Video Article

Self-reporting Scaffolds for 3-Dimensional Cell Culture

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Abstract

Culturing cells in 3D on appropriate scaffolds is thought to better mimic the *in vivo* microenvironment and increase cell-cell interactions. The resulting 3D cellular construct can often be more relevant to studying the molecular events and cell-cell interactions than similar experiments studied in 2D. To create effective 3D cultures with high cell viability throughout the scaffold the culture conditions such as oxygen and pH need to be carefully controlled as gradients in analyte concentration can exist throughout the 3D construct. Here we describe the methods of preparing biocompatible pH responsive sol-gel nanosensors and their incorporation into poly(lactic-co-glycolic acid) (PLGA) electrospun scaffolds along with their subsequent preparation for the culture of mammalian cells. The pH responsive scaffolds can be used as tools to determine microenvironmental pH within a 3D cellular construct. Furthermore, we detail the delivery of pH responsive nanosensors to the intracellular environment of mammalian cells whose growth was supported by electrospun PLGA scaffolds. The cytoplasmic location of the pH responsive nanosensors can be utilized to monitor intracellular pH (pHi) during ongoing experimentation.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50608/>

Introduction

A key strategy in tissue engineering is the use of biocompatible materials to fabricate scaffolds whose morphology resembles the tissue that it is going to replace and is also capable of supporting cell growth and function^{1,2}. The scaffold provides mechanical support by allowing cell attachment and proliferation yet allows cell migration throughout the interstices of a 3D cellular construct. The scaffold must also allow for the mass transport of cell nutrients and not inhibit removal of metabolic waste³.

Electrospinning has emerged as a promising method for the fabrication of polymeric scaffolds capable of supporting cellular growth⁴⁻⁶. The nonwoven electrospun fibers produced are suitable for cell growth as they are often porous and allow cell-cell interaction as well as cell migration throughout the interstices of a 3D cellular construct⁷. It is important to monitor cell viability during the period of culture and to ensure that cell viability is maintained throughout the whole of the 3D construct. For example, culture conditions such as oxygen and pH require careful control, as gradients in analyte concentration can exist within the 3D construct. Bioreactors or perfusion systems can be employed to mimic *in vivo* conditions of interstitial flow and as a result increase nutrient transfer and metabolic waste removal⁸. The question of whether such systems are ensuring constant microenvironmental conditions can be addressed by assessing the cellular microenvironment in real-time.

Key microenvironment metrics that could be monitored in real-time include: temperature, chemical composition of cell media, concentration of dissolved oxygen and carbon dioxide, pH, and humidity. Of these metrics, temperature can be most readily monitored using *in situ* probes. Methods for monitoring the remaining listed metrics commonly involve removal of an aliquot for sampling and therefore disturb the cell culture and increase the contamination risk. Continuous, real-time methods are being sought. Current monitoring methods usually rely upon instruments that physically probe the cellular construct such as a pH monitor or oxygen probe. However, these intrusive methods can damage the cellular construct and disturb the ongoing experiment. Noninvasive monitoring of analyte concentrations within the 3D construct could enable real-time monitoring of various environmental aspects such as nutrient depletion⁹. This would allow assessment of nutrient supply to deeper regions within the structure and determine whether metabolic waste was being removed effectively^{10,11}. Systems that attempt to address the issue of invasiveness generally involve the use of a perfusion chamber that passes culture medium through both the culture vessel and to external sensors to monitor pH, oxygen and glucose¹². There is increasing interest in developing sensors that can be directly integrated into the culture vessel that do not require removal of an aliquot for sampling and as such would provide *in situ* monitoring.

To address such shortcomings for *in situ* and noninvasive monitoring of microenvironmental conditions we have incorporated analyte responsive nanosensors into electrospun scaffolds to produce self-reporting scaffolds¹³. Scaffolds that act as sensing devices by monitoring fluorescence activity have been prepared previously, where the sensing device was either the actual polymeric scaffold created by electrospinning or through the use of an analyte responsive dye which is incorporated into the polymer prior to scaffold formation^{14,15}. However, these sensing devices have the potential to give erroneous optical outputs caused by possible interference from other analytes. The use of a ratiometric sensing device such

as those prepared in the described protocol holds the potential to eliminate these possible adverse effects and provide a response specific to the analyte in question.

