Conformal coating of orthopedic plates with x-ray scintillators and pH indicators for x-ray excited luminescence chemical imaging through tissue.

U. Uzair[1], C. Johnson[1], S. Beladi[2], D. Benza[1,3], Y. Raval[2], M. Ranasinghe[1], G. Schober[1], T.-R. J. Tzeng[2] and J. N. Anker[1*]

[1]Departments of Chemistry and Bioengineering, Center for Optical Materials Science and Engineering Technology (COMSET), Clemson University,
[2]Department of Biological Sciences, Clemson University,
[3]Department of Electrical and Computer Engineering, Clemson University.
*Corresponding author janker@clemson.edu

ABSTRACT

We describe a material that allows for high spatial resolution pH mapping through tissue using X-ray excited luminescence chemical imaging (XELCI). This is especially useful for detecting implant associated infection and elucidating how the local biochemical environment changes during infection and treatment. To acquire one pixel in the image, a focused X-ray beam irradiates a small region of scintillators coated on the implant and the X-ray excited optical luminescence spectrum is modulated by indicator dyes to provide a chemically sensitive measurement with low background. Scanning the X-ray beam across the implant surface generates high spatial resolution chemical measurements. Two associated challenges are 1) to make robust sensors that can be implanted in tissue to measure local chemical concentrations and specifically for metal orthopedic implants, and 2) to conformally coat the implant surface with scintillators and pH indicator dyes in order to make measurements over a large area. Previously, we have physically pressed or glued a pH-sensitive hydrogel sensor to the surface of an implant, but this is impractical for imaging over large irregular device areas such as an orthopedic plate with holes and edges. Herein we describe a chemically sensitive and biocompatible XELI sensor material containing scintillator particles (Gd_2O_2S:Eu) and a pH sensitive hydrogel coating using a roughened epoxy coating. A two-part commercial grade epoxy film was tested and found to make the coating of pH sensitive layer adhere better to the titanium surface. Sugar and salt particles were added to the surface of the epoxy as it cured to create a roughened surface and increase surface area. On this roughened surface, a secondary layer of diacrylated polyethylene glycol (PEG) hydrogel, containing a pH sensitive dye, was polymerized. This layer was found to adhere well to the epoxy-coated implant unlike other previously tested polymer surfaces which delaminated when exposed to water or humidity. The focused X-ray beam enabled 0.5 mm spatial resolution through 1 cm thick tissue. The pH sensor coated orthopedic plate was imaged with XELCI through tissue with different pH to acquire a calibration curve. The plates were also imaged through tissue with low pH region from a *Staphylococcus aureus* biofilm grown on one section. These studies demonstrate the use of pH sensor coated orthopedic plates for mapping the surface pH through tissue during biofilm formation using XELCI.

INTRODUCTION

Fracture fixation surgeries uses orthopedic plates and screws to hold fractured bones in place and allow them to heal. While these surgeries restore ability and improve quality of life,

introducing implants increases the risk of infection, and more importantly bacteria can form biofilms on the implant surface which are resistant to antibiotics and the host's immune system. Roughly 5% of 2 million fracture fixation devices installed annually in the US become infected, with higher rates for immune compromised patients and traumatic injuries including about 40% of internally fixated battlefield injuries which often have debris in the wounds.[1,2] If the infections are caught early, they can often be treated with retention of the hardware using surgical irrigation and debridement, and antibiotics. After about 3 weeks, however, the device usually needs to be removed to treat the infection, followed by replacement after eradicating the infection.[3,4] Imaging techniques to catch these infections within the first 3 weeks of growth could help prevent risks associated with the infection, additional surgeries, and hospitalization. While some imaging methods such as plain radiography, computed tomography (CT), ultrasound, positron emission tomography (PET) and single photon emission computed tomography (SPECT) have been used in diagnosis, they either have a low resolution to accurately image the surface of the implant, or are only effective at later infection stages.[5,6] They also do not provide chemical sensitivity and specificity to the implant surface to study the local environment at the nidus of resistant infections.

