanding of developmental biology, disease mechanisms, regenerative medicine, drug discovery, large-scale protein production and tissue engineering. However, potential inadequacies of 2D culture systems have emerged, especially with respect to their inability to emulate *in vivo* conditions and provide physiological relevance. Important physical, mechanical, and biochemical cues are missing under such simplified conditions. Thus, creating a three-dimensional cell culture could be more physiologically relevant for mimicking extracellular environments. The development of 3D cell culture systems has greatly benefited tissue engineering and models for drug screening (1, 2). There are many challenges associated with employing 3D cell cultures, including monitoring the interior of tissue constructs. Among key indicators of metabolism, pH is one of the most pivotal parameters requiring optimization since pCO_2 , lactate, ammonium and medium compositions could all impact pH (3–5). The monitoring of accurate and reliable pH during bioprocesses is vital because cell culture media pH directly affects cellular growth, proliferation and target protein productivity. Extracellular pH changes can tremendously influence the synthesis of cellular matrix components. Additionally, in pharmaceutical applications extracellular pH monitoring is used for cell-based assays to examine perturbations in metabolic activity as the result of drug efficacy, metabolism and toxicity. Therefore, a pH sensor platform for monitoring within 3D cell cultures and other cellular microenvironments in a non-invasive and non-interactive manner needs to be developed. The design, construction and testing of such a sensing platform is the topic of this article.

Approaches to pH measurement, such as the determination of hydrogen ion levels with a standard pH electrode, are well developed (6). Conductometric/capacitive chemical pH sensors (7) and ion-sensitive field-effect transistor (ISFET)-based sensors (8) have also become standard methods for determining pH in solutions and small samples. Although these electrochemical-based methods are reliable and reproducible (9), with unmatched temporal response, their use for obtaining multi-point spatial information in cellular environment is limited. Here, we report on an optical pH-responsive NFS platform, for sensing pH with spatio-temporal information.

Optical methods offer an ideal approach for pH sensing in cellular microenvironments due to their advantages in cost, size, multiplexability, and the possibility of remote sensing (10, 11). Conventional pH-sensitive fluorophores, such as fluorescein (12) and Oregon green (13), are most extensively used for cellular applications. By pairing these fluorescent indicators with confocal microscopy, intracellular pH distribution can be mapped in three dimensions (14). However, it is difficult to use these indicators for extracellular measurements due to their rapid diffusion in solution. Chemically or physically immobilizing molecular indicators in a polymer matrix has been demonstrated to prevent diffusion and is currently employed in nanoparticles, microparticles and larger constructs (15, 16). The polymer matrix shields the dyes from cellular interferences and may protect cells from toxicity (17).

Some researchers have immobilized pH-responsive dyes in polymeric microspheres or hydrogels to measure acidic pH environments in tissue engineering scaffolds and other

relevant areas. For example, pH levels within PLGA microspheres have been measured by entrapping two pH-sensitive fluorophores within the polymer. Confocal microscopy was used to visualize the spatio-temporal distribution of the pH within the polymer microspheres. However, particles are more difficult to use for probing pH changes of engineered tissue. Microspheres can potentially diffuse out of tissue and endocytosed by cells, depending on their size. Moreover, this type of probe has a limited pH range due to the chemical nature of the fluorophore, which limits its dynamic response to pH changes determined mainly by the pK_a of the fluorophore (18). Thus, a pH sensor with a tunable dynamic range that is also an integral component of the polymeric scaffold itself could prove to be a vital tool in cellular or tissue microenvironment.

Ionophore-based ion selective optical sensors, also called ion selective optodes, are being considered as a promising modality for optical sensing since they have many advantages in comparison to conventional optical methods (19, 20). Ion selective optodes are well developed, versatile optical sensing tools for the detection of ions including sodium, potassium, and chloride at the cellular level. Optodes have been successfully miniaturized into nanoparticles for intracellular monitoring (21). This approach prevents the rapid diffusion of sensing components into the surrounding environment, shows improved response characteristics, and offers tunable dynamic range and selectivity in comparison with conventional molecular indicators (10, 20). Here, the proton optodes are composed of a pH-sensitive fluorophore, an ionic additive, a plasticizer, and a polymer to impart mechanical stability. The plasticizer uniformly distributes the optode and increases the mobility of analyte transport between the sensing and sample phases. The mechanism of the hydrogen optode-based sensor has been previously described (16). The hydrogen NFSs mechanism is different from traditional optode sensor mechanisms that are based on ion-exchange or ion-coextraction. Our optodes are based on the partition of ions between the sample phase and the sensing phase (22). In particular, the optode construct is based on the distribution of protons (19) and a chromoionophore serves as both proton ionophore and optical reporter. The hydrogen concentration in the sensing phase is in equilibrium with the sample phase, making the measurements dynamic. Our sensor is not responsive or altered by the presence of cations and anions. The advantage of this system, compared to other fluorescent molecular indicators, is the addition of a negatively charged additive to the lipophilic matrix that enables the user to tune the dynamic range of the sensor. Moreover, a second fluorescent reporter can be added to the lipophilic polymer matrix as an internal standard so that ratiometric quantitative measurements can be obtained on the cellular level (23).