The electrospun scaffolds presented here have been prepared from the synthetic co-polymer poly(lactic-co-glycolic acid) (PLGA), selected due to having Food and Drug Administration (FDA) approval, owing to its biodegradable and biocompatible properties and a track record of supporting the growth and function of various cell types¹⁶⁻¹⁸. The prepared ratiometric analyte responsive nanosensors are responsive to pH. The nanosensors incorporate two fluorescent dyes into a biocompatible sol-gel matrix where one dye, FAM is responsive to pH and the other, TAMRA acts as an internal standard as it is not responsive to pH. Furthermore the fluorescence of both FAM and TAMRA can be analyzed separately as they do not significantly overlap. Determining the ratio of the fluorescence emission of both dyes at specific wavelengths gives a pH response independent of other environmental conditions. The self-reporting scaffolds could allow repeat assessment of pH *in situ* and in real-time without disrupting the developed 3D model. We have demonstrated that these scaffolds are capable of supporting cell attachment and proliferation and remain responsive to the analyte in question. The kinetics of acidic by-products in engineered constructs remains understudied and as such using the pH responsive scaffolds could greatly facilitate such studies¹⁹. Furthermore, the use of the self-reporting scaffolds for tissue engineering applications presents the opportunity to fully understand, monitor and optimize the growth of 3D model tissue constructs *in vitro*, noninvasively and in real-time.

The pH responsive nanosensors have also been delivered to the intracellular environment of fibroblasts cultured upon electrospun PLGA scaffolds. The ratio of the fluorescence emission from the dyes were used to monitor pH_i and compared to a self-reporting scaffold incorporating pH nanosensors. The delivery of nanosensors to cells cultured in a 3D environment could enable monitoring of analyte concentration deep within the construct in a nondestructive manner. Therefore nanosensors may be a viable imaging tool to nondestructively assess cell behavior throughout 3D constructs allowing long-term analysis. Screening the analyte concentration of individual cells within a 3D construct could ensure that they are receiving sufficient nutrient and oxygen concentrations. Monitoring process parameters could assist in the development of standardized techniques for the effective mass transport of oxygen and nutrients. The delivery of nanosensors to the intracellular environment and incorporation of nanosensors into polymeric scaffolds could be combined to allow assessment of cell viability as well as scaffold performance within 3D constructs during the tissue growth process. This may lead to increased knowledge of these constructs and progress the fabrication of biologically relevant tissue substitutes.

Protocol

Overview

Section 1 describes the preparation of pH responsive nanosensors and characterization of nanosensor response to pH using fluorescence spectrometry and their size using SEM.

Section 2 describes the preparation of electrospun polymer scaffolds and characterization of their morphology and size using SEM. Section 2 also describes the preparation of self-reporting scaffolds which are electrospun scaffolds with the inclusion of the pH responsive nanosensors. The resulting self-reporting scaffolds are characterized by SEM to assess whether any morphological changes in the electrospun fibers has occurred. The fluorescence from the self-reporting scaffolds resulting from the incorporated nanosensors is assessed by confocal microscopy.

Section 3 describes the culture of cells upon the electrospun scaffold and the self-reporting scaffold. The scaffolds are first sterilized in preparation for cell culture following which the scaffolds are assessed to determine whether sterilization and the culture of cells affect the fluorescence capability of the self-reporting scaffolds. The protocol also describes the delivery of nanosensors to cells that are cultured upon electrospun scaffolds. LysoTracker dye is used to ensure that nanosensors have not been taken into cellular acidic compartments.

1. Preparation and Analysis of pH Responsive Sol-gel Nanosensors

1.1 Preparation of pH Responsive Sol-gel Nanosensors

Note: This preparation should be performed in a fume hood.