To noninvasively detect and monitor infection localized on the surface of an implanted medical device, and to study the local chemical environment, sensors must be placed on that surface and interrogated remotely. XELCI uses indicator dyes encapsulated in polymer films to determine the local chemical concentration. An implant (usually titanium alloy or stainless-steel plate) is coated with X-ray scintillator in a polymer matrix and this scintillator film is further coated with a film containing pH indicator dyes. After implantation, the scintillator layer is excited by a focused beam of X-rays and the resulting light emission diffuses through the tissue and is collected by a spectrometer or two bandpass-filtered photodetectors to find the spectral peak ratio. To generate an image, the beam is scanned over the sample, while the ratio is measured point by-point. This provides a chemically specific measurement with high spatial resolution (because of the small X-ray beam spot size) and low background signal (because of minimal X-ray luminescence from the tissue). High spatial resolution provided by XELCI is important for mapping local changes during bacterial biofilm growth and treatment, especially for heterogeneous pH distributions (including spots and edges). High spatial resolution also allows separate regions of the implant to be distinguished, including spectral reference regions and potentially arrays of different sensor types. There is ongoing research in developing imaging and theranostic techniques based on the use of x-rays, for example, Wang et al, reported x-ray irradiated luminescence as a potential tool to inhibit bacterial growth but does not provide any information on the spatial resolution[7] while other research reports are focused on increasing the spatial resolution of x-ray luminescence computed tomography.[8–13]

We are particularly interested in monitoring pH as a method to detect and track infections on implanted medical devices. Previously, we have shown pH changes occurring during bacterial culture in vitro for silica based pH sensors deposited on glass slides.[5,14] We also improved our pH sensor to be more robust and biocompatible by shifting to a hydrogel based sensor and evaluated the performance by imaging it at various pH through different thicknesses of chicken tissue and human cadaveric tissue.[15] The goal in this study was to develop the pH sensor into a conformal coating which could be applied to titanium orthopedic implants eventually for studies in small animals.

In order to image these implants with XELCI, they must be coated with scintillator particles. While these particles can be effectively added into a biocompatible polydimethylsiloxane (PDMS) coating, this hydrophobic coating does not stick well to the surface of the implant and the subsequent hydrophilic polyethylene glycol (PEG) film containing

the dye. Scintillator particles can also be sintered. However, the temperatures needed are too high for the implants themselves to withstand (sintering temperature of ~1700 ºC versus the melting point of titanium at ~1650 ºC).[16,17] Even once the temperature is lowered with the use of sintering agents, such as sodium fluoride, the scintillator particles can diffuse together without adhering to the titanium implant. Therefore, we tested a roughened epoxy polymer coating to serve both as a scintillator layer and an anchor layer for PEG hydrogel that contain the pH dye. We used sugar and coarse sea salt to create a roughened surface that allows for better adhesion of the hydrogel coating. With this method, our group achieved results that could lead to an applicable whole implant coating for imaging through tissue.

EXPERIMENTAL METHODS

We first fabricated stand-alone hydrogel-coated epoxy films, characterized their spectral response, calibration, imaging response through tissue, and toxicity. We then coated titanium plate with the epoxy-hydrogel films and tested their ability to withstand simulated irrigation and tissue-friction, and used them for imaging in vitro biofilm growth with and without tissue.