Although these approaches for pH measurement have been developed, there are few experimental techniques to attain continuous pH measurements and three-dimensional spatial information in scaffolds (24). Acid-base homeostasis is critical for the performance of cell proliferation and differentiation, and acidification is a common occurrence within the tissue constructs (25). The potential causes of acidification are twofold: the degradation of the polymeric scaffold itself due to spontaneous or enzyme-catalyzed hydrolysis can create an acidic environment (26), and in larger constructs cells in the core could become necrotic owing to inadequate mass diffusion (27). Also, nutrient transport and the removal of metabolic waste generated by the cells can be limited and thus acidic byproducts accumulate, resulting in a drop of the environmental pH level from pH 7.4 to as low as pH 5

(3, 28). This acidification could be detrimental to cell proliferation, cell viability, and the stability of biomolecules. Furthermore, acidification could potentially invoke strong inflammatory responses, ultimately causing the failure of the engineered tissue when implanted *in vivo* (29). Thus, quantitative analysis of pH within the microenvironment with temporal and spatial information from both cells and scaffold on which they grow is highly desired.

A variety of NFS fabrication techniques are currently used to develop complex two- and three-dimensional structures including drawing, template synthesis, self-assembly and phase separation (30, 31). Electrospinning has emerged as a convenient and cost-efficient method for polymeric scaffold fabrication. Since electrospun NFSs of biocompatible and biodegradable polymers have sufficient mechanical properties and can create scaffold for cellular growth, electrospinning has been widely used in tissue engineering (32). This process is capable of providing a fiber-based matrix on the micro- and nano- scales with high porosity, high surface area, and possibilities for surface functionalization (31, 33). Given the advantages of NFSs provided by electrospinning, we employ this technique to combine an optode-based pH sensor and polymeric tissue-engineering scaffold into one platform.

Herein, we compare two pH-sensitive NFSs: PCL-NFSs and PLGA-NFSs. PCL and PLGA were chosen as the platform matrices due to their biocompatibility, biodegradability, and wide use in 3D cell culture and tissue engineering fields (34, 35). For example, these polymers have been investigated as biodegradable implantable tissue scaffolds meant to promote tissue regeneration by eventual population of cells and deposition of extracellular matrix. The incorporation of a pH-sensing element into these biodegradable polymers would enable their use in tissue regeneration applications with monitoring of the nascent tissue as it grows.

We show the NFSs take accurate and quantitative measurements of pH levels with reversibility and long-term stability in solution. We demonstrate this by seeding Human Embryonic Kidney (HEK) cells on the PCL-NFSs. Furthermore, the ability for the PCL-NFSs to monitor pH in 3D cell culture environments is confirmed, with the addition of bacteria as a model for culture contamination or tissue infection demonstrating a sharp, measurable decrease in pH. Therefore, this platform is the first step towards the development of pH sensing cellular environment that could serve as a tool for quantitative detection of chemical dynamics and mapping the future *in vitro* pharmaceutical screening applications.

MATERIALS AND METHODS

Materials

Poly(*D,L*-lactide-*co*-glycolide) 85:15 (Lactide:Glycolide) ester terminated, (MW 100,000–200,000) was obtained from PolySciTech (West Lafayette, Indiana). Citroflex[®]A6 plasticizer (Acetyltri-*n*-hexyl citrate) was obtained from Vertellus (Indianapolis, Indiana). Poly(ϵ -caprolactone) (PCL) (MW 70,000–90,000), ChromoionophoreII(CHII), tetrahydrofuran(THF)(99.9%), Sodium tetrakis(3,5-bis(trifluoromethyl)phenyl)borate) (NaTFPB), Tris Base and *N,N*-dimethylformamide(DMF) were purchased from Sigma-

Aldrich (St. Louis, Missouri). Octadecyl Rhodamine B Chloride (R18) was purchased from Invitrogen, LifeTechnology (Carlsbad, California). High vacuum grease was obtained from Dow Corning (Midland, Michigan). Cover Glass Discs (1mm) were purchased from Deckglaser (Braunschweig, Germany). Human embryonic kidney 293 (HEK 293) cells and MC3T3-E1 subclone 4 murine preosteoblast cells were obtained from ATCC (Manassas, Virginia). 4,6-diamidino-2-phenylindole (DAPI) solution and phalloidin solution was purchased from Thermo Scientific (Cambridge, Massachusetts) and Abcam (Cambridge, Massachusetts), respectively. 50mm glass bottom dish with poly-D-lysine coated was purchased from MatTek Corporation (Ashland, Massachusetts). Protanal LF 20/40 alginate was a generous gift from FMC BioPolymer (Philadelphia, Pennsylvania). G4RGDSP peptide was obtained from Mimotopes (Victoria, Australia).