1. Prepare the fluorophores for use within the pH nanosensors by dissolving 5-(and-6)-carboxyfluorescein, succinimidyl ester (FAM-SE) (1.5 mg) in dimethylformamide (DMF) (1 ml) in a round bottomed flask and 6-carboxytetramethylrhodamine, succinimidyl ester (TAMRA-SE) (1.5 mg) in DMF (1 ml) in another round bottomed flask. Add 3-aminopropyltriethoxysilane (APTES) (1.5 mL) to each flask and stir under a dry nitrogen atmosphere (21 °C) for 24 hr in the dark *i.e.* wrap the samples in foil.
2. Add both of the above dyes (250 µl) to a mixture of ethanol (6 ml) and ammonium hydroxide (30 wt %, 4 ml) contained in a round-bottomed flask and stir for 1 hr (21 °C).
3. Slowly add tetraethylorthosilicate (TEOS) (0.5 ml) to the mixture, continue stirring for a further 2 hr. The mixture will turn cloudy. The nanosensors can be collected by rotary evaporation. Nanosensors can be stored in a glass vial at 4 °C for future use.

CAUTION: further handling of nanosensors in their dry form should be performed in a fume hood or in the case of weighing them the balance should be enclosed, for example in a Weighsafe.

1.2 Nanosensor Response to pH

1. Prepare Sørensen's phosphate buffer solutions ranging from pH 5.5-8.0 by mixing specific ratios of sodium phosphate monobasic (0.2 M) and sodium phosphate dibasic (0.2 M) stock solutions. Check the final pH using a pH meter. Make any minor adjustments to the pH using sodium hydroxide (NaOH) (4 M) or hydrochloric acid (HCl) (2 M).

2. Suspend the pH nanosensors in the pH buffers (5 mg/ml) by firstly holding the vessel containing the nanosensor solution on a vortex for 3 min then submerging the vessel in an ultrasonicator until the solution becomes cloudy (approximately 5 min). Repeat these steps to fully suspend the nanosensors in solution.
3. Place the first solution into an optical cuvette and use excitation wavelengths of 488 nm for 5-(and-6)-carboxyfluorescein (FAM) and 568 nm for 6-carboxytetramethylrhodamine (TAMRA). Collect the emission wavelengths at 500-530 nm for FAM and 558-580 nm for TAMRA using a fluorescence spectrometer.
4. Change the solutions in the optical cuvette so that the pH can be observed at different pH and continue collecting the emission spectra.
5. Calculate the ratio of the emission maxima produced at each pH value to produce a calibration curve.

1.3 SEM of Nanosensors

1. Place a sample of the nanosensors onto a carbon coated electron microscope stub and sputter coat with gold for 5 min under an argon atmosphere.
2. Observe by scanning electron microscopy (SEM). During imaging adjust the working distance, voltage and magnification to minimize electron charging (**Figure 1**).

2. Preparation and Analysis of PLGA Scaffolds

2.1 Electrospinning PLGA Scaffolds

1. In a fume hood dissolve poly(lactic-co-glycolic acid) (PLGA) in dichloromethane (DCM) (20% (w/w)) with pyridinium formate (PF) (1% (w/w)). Place the solution into a 10 ml syringe with an 18 G blunt fill needle and securely fit to a syringe pump.
2. Distance the needle tip 20 cm away from a 20 cm x 15 cm aluminum collecting plate.
3. Attach the electrode of a high voltage power supply to the tip of the syringe and the earth to the aluminum collecting plate.

CAUTION: Care should be taken as high voltage is used *i.e.* always turn the electrical supply off before handling any of the electrospinning equipment.

4. Deliver the solution using a constant flow rate of 3.5 ml/hr at 12 kV for 2 hr. Electrospinning using these conditions will give a scaffold depth of approximately 60 μ m.
5. Leave the scaffold in a fume hood for 24 hr to allow solvent residue to evaporate.

2.2 Electrospinning pH Responsive PLGA Scaffolds

1. Prepare scaffolds incorporating pH responsive nanosensors as described in section 2.1 but with the modification of adding nanosensors to the polymer solution (5 mg/ml). Suspend the nanosensors in the PLGA solution with the assistance of ultrasonication (approximately 5 min) prior to placing into a 10 mL syringe.

2.3 SEM of PLGA Scaffold and pH Responsive Scaffold

1. Place a sample of the PLGA scaffold or pH responsive scaffold onto a carbon coated electron microscope stub and proceed as described for SEM of nanosensors (**Figure 1**).