Epoxy Film Coated with pH-Indicator Loaded Hydrogel

The epoxy film was prepared by mixing Gd_2O_2S:Eu scintillator particles (UKL63/N-R1, Phosphor Technologies Inc., Stevenage, England) with two-part epoxy (Loctite Epoxy Quick Set, Loctite® Brand, Henkel Corporation, Westlake, OH, USA) in 1:4 ratio. These were stirred together on Aluminum foil using a disposable pipette. The epoxy was then spread on aluminum foil to make 12 small samples (roughly 1 cm x 2 cm). To create a rough surface, six of the samples were then coated with granular sugar and six samples were coated with coarse sea salt. Particle size of around 500 microns is typical for fine sugar crystals while the coarse sea salt grains are a little larger, up to 2 mm. The samples were covered and left to cure 24 hours. Once cured, the samples were put in a beaker of water to dissolve the sugar and salt off the surface. These were then dried and dip coated in PEG hydrogel solution described earlier [15] at concentrations of 10%, 50%, and 80% (w/w) PEG in a water/glycerol solution containing a pH dye (either bromocresol green or bromothymol blue). A sugar (sucrose) and salt (sodium chloride) roughened epoxy sample was used for each concentration, the sample being dip coated in polymer solution under nitrogen atmosphere and cured under UV for one minute. A second coating was then applied, and the sample left to cure under UV for 5 minutes. The final cured samples were left in an inert atmosphere for at least two hours and then taken out and put in water to test adherence of the polymer layer to epoxy-scintillator layer. One half of the sugar-roughened epoxy sample coated with the 10% PEG hydrogel was dipped in a pH 4 buffer and the other half in a pH 7 buffer until half of the sample was green and half blue (pH 4 and pH 7 respectively). This sample was then imaged using XELCI.

Spectra

The spectra of the uncoated epoxy-scintillator film and the pH dye containing hydrogel coated epoxy-scintillator layer were taken using an inverted Leica DMI-5000 microscope (Leica Microsystems, Germany) with no emission filter in the beam path. An X-ray source (Mini-X with a silver target, Amptek, Inc., Bedford, MA) was used to excite the sample (source placed at about 6.7 cm from the sample and no collimator in place). Radioluminescence was directed to a spectrometer (DNS 300, DeltaNu, Laramie, WY, United States), equipped with a cooled CCD camera (iDUS-420BV, Andor, South Windsor, CT, United States). The CCD exposure time was 0.1 seconds in full vertical binning mode.

XELCI Imaging

The sample of interest (epoxy-hydrogel coating or the pH sensor coated orthopedic plate) was placed on a piece of porcine tissue and imaged using XELCI with no tissue covering and with a second piece of 6 mm or 1 cm thick porcine tissue on top of the sample. The XELCI imaging set up consists of a focused x-ray beam (iMOXS, Institute for Scientific Instruments GmbH, Berlin, Germany) focused on a sample on an x-y movable stage (Models: LTS300 and LTS150, Thorlabs Inc., Newton, NJ, United States for x and y axis and Motorized Linear Vertical Stage Model AT10-60, Motion Control, Smithtown, NY, United States for the z-stage) and employs the use of photomultiplier tube as photo detectors to detect the light of different wavelengths of interest using optical filters. This sample was imaged at 5 mm/s with a 250 μm step size. The scintillator particles used (Gd_2O_2S:Eu) emit 620 and 700 nm wavelengths of light. The pH dyes used absorb the 620 nm light at basic pH but the absorbance blue-shift at acidic pH resulting in higher intensity of the 620 nm light under acidic conditions. The 700 nm light however remains unaffected at all pHs and is not absorbed by the dye. Images were taken at wavelengths of 620 nm and 700 nm, the 620 nm showing changes in light intensity, due to absorption by the pH dye containing hydrogel layer, and the 700 nm showing a constant light intensity. The 700 nm light intensity is used to normalize the image (calculating the ratio of 600 nm to 700 nm light at each pixel) to account for variation in optical collection efficiency and X-ray intensity from sample to sample.

Toxicity Study

Three sets of studies were conducted to evaluate the potential toxicity of the glues and the epoxy film without any pH indicator coating. The studies include Study 1: Toxicity study of different adhesives with bacterial cells. Study 2: Toxicity comparison of pre-leached and non-leached regular (quick set) epoxy film samples with bacterial cells. Study 3: Toxicity comparison of pre-leached and non-leached regular (quick set) epoxy samples with mammalian cells.