Optode composition

Both polymeric electrospun NFSs were produced from ratiometric optode cocktails containing all sensing components. The general hydrogen optode formulation was: 0.25 mg CHII, 0.1 mg NaTFPB and 0.01 mg R18 in a total volume of 1 mL electrospinning solution. 12.5% (w/v) of PCL and 8% (w/v) Citrofex® A6 were dissolved in THF for the production of the PCL-NFSs. For the PLGA electrospinning solution, 5% (w/v) of PLGA and 8% (w/v) Citrofex® A6 were prepared in a final volume of 1 mL.

Fabrication of the NFSs

Electrospinning was performed on a Nanospinner (Inovenso, Istanbul, Turkey) equipped with a syringe pump. For the PCL-NFSs, the optode solution was electrospun at a distance of 12 cm with a 4 mL/h flow rate under a high voltage of 25 kV for 5 min. As for the PLGA-NFSs, the electrospinning distance was 15 cm at a flow rate of 1 mL/h and an applied voltage of 25 kV. NFSs were collected on the grounded collector covered with aluminum foil with silanized glass discs or a petri dish adhered with high vacuum grease.

Scanning electron microscopy

Images of the PCL- and the PLGA-NFSs were acquired on a Hitachi S4800 scanning electron microscope with a 3 kV accelerating voltage. The working distance was 8300 µm. The samples were not sputter coated. Fiber diameters were measured using Quartz PCI (Quartz Imaging Corp.) software. The average diameter of the fibers was estimated based on the statistical average value of 50 fibers from different batches of the NFSs. Pore diameter was analyzed using SEM images at 5000x magnification. One hundred pores were randomly selected and used to calculate the mean and standard deviation using ImageJ.

Confocal microscopy imaging

The NFSs were imaged using a Zeiss Confocal Microscope (Thornwood, New York) using laser lines of 405 nm, 488 nm, 555 nm and 639 nm with 63×1.4 N.A oil immersion objective. Pixel dwell time was 1.58 µs.

The NFSs responses to pH

To determine the NFS response to pH, the NFSs on glass discs were removed from the aluminum foil and set down into a transparent bottom 96-well plate. 100 μ L of pH=5, 6, 7, 8, 9, 10, 11, 12 solutions in 10 mM HEPES buffer solution were added to individual wells. pH of the calibration solutions were adjusted with 0.1 M HCl, 0.2 M Tris Base and 1 M NaOH. Fluorescence measurements were taken with Molecular Devices SpectraMax Gemini EM (Sunnyvale, California) in bottom read mode. Each pH measurement was performed in triplicate from three different discs of the NFSs from the same batch. Two wavelength setups were used: CHII (ex: 660 nm; em 705 nm) and R18 (ex: 550 nm; em 585 nm). The fluorescence intensity ratio for CHII: R18 was calculated as below:

$$\text{Fluorescence Intensity Ratio} = \frac{I_{[CHII]}}{I_{[R18]}}$$

Where $I_{[CHII]}$ is the fluorescence intensity of CHII as read by the plate reader and $I_{[R18]}$ is the fluorescence intensity of the reference dye R18.

To calibrate the response of the NFSs, the pH value (reflecting the log of proton concentration) was plotted against α defined by:

$$\alpha = \frac{R_{[pH]} - R_{min}}{R_{max} - R_{min}}$$

Where $R_{[pH]}$ is the intensity ratio of the NFSs in a specific pH solution, R_{min} is the ratio when the pH equals 12 which represents the fully deprotonation status and R_{max} is the ratio at pH = 5. The error was determined from the ratios according to the laws of error propagation. The center of the dynamic range was determined as the pH value, where $\alpha = 0.5$ and the sensitivity was calculated as the change in pH with 1% change of α . The experiments were conducted in triplicate.

Stability of the NFSs

The solution stabilities of both PCL and PLGA NFSs were evaluated by comparing their response calibration curves over 15 days. Both types of NFSs were fabricated and stored in 10 mM HEPES buffer solutions with pH 5, 6, 7, 8, 9, 10, 11, 12 at room temperature while protected from light exposure by covering with aluminum foil. Determination of the NFSs response to pH was performed at day 1, 2, 4, 6, 8, 10, and 15. On each day, the storage solution was removed and fresh calibration solution was added in each well before performing the reading. The NFSs were calibrated under the same conditions and methods as mentioned above. The experiments were conducted in triplicate and error was calculated based on the error propagation.