2.4 Calibration of pH Responsive Scaffold

1. Place a sample of pH responsive scaffold into a 35 mm culture plate and immerse in appropriate buffer solutions.
2. On a confocal microscope use a 488 nm argon laser to excite the FAM and a 568 nm krypton laser to excite the TAMRA within the nanosensors. Observe the fluorescence emission for pH responsive scaffolds at 500-530 nm for FAM and 558-580 nm for TAMRA. (**Figure 2**). Use sequential scanning to avoid collection of excitation wavelengths.

3. Cell Culture upon PLGA Scaffolds and pH Responsive PLGA Scaffolds

Note: The following should be performed in a cell culture cabinet.

3.1 Preparation of PLGA Scaffolds for Cell Culture

1. Cut the PLGA or pH responsive PLGA scaffolds into 2 cm² pieces.
2. Sterilize scaffolds by irradiating with ultraviolet (UV) light at a distance of 8 cm for 15 min each side.
3. Transfer the scaffolds to a 12-well culture plate and place a steel ring on top. Add a solution of Penicillin/Streptomycin (5% v/v) in phosphate buffered saline (PBS) to the inside and outside of the steel ring, incubate overnight (37 °C, 5% CO₂). Note the steel ring was manufactured at The University of Nottingham and has the following dimensions: 2 cm outer diameter, 1 cm inner diameter, 1 cm depth.
4. Remove the sterilizing solution and wash with PBS.
5. Add cell culture media to the inside (500 μ l) and outside (500 μ l) of the steel ring, place in an incubator (37 °C, 5% CO₂) whilst preparing a cell suspension.
6. Prepare a cell suspension and perform a cell count using a Trypan Blue exclusion assay.
7. Create a cell suspension to achieve a cell number of 1 x 10⁶ cells/ml.
8. Remove media from the inside and outside of the steel ring.
9. Replace with prewarmed cell culture media on the outside of the steel ring (1 ml).
10. Add 300 μ l of the cell suspension to the inside of the steel ring - rock plate backwards and forwards gently to distribute cells.

- Place the cell culture plate into an incubator (37 °C, 5% CO₂) - cell attachment should have taken place within 24 hr, however continue culture for as long as the experiment requires - refreshing the media every 2-3 days.

3.2 Nanosensor Delivery to Cells Cultured upon PLGA Scaffold

- Seed cells onto PLGA scaffold as described in section 3.1. For this study a blank PLGA scaffold (not containing nanosensors) was used due to overlap of fluorescence emission when imaging.
- Place the cell seeded scaffold into an incubator (37 °C, 5% CO₂) for 72 hr to allow the cells to grow and migrate throughout the scaffold prior to nanosensor delivery.
- Prior to nanosensor delivery wash the cells with PBS and change the media from standard culture media to antibiotic and serum free media, incubate (37 °C, 5% CO₂) for 1 hr. Note: standard culture media for 3T3 cells comprises of Dulbecco's Modified Eagle's medium supplemented with fetal calf serum (10% (v/v)), L-glutamine solution (2 mM), penicillin/streptomycin (1% (v/v)).
- During the incubation period prepare the nanosensor-liposome complex by briefly sonicating nanosensors (5 mg) with Optimem (50 µl) to create solution A.
- Create solution B by adding Lipofectamine 2000 (5 µl) to Optimem (45 µl), incubate at room temperature for 5 min.
- Add solution A to solution B, incubate at room temperature for 20 min to form solution C which is the nanosensor-liposome complex.
- Add solution C (100 µl) to cells and incubate (37 °C, 5% CO₂) for 3 hr.
- Remove the cell seeded scaffolds from the incubator, aspirate media and wash twice with PBS before adding PBS (1 ml) to allow live cell visualization by confocal microscopy. Note: PBS was used in this protocol so that the phenol red indicator present in cell culture media did not cause autofluorescence and also because a calibration at different pH was being performed. However phenol red free media could be used for longer term analysis of cell cultures.
- Immerse the cell seeded scaffold into buffers of different pH and monitor the response of the nanosensors associated with the cells.
- Monitor the pH_i by determining the ratio of the fluorescence intensities from FAM and TAMRA by observing fluorescence using confocal microscopy (**Figure 3**).