Reversibility of the NFSs

For reversibility experiments, solutions of pH 5 and 8 were added to each well in the 96-well plate alternatively for three cycles. Fluorescent measurements were taken every hour with

the same excitation and emission settings as in the calibration experiment. The experiments were conducted in triplicate. Data were normalized by the initial ratio and error was calculated based on the error propagation.

Seeding human embryonic kidney 293 (HEK 293) cells on the PCL-NFSs

Electrospun PCL-NFSs were collected to a poly(lysine)-coated 50 mm glass bottom dish directly with a collection time of 1 min then the solvent residue in the PCL-NFSs was evaporated for 1h at room temperature. The PCL-NFSs were sterilized with 70% ethanol and completed dried overnight before the cells were seeded onto the NFSs. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin) with a 30% original confluency and maintained at 37°C in a humidified, 5% CO₂ atmosphere for 48 hours when the cell confluency reached 80%. Cells were fixed with 4% formaldehyde for 30 min. Then DAPI (360 nM) and Phalloidin (330 nM) in PBS buffer were incubated with cells at room temperature for 40 min. Cells were washed with PBS three times after staining. Cell morphology was observed using a fluorescent confocal microscope (ZEISS, LSM700).

Alginate preparation

The peptide glycine(x4)-arginine-glycine-aspartic acid-serine-proline (G4RGDSP), which contains the RGD cell-binding domain, was covalently coupled to the alginate as previously described (36). The alginate was then dialyzed for 4 days, treated with activated charcoal, sterilized through a 0.22 µm filter, and lyophilized. The alginate was reconstituted in sterile phosphate buffered saline (PBS) at 2% w/v for these studies.

pH sensing studies of cells cultured in alginate hydrogels

PCL NFSs were punched into 6-mm diameter circles, sterilized in 70% ethanol, and allowed to air dry in a biosafety cabinet. The PCL NFSs were then transferred into the wells of a 96-well plate with a thin layer of sterilized vacuum grease on the bottom of each well to secure the NFSs in place during the study. Cell-laden hydrogels were fabricated by blending MC3T3 cells in the 2% w/v alginate solution at a concentration of 1×10^6 cells / ml alginate. The alginate was then crosslinked with a slurry of calcium sulfate (210 mg/ml in distilled water) at a ratio of 25:1, and then cast between two glass plates spaced 0.4 mm apart. After 20 minutes, 6-mm diameter disks (N=7) were punched out. Hydrogels without cells were fabricated as controls by the same method. The hydrogel disks were placed on top of the PCL NFSs in the 96-well plate. 150 µl of α-MEM was added to each well, and the plate was placed in a humidified incubator at 37 °C and 5% CO₂. At pre-determined timepoints, the fluorescence for CHII and R18 were measured on a plate reader (Synergy H1 Multi-Mode Reader, BioTek).

After 9 days of culture, a concentrated drop of 27 µl of 1×10^7 *E. coli* cells was added to the wells. The fluorescence intensities for CHII and R18 were measured immediately before and then 4, 22, 30, and 45 hours after the bacteria was added.

To obtain images of the MC3T3-laden hydrogels being cultured over the NFSs, the system was assembled as described above but with disks of MC3T3-laden alginate hydrogels that

were 10-mm in diameter. On days 1, 4, and 7, images were obtained with a confocal microscope (Olympus Fluoview 10i) at 10x magnification. First, single images were taken of the fibers, and then the cells were stained with a live/dead stain comprised of 1.5 mg/ml fluorescein diacetate and 1 mg/ml ethidium bromide, and images taken of the cells. Pre-programmed excitation/emission wavelengths were used: FarRed-Narrow for CH11, DS-Red2 for R18, FITC for live cells, and Red-Narrow for dead cells. The Sensitivity and Laser values that were used were 32.0% and 5.0% for the DS-Red2, 65.0% and 80.0% for the FarRed-Narrow, 27.0% and 3.0% for the FITC, and 37.0% and 5.0% for the Red-Narrow for all images across each point in time.

Statistical analysis

Values were represented as mean $\pm$ standard deviation. All significance was set as 95% confidence ($\alpha=0.05$)

RESULTS

Morphology of the PCL- and PLGA-NFSs

To determine the formulation of spinning solution to produce uniform PCL NFSs, solutions of 8%, 12.5% and 15% (w/v) PCL in solvents such as THF, acetone, and HFIP were tested. THF was chosen as the preferred solvent since the fibers produced did not have unwanted beading effects (data not shown). In addition, the viscosity of 8% PCL solution was too low for fiber formation via electrospinning. Conversely, the higher concentration polymer solution of 15 % clogged the feeding tubes on the electrospinner. Thus, a concentration of 12.5% (w/v) PCL in the spinning solution was determined to produce the most uniform fibers.

The electrospun NFSs were imaged by confocal microscopy to confirm the formation of the scaffolds. The morphology and fluorescence of the PCL-NFSs was shown in Fig. 1a–c. The image was a representative of the NFSs throughout the sample, but a less-dense region was shown for visual clarity. The images suggested that the fluorophores were incorporated homogeneously within the polymer matrix as can be seen by the uniform fluorescence of the NFSs. The SEM image of the PCL-NFSs confirmed that long and continuous fibers were formed with diameters in the range of 900 ± 200 nm (Fig. 2a). The NFSs were free from beading and wetting issues. In this SEM image, the NFSs were also shown to be dense and continuous, and the orientation of the fibers was random. Moreover, a porous surface structure was observed as shown in the inset SEM image with higher magnification (Fig. 2a). The pore diameter of the PCL-NFSs surface was 54 ± 23 nm, and a histogram of the diameter distribution is shown in Fig. S3.

As for the PLGA-NFSs, the concentration of the PLGA in the spinning solution was chosen to be 5 % (37). The solvent chosen for the PLGA-NFSs was THF/DMF with a 1:1 ratio. This formulation produced the most uniform NFSs compared to 1:2 and 1:3 ratios, which caused surface wetting issues since solvents did not evaporate fast and effectively. The fluorophores were co-located and evenly distributed throughout the PLGA-NFSs (Fig. 1d–f). Due to the high density of the NFSs, there were multiple layers of NFSs in this image

causing some fibers to be out of focus. The scanning electron photomicrographs showed smooth and regular PLGA-NFSs with an average diameter of 250 ± 50 nm. The density was confirmed to be higher compared to the PCL-NFSs. Also, the PLGA-NFSs did not have an evident porous surface at this magnification (Fig. 2b).

Response characterization

The practical applications of the pH-responsive NFSs require an appropriate dynamic range with a maintained high sensitivity for cellular studies. The desired working pH range for the NFSs is from pH 8 to as low as pH 6 owing to the acidic microenvironment caused by cell growth and metabolite accumulation (38). When the proton concentration is high in the calibration solution, the sensing components will have a larger α . As the pH increases, α will decrease correspondingly. The polymer-based NFSs responded to pH in a sigmoidal manner (Fig. 3). The center of the dynamic range of PCL-NFSs was 7.8 ± 0.1 and sensitivity was 0.2 pH per 1% α change in the dynamic range of pH 6.9 to pH 8.5. Currently, the dynamic range of the PCL-NFSs fell in this working range (pH 5 to pH 8) and the sensitivity of the NFSs enabled the detection of changes in pH fluctuation in the cell studies as shown later.

Meanwhile, the results from the PLGA-NFSs showed suboptimal dynamic range of pH 8.3 to 11.0 with the center value of 9.6 ± 0.2 (Fig. 3b). The sensitivity of the PLGA-NFSs was 0.3 pH in 1% change of α . Both the dynamic range and the sensitivity indicated that the PLGA-NFSs were not as sensitive as PCL-NFSs for pH measurements for cell culture or most physiological environments.

To investigate the interference of anions and cations on the pH-sensing NFSs, phosphate, perchlorate and chloride anions were compared based on Hofmeister Series (Fig. S1) (39). Also, the concentrations of background cations (sodium) did not alter the pH-responsive NFSs performance (Fig. S2).

Reversibility

To evaluate the reversibility of the PCL- and the PLGA-NFSs, the NFSs were subjected to three cycles of alternating between pH 5 and pH 8 solutions. The PCL-NFSs displayed evident reversibility over three cycles (Fig. 4a). The response towards both pH 5 and pH 8 solutions remained without any significant difference among each cycle measurements. This indicated that the PCL-NFSs retained their functionality when pH value varies over time.

The PLGA-NFSs also exhibited some degree of reversibility over multiple cycles between pH 5 and pH 8 (Fig. 4b). However, the response to the pH 5 solution did show a change over time with a significant difference (p-value of 0.041). We observed that the ratio of CHII to R18 became slightly higher during the cycles. Meanwhile, the responses to the pH 8 solution did not show significant differences (p-value greater than 0.05). Thus, the dynamic range associated with the PLGA-NFSs was suitable for pH measurement in more basic microenvironments; however, it may not be suitable for measurements at acidic pH.