3.3 Nanosensor Location within Mammalian Cells

- Incubate cells with nanosensors containing only the FAM dye as described in section 3.2 (this is because the fluorescence emission of TAMRA is in the same spectral region as the LysoTracker Red). Prepare these nanosensors as described in section 1.1 but with the omission of preparing and adding TAMRA.
- Following the nanosensor delivery period dilute LysoTracker Red stock solution to 50 nM.
- Add an aliquot of the diluted solution (2 µl) to cells cultured upon PLGA scaffolds.
- Incubate the cells with LysoTracker Red for 1 hr (37 °C, 5% CO₂).
- Following the incubation period remove the media and wash the cells twice with PBS (pH 7.4) before changing the media to PBS (pH 7.4) (2 ml) in preparation for viewing using confocal microscopy. Note: Phenol red free media could be used instead of PBS.
- Add the nuclear stain Draq5 to the PBS so the final concentration is 5 µM and incubate with the cells for 3 min (37 °C 5% CO₂). It is not necessary to change the media following the incubation period with Draq5.
- Use a 488 nm argon laser to excite the FAM only nanosensors, a 568 nm krypton laser to excite the LysoTracker Red and 633 nm helium/neon laser to excite the Draq5 chromophore and observe fluorescence at 500-530 nm, 580-600 nm, 650-750 nm respectively (**Figure 3**). Use sequential scanning to avoid collection of excitation wavelengths.

Representative Results

The size distribution of the prepared pH responsive nanosensors was characterized using SEM, where the population of nanosensors imaged were measured and found to have nanometer dimensions in the range of 240-470 nm (**Figure 1A**). The achievement of a narrow and reasonably small diameter is consistent with using the Stöber method to prepare nanoparticles. It has been found that using a basic pH environment during the synthesis of nanoparticles *i.e.* nanoparticles prepared using the Stöber method, allows good control of the size, whereas preparation using acidic conditions produces a wide dispersion in particle size. Representative SEM micrographs of the PLGA electrospun fibers fabricated using the described methods are shown in **Figure 1B** demonstrating that the produced PLGA scaffold consists of nonwoven fibers displaying minimal beading or breakage where the fibers have nanometer dimensions. **Figure 1C** demonstrates that the addition of nanosensors to the PLGA solution at a concentration of 5 mg/mL produces fibers that are comparable to the control sample in **Figure 1B**. The SEM micrographs provide evidence of nanosensor association on the surface of the fibers however they may also be incorporated within the scaffold fibers, degradation studies of these scaffold/nanosensor scaffolds could be carried out in conjunction with SEM and/or confocal microscopy to determine if this affects nanosensor performance.

The capability of the nanosensors to retain their optical activity following incorporation into the PLGA fibers was verified by examining the self-reporting scaffolds using confocal microscopy. Retention of the ability to remain optically and chemically active when incorporated into the scaffold fibers could enable analyte concentrations to be monitored. The fluorescence emitted from the nanosensors demonstrated that the nanosensors retained their optical activity and are associated with the scaffold fibers. Fluorescence emitted from the dyes FAM (green) and TAMRA (red) incorporated in the pH responsive nanosensors is shown in **Figures 2A** and **2B** respectively. This is a positive result that makes it feasible for analyte concentrations to be monitored *in situ*. It is also the first time that optically active ratiometric nanosensors have been incorporated into PLGA scaffold fibers using the electrospinning process. Having confirmed that the nanosensors can be visualized using fluorescence microscopy following incorporation into PLGA fibers, the nanosensor response to changes in analyte concentration was verified. Immersing the scaffold into buffers of differing pH resulted in a change of fluorescence intensity from the FAM dye whilst the fluorescence emitted by the TAMRA reference dye did not noticeably change. The ratio of fluorescent intensity of both dyes can be used to produce a calibration curve as shown in **Figure 2C**. The calibration curve for nanosensors assessed alone and when incorporated into electrospun PLGA scaffold is comparable and demonstrates that the nanosensors retain their optical activity following incorporation into the self-reporting scaffolds. The ability of the self-reporting scaffold to reversibly respond to different pH values was assessed by alternately immersing the scaffold in buffers with pH values of 5.5 and 7.5. The reversibility was found to be very good as shown in **Figure 2D** where the self-reporting scaffold can respond to numerous cyclic changes in pH. Long-term studies have not been performed to assess the effect of PLGA degradation upon the nanosensor

incorporation; it is thought that because the nanosensors provide a ratiometric response the quantity of nanosensors present will not affect the result.