Stability and lifetime

The response stability of the NFSs was assessed by determining the dynamic range and sensitivity of NFSs. Sensors were fabricated and stored for two weeks, and the dynamic range was measured repeatedly over this timeframe. The results showed a stable response over 15 days of solution storage for PCL-NFSs (Fig. 5a). On day 1, the PCL-NFSs detected pH changes with a center value of dynamic range of 7.8 ± 0.1 . The value then changed to 8.1 ± 0.2 by day 15, representing an overall change of 3.8% (Fig. 5b), indicating no significant differences among the dynamic range. The fluorescent signal was retained without significant drifts. This demonstrated that the PCL-NFSs maintained their pH-responsive character over 15 days. The PCL polymer structure imparted the mechanical stability and helped retain the active sensing function. The dynamic range of the PLGA-NFSs, however, drifted significantly during the 15-day evaluation (Fig. 5c). After the first two days, there was an 8.4% decrease of center value of dynamic range. The overall change reached 13.4% after 15 days (Fig. 5d). Unlike the PCL-NFSs, the dynamic range shifted to the left gradually every day under the same conditions as with the PCL-NFSs.

Spatial Distribution and morphology of seeded HEK cells on the PCL-NFSs

To evaluate the PCL-NFSs as a potential cellular scaffold, HEK 293 cells were seeded onto PCL-NFSs spun directly into a 50 mm glass bottom dish. Both Phalloidin and DAPI were used to stain the cells. Phalloidin binds selectively at the interface of F-actin subunits and is commonly used as stain for actin filament in the plasma membrane, and DAPI is an effective nucleus stain (40). We chose to focus on an area with fewer fibers to better demonstrate the cell-scaffold interaction, but cells were observed throughout the sample.

HEK 293 cells seeded onto the PCL-NFSs maintained a normal phenotypic spindle-like shape in a stationary culture (Fig. 6), indicating that the cells can survive and interact with the NFSs. The PCL-NFSs also maintained their morphologies in a 37°C incubator with a humidified atmosphere of 5% CO₂ in air. The integrity of PCL-NFSs was evaluated by assessing changes in fluorescence and structure after 48 hours of incubation at 37°C. Confocal and SEM images show the structure and fluorescence of the fibers was preserved (Fig. S4). The shape of nanofibers incubated in the presence of HEK293 cells showed little change with an average diameter 605 ± 150 nm, compared to fibers before incubation.

Studies of cells cultured in hydrogels with PCL-NFSs

Next, a 3D culture system was fabricated and the ability to detect the pH of this system with the NFSs was investigated. MC3T3 preosteoblast cells were cultured within RGD-alginate hydrogels. To view the cell-laden hydrogels cultured on the NFSs, confocal images were obtained (Figure 7). Each row represents a different day of culturing, and each column represents a different wavelength observed. The first column (705 nm wavelength) was used to determine the pH of the system based on its fluorescence intensity. The relatively constant intensities of NFSs observed in Fig. 7a, e, i indicated no observable changes in pH. The second column (585 nm wavelength, Fig. 7b, f, j) is a measurement of the internal standard, representing the relative density of the NFSs, and also appeared constant over time. The third and fourth columns show live (green) and dead (red) cells in the 3D culture system

over time. The cells were shown to have high viability throughout the 7 days, with live cells present (Fig. 7c, g, k) and very few dead cells (Fig. 7d, h, l).

The fluorescence of the NFSs over time when cultured in the presence of both empty and cell-laden hydrogels was monitored in a high-throughput 96-well format with measurements obtained from a plate reader. The readings, reported as α -values, were stable over time (Fig. 8) as expected within the buffered cell culture system. However, shortly after the addition of *E. coli* bacteria cells to these cultures, the α -values began to increase dramatically, reflecting an acidification of the system. There was significant variance between the samples due to variations in the system, such as nanofiber densities or exact placement of the nanofibers and hydrogels within the well, and thus the error bars are not displayed on this graph but shown in Table. SI. However, the trend towards an increased α -value, and therefore decreased pH, is clear upon addition of the bacteria to the culture system.

DISCUSSION

The pH-responsive NFS platform is designed to monitor pH in microenvironments of interest. The compositions of optode determine sensor response and reversibility. In the optode formulation, it is important to note that the properties of chromoionophore play a significant role in determining the response range of a specific sensor. The working range of the sensor is primarily determined by selecting a chromoionophore with an appropriate pK_a . In our pH fiber sensors, CHII is chosen mainly because its pK_a , in a previously reported system based on polyvinyl chloride PVC plastizied with dioctyl sebacate, was previously reported to be 9.16 ± 0.02 (41) which is close to our desired working range for pH measurement. The dynamic range of the NFSs can be fine-tuned by varying the ratio of the components in the optode. By increasing the amounts of chromoionophore and charged ion-exchanger additives, the dynamic range will shift to a lower pH range.