Analyte responsive nanosensors were delivered using a liposomal transfection agent to the intracellular environment of 3T3 fibroblasts whose growth was supported by blank PLGA scaffolds. Confocal microscopy images of 3T3 fibroblasts cultured upon blank PLGA scaffolds demonstrate that nanosensors are associated with the fibroblast cells with fluorescence observed from FAM and TAMRA shown in **Figures 3A** and **B** respectively. Overlaying the fluorescence from these channels shows that the fluorescence from the two dyes is co-localized, suggesting that the dyes have remained entrapped within the nanosensors' biocompatible matrix (as depicted by yellow fluorescence in **Figure 3C**). The nanosensors are thought to be within the cytoplasm of the 3T3 fibroblasts and not contained within acidic compartments as evidenced by the lack of co-localized fluorescence from FAM only nanosensors and Lysotracker Red dye depicted in **Figure 3D**. The fluorescence intensity of pH responsive nanosensors delivered to 3T3 fibroblasts was monitored whilst the cells were subjected to changes in pH the results of which are shown in **Figure 3E**. The graph produced demonstrates that the cells cultured upon the electrospun PLGA scaffold have maintained a pH in the range 6.8-7.0.

Figure 1. SEM micrographs of nanosensors, PLGA fibers and self-reporting scaffolds. (A) pH responsive sol-gel nanosensors showing spherical nanometer sized particles (B) PLGA electrospun nonwoven fibers where fiber morphology is regular with minimal beading and breakages (C) pH responsive self-reporting scaffolds where the nanosensors can be observed to be protruding from the PLGA yet fiber morphology remains comparable with control PLGA fibers that do not contain nanosensors. [Click here to view larger image.](#)

Figure 2. Confocal microscopy images of pH responsive self-reporting scaffolds where fluorescence can be observed from the nanosensors associated with the PLGA scaffold fibers. (A) FAM (green) the pH responsive dye and (B) TAMRA (red) the reference dye. (C) Calibration graphs of pH responsive sol-gel nanosensors and pH responsive scaffolds demonstrate that the optical and chemical response of the nanosensors has not been affected when incorporated within the PLGA scaffold fibers. (D) Reversibility of the self-reporting scaffold performed by immersing the scaffold in alternate buffer solutions of pH 5.5 and pH 7.5. [Click here to view larger image.](#)

Figure 3. Confocal microscopy images of cell internalized nanosensors and graphs of pH and pHi response. Fluorescence from the internalized nanosensors (A) FAM (green) (B) TAMRA (red) (C) co-localization of fluorescence from FAM and TAMRA (yellow) (D) FAM only nanosensors (green) delivered to 3T3 fibroblasts cultured upon PLGA scaffold with Draq5 (magenta) labeling of the nucleus and LysoTracker Red (red) labeling of lysosomes. The fluorescence of FAM and LysoTracker red are not co-localized therefore demonstrating that it is unlikely that nanosensors have been internalized into cellular acidic compartments (E) pHi measurements taken from nanosensors delivered to 3T3 fibroblasts cultured upon a blank PLGA scaffold compared to the pH calibration of a pH sensing scaffold without the culture of cells. This also demonstrates that the pH measurements performed using the internalized nanosensors are not acidic as the cells cultured upon the electrospun PLGA scaffold have maintained a pHi in the range 6.8-7.0. [Click here to view larger image.](#)

Discussion

Tissue engineering aspires to create biological substitutes that can be used both as *in vivo* like *in vitro* tissue models and in tissue replacement therapy to repair, replace, maintain or enhance the function of a particular tissue or organ. Synthetic substitutes have been used for many years to replace or aid repair of tissues but these often fail due to poor integration with the host tissue and/or infection, which ultimately leads to rejection or further revision surgery. Generating living tissue in the laboratory prior to implantation may address the issue of achieving full integration and reduce the need for revision surgery. However, for this goal to be realized appropriate *in vitro* propagation and manipulation of cells within the 3D constructs should be coupled with appropriate physical conditioning of the tissue *in vitro*. This can be achieved in a number of ways one being to continue research into the fundamental biology involved but also to research the engineering and manufacturing issues related to the production of synthetic substitutes. It is this latter issue that the described protocol aims to contribute.