The high surface area to volume ratio of the NFSs facilitates the proton exchange between the sensing and sample phases. Also, the highly porous nature of the PCL-NFSs may potentially improve the response time of these sensors. The collection time of the electrospinning process leads to different densities and thickness that will affect fluorescence intensity of NFSs. To overcome these differences, a ratiometric detection scheme that enables correction for unequal sensor loading, leakage, scaffold thickness, or photo-bleaching issues was used (42, 43). The encapsulation of a reference dye R18 enables the calculation of a correction factor using the ratio of fluorescence value (CHII:R18) (Fig. 1a and Fig. 1d). Here, the internal reference fluorophore, R18 is chosen for two reasons. First, a sufficient fluorescence quantum yield allows it to work as a bright internal standard and both its excitation and emission spectrums are far from those of chromoionophore II. Therefore, there is no interference with the chromoionophore II response (44). Also, the signal of R18 will increase slightly in basic conditions. Thus, as the system acidifies, R18 has an upward trend in fluorescence compared to the response of CHII, which decreases. By taking the ratio of CHII and R18 emission, the sensitivity is amplified from 9% to 34% and 24% to 37% per pH unit change for the PCL-NFSs and the PLGA-NFSs, respectively. Therefore, incorporating two dynamic fluorophores improves the measurement sensitivity of the NFSs, with a more significant improvement noted for the PCL-NFSs.

There is a notable difference in the dynamic range between the PCL- and the PLGA-NFSs even though the formulations of the two types of NFSs are similar. Compared to the PCL-NFSs, the dynamic range of the PLGA-NFSs is more challenging to tune by modifying the ratio of the sensing components in the optode. The pK_a of PLGA is acidic (45), which makes it an acidic polymer matrix for the optode compared to PVC that is the most commonly used polymer in optode formulations. Due to this acidic nature of PLGA, high pH solution is needed for PLGA-NFS to reach its deprotonation state during equilibrium process, and the dynamic range of the PLGA-NFSs tends to be within higher pH ranges. Unlike PLGA, the neutral PCL polymer does not interfere with the optode response, making the PCL-NFSs more suitable for pH measurement of polymer scaffolds for cellular applications.

The dynamic range of the NFSs calibrated in HEPES buffer adjusted by different acids did not show significant differences. In addition, primary cations like sodium did not response to the pH-NFSs, which further proves that the response mechanism of the pH-NFSs is primarily based on the distribution of protons between the sensor and the sample segments and is not ion-exchange between sample cations and the hydrogen ions, thus the resulting sensor is free from cation interference.

Although the surface of the PCL-NFSs appeared to be more porous when visualized by SEM, the porous surface did not significantly affect the response time of the sensors compared to PLGA-NFS. However, increasing the surface area to volume of sensors is a common method for increasing the sensitivity or response time of sensors (33, 46), so further studies to investigate the fiber thickness and porosity in the future are warranted. Reversibility is essential to characterizing pH fluctuations in cellular studies. Reversible pH sensors have the potential to measure the increase or decrease of pH in the extracellular environment of the NFSs and can provide insight into the degradation of the NFSs themselves. The PCL-NFSs exhibit fully reversibility while PLGA-NFSs suffer from poor reversibility. It is known that both PCL and PLGA can generate acidic metabolites when ester bonds are converted to carboxylic groups by hydrolysis (47), but they have different biodegradation rates (48). PCL follows a slow degradation profile due to its hydrophobic- CH_2 moieties and crystallinity. Conversely, PLGA degradation begins with water uptake and has a significantly faster degradation rate.

The PLGA-NFSs are more likely to generate an acidic microenvironment in the time frame of our experiments, so it is likely that their effect on the sensor response would be greater. Indeed, these NFSs exhibited issues with reversibility, which are attributed to changes in the pH microenvironment since leaching of the fluorophores was not observed by confocal microscopy. This may be due to the acidic microenvironment of the degrading PLGA causing the dynamic range of the sensor to shift. In addition, the fifteen-day stability test demonstrated a shift in the calibration curve that indicated an acidification of the NFS microenvironment, which further supports our conclusions. These two polymers, as representatives for our NFS platform, could be used in different applications depending on the time requirements for tissue growth and pH sensing.

In summary, the PCL-NFSs display a sufficient sensitivity in desired dynamic range and full reversibility, and PLGA-NFSs, on the other hand, exhibit a dynamic range which is not within the sensor ideal working range with lower sensitivity compared to PCL-NFSs and suffer from poor reversibility. Therefore, we choose the PCL-NFSs to demonstrate the potential as a scaffold for cell growth.

Although PCL is routinely used as a support for cellular growth, the adding of plasticizer and sensing components had the potential to prevent cellular growth. We showed, in preliminary testing, that HEK 293 cells were able to adhere to the NFSs and maintained their viability. Additionally, cells cultured in 3D hydrogels placed on top of these NFSs demonstrated high cell viability when stained with live/dead stain over time. Therefore, it was confirmed that the PCL-NFSs are compatible with cell culture systems as they do not impact cell viability.