The intended use of the described PLGA scaffolds is to provide a temporary support to mammalian cells to assist their growth into a biologically relevant structure. Incorporation of analyte responsive nanosensors into the PLGA scaffolds could allow local analyte concentrations to be monitored throughout a 3D construct during the tissue growth process. The use of pH responsive scaffolds could allow the determination of whether acidic by-products produced as the scaffold degrades and cells grow are being effectively removed from within a 3D construct. This is a step towards addressing the current lack of process monitoring and control of *in vitro* tissue regeneration processes. The utilization of scaffolds that are themselves sensing devices may enable *in situ*, real-time microenvironmental assessment. Monitoring the construct microenvironment without damaging the tissue is essential and will allow process monitoring at all stages of tissue production.

The capability of the produced pH responsive scaffold to report the local analyte concentration was assessed by monitoring the fluorescence output from the nanosensors using confocal microscopy. A calibration experiment was performed for pH sensing scaffold and proved comparable to that of the pH responsive nanosensors not incorporated into PLGA fibers. The ability to produce a calibration curve demonstrates that the nanosensors remain responsive to changes in pH when incorporated into the polymer scaffold and also that the process of sterilization of the scaffolds did not cause any detrimental effects to the sensing ability of pH nanosensors. Furthermore, nanosensors were delivered to the intracellular environment of cells cultured upon PLGA scaffolds where cellular response to changes in pHi can be monitored. The ratio of the fluorescence intensities from FAM and TAMRA were used to monitor the pHi and compared to a pH responsive scaffold, suggesting that the 3T3 fibroblast cells have maintained a pHi in the range pH 6.8-7.0. Incubating 3T3 fibroblasts transfected with nanosensors with the nuclear stain Draq5 indicated that the majority of cell associated nanosensors were not located in the nuclear region. Nanosensor location does not appear to be within acidic compartments such as endosomes or lysosomes as determined by incubating the nanosensor transfected 3T3 fibroblasts with LysoTracker Red, where co-localization of FAM and LysoTracker Red would be depicted by yellow pixels in confocal images. Further studies such as analysis by transmission electron microscopy (TEM) and identification of biological markers for internalization pathways could verify this

further. In addition, long-term studies such as time-resolved confocal microscopy could determine the long-term fate of the nanosensors within cells cultured upon self-reporting scaffolds. If nanosensors were to be delivered to other cell types then their location should be identified as the result may not be the same as that shown here.

The use of self-reporting scaffolds have the potential to noninvasively monitor microenvironmental conditions within tissue engineered constructs. Having the capability to perform *in situ* measurements could enable optimizing the growth of 3D tissue constructs *in vitro* noninvasively and in real-time. The cytoplasmic location of the pH responsive nanosensors can be utilized to monitor pH during ongoing experimentation. The nanosensors delivered to the internal environment of cells or self-reporting scaffolds can both be used within static or dynamic tissue culture systems and can lead to determining the culture conditions required for optimum growth of tissue engineered constructs *in vitro*. Further development can lead to an on-line system that act upon nutrient depletion ensuring a consistent supply of nutrients and oxygen throughout the construct, ultimately giving a highly reproducible method for the culture of tissue constructs.

The described protocols have prepared scaffolds with dimensions suitable to allow imaging using confocal microscopy however, to enable larger 3D constructs to be imaged multi-photon excitation may be required to allow imaging within deeper constructs. The optical equipment used such as the microscope lens should be selected to determine the optimal working distance for the lens and the construct dimensions.

Disclosures

The authors declare that they have no competing financial interests.

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