The ability to detect pH shifts in three dimensional cell culture conditions was examined. First, the ability to detect the fluorescence of the NFSs when placed underneath empty or cell-laden hydrogels was confirmed. The fluorescence remains relatively constant over time, as expected within this buffered culture system with cells of high viability. This was also confirmed by quantitative measurements obtained for the system with a high throughput plate reader format, where the readings stayed relatively constant over time in culture. Cell culture media has a high buffering capacity to maintain the cell environment close to physiologic pH 7.4. *E. coli* bacterial cells were added to the 3D mammalian cell culturing system to mimic a culture contamination or tissue infection. These bacteria with a doubling time on the order of 30 minutes proliferate substantially faster than mammalian cells and rapidly overwhelm the culture system, depleting nutrients and decreasing the pH. Indeed, the NFSs were readily able to detect this decrease in pH. These results demonstrate that these NFSs are promising tools for the detection of pH shifts that may influence cellular environments. Furthermore, a high throughput format for rapid screening of various culture conditions may be utilized if these scaffolds were to be placed in 96-well plates.

CONCLUSIONS

In this paper, we designed and validated a novel optical NFS platform with two types of polymers to measure pH. We created a pH-responsive structure, which consists of a polymer scaffold with an optode sensor, to support cellular growth as well as continuously monitor pH of the environment. Both PCL- and PLGA-NFSs have a long lifetime of at least 15 days with stable pH responsive properties. The PCL-NFSs exhibit a response to *in situ* pH change within a desired working range while the PLGA-NFSs have a dynamic range of pH 8.3 to 11.0 due to the acidic nature of the polymer itself. Thus, the PCL-NFSs have a better potential to monitor acidic microenvironments that could form within 2D or 3D cell cultures. Therefore, they are promising for the use in measuring pH fluctuations in the extracellular microenvironment of interest, such as in cultured cell systems. Also, our tunable, reversible, and ratiometric NFS platform can be extended for monitoring different analytes such as oxygen, and one could envision a high-throughput screening tool for monitoring the effects of drugs on cellular metabolism and toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

PCL-NFS	poly(ϵ -caprolactone) electrospun nanofiber scaffold
PLGA-NFS	poly(lactic- <i>co</i> -glycolic acid) electrospun nanofiber scaffold
CHII	ChromoionophoreII
THF	tetrahydrofuran
NaTFPB	Sodium tetrakis(3,5-bis(trifluoromethyl)phenyl]borate)
DMF	Dimethylformamide

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Fig. 1.
Confocal images of the electrospun PCL-NFSs (a–c) and PLGA-NFSs (d–f). a: R18 channel; b: CHII channel; c: overlay of (a) and (b). Scale bar: 10 μ m

Fig. 2. SEM images of the electrospun PCL-NFSs (a) and PLGA-NFSs (b), respectively. The insets show enlarged SEM images of these NFSs. Scale bar: 2 μ m

Fig. 3. Comparison of the PCL- (black) and the PLGA- (red) NFSSs. The center values of dynamic range are 7.8 ± 0.1 and 9.6 ± 0.2 , respectively. Data represented as mean values with error bars for standard deviations ($n=3$).

Fig. 4. Reversibility data from the PCL-NFSs (a) and the PLGA-NFSs (b). The NFSs were secured in 96-well plate and imaged at 1 hour intervals with alternating solutions of pH 8 and pH 5. The ratio of the CHII to R18 was computed for each time point and normalized. Data represented as mean values with error bars for standard deviations (n=3).

Fig. 5. Calibration curves for the PCL-NFSs (a) and the PLGA-NFSs (c), showing changes in sensitivity to pH over 15 days when stored in different pH solutions at room temperature. The K_d values of the PCL-NFSs (b) and the PLGA-NFSs (d) are shown for each recorded day. Data represented as mean values with error bars for standard deviations ($n=3$).

Fig. 6. HEK cells seeded on the PCL-NFSs after incubation at 37 °C for 48 hours. Cells were fixed and a nucleus was stained with DAPI (blue) and actin filaments were stained with phalloidin (green). The fluorescent of the PCL-NFSs shown were the overlay of CHII and R18 (orange). Scale bar: 10 μ m.

Fig. 7.

Grouping of confocal images taken at 1, 4, and 7 days of culture for the wavelengths labeled 705 nm for pH dependent fluorescence, 585 nm for fiber density, 520 nm for live cell staining, and 620 nm for dead cell staining. The scale bar represents 0.02 cm

Fig. 8. Scatter plot depicting the α -values for the systems with and without MC3T3s in the system. *E. coli* was added to the system at hour 216.