Toxicity Study 1: Samples of different types of adhesives were prepared according to manufacturer instructions for toxicity study with bacterial and mammalian cells. The different types of adhesives tested were: (i) Marine Epoxy (Loctite Epoxy Marine, Loctite® Brand, Henkel Corporation, OH, USA); (ii) Regular Epoxy (Loctite Epoxy Quick Set, Loctite® Brand, Henkel Corporation, OH, USA) ; (iii) Vinyl Adhesive (Vinyl, Fabric and Plastic Clear Fabric Repair Adhesive, Loctite® Brand, Henkel Corporation, OH, USA); (iv) Gorilla Glue (White Gorilla Glue Pen, The Gorilla Glue Company, OH, USA). These samples were cut into discs 9 mm in diameter and 1 mm thick (unless otherwise stated). The samples were placed in a 24 well plates. 1 mL of fresh TSB (tryptic soy broth) medium was added to each well containing 5000 of *S. aureus* cells as inoculum. The plate was incubated at 37 °C for 24 hours. Then the media was diluted in to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} and cultured on TSA (tryptic soy agar) plates. After 24 hour of incubation the colonies were counted and based on the formula below, the concentration of bacteria given as colony forming units per mL (CFU/mL) in each well was calculated. If the number of colonies on all the plates are more than 300, more dilutions are needed. CFU/mL = (Number of colonies/ Volume plated) x (1/Dilution factor).

Toxicity Study 2 (Bacterial Culture): Toxicity comparison of pre-leached and non-leached regular (quick set) epoxy samples. This study was done to compare the effect of pre-leaching the cured epoxy resin with different surface roughness to non-leached cured epoxy resin with different surface roughness. The different samples tested are as follows. (1) Pre-leached smooth surface epoxy; (2) pre-leached roughened surface epoxy with small pore size (sugar roughened); (3) pre-leached roughened surface epoxy with large pore size (salt roughened); (4) non-leached smooth surface epoxy; (5) non-leached roughened surface epoxy with small pore size (sugar roughened); (6) non-leached roughened surface epoxy with small pore size (salt

roughened). These were cut into discs 5 mm in diameter and 1 mm thick and were placed in a 24 well plates. 1 mL of fresh TSB medium was added to each well containing 5000 of *S. aureus* cells as inoculum. The plate was incubated at 37 °C for 24 hours. Then the media was diluted in to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} and cultured on TSA plates. After 24 hour of incubation the colonies were counted and the concentration of bacteria given as colony forming units per mL (CFU/mL) in each well was calculated the same way as described for toxicity study 1.

Toxicity Study 3 (Mammalian Cells): Fibroblasts L929 Cells (T192755Z) were used with an initial cell concentration of 2.5 x 10^4 cells/mL per well in a 24 well microtiter plate and exposed to the different types of epoxy discs for 24 hours in 37°C. The cells were then imaged using a fluorescent microscope. The final concentration of cells after exposure to epoxy discs calculated using a hemocytometer.

Calibration

pH calibration curves were constructed first for a bromocresol green (BCG) indicator dye, and then for bromothymol blue (BTB). A sample of epoxy-PEG film containing the pH dye, was prepared as described in the epoxy and hydrogel coating section and cut into either 5 mm or 7 mm diameter discs using a hole punch. These discs were equilibrated overnight in pH 3, 4, 5, 6, 7, and 8 buffers to achieve the color change response associated with the respective pH. A reference disc was also prepared that consisted of just the scintillator particles in the epoxy matrix without any hydrogel and pH dye coating. These discs were then placed in 3D printed holder containing the different pH buffers. Reference disc was placed in PBS. This was then imaged using XELCI through 6 mm and 11 mm of porcine tissue and the 620 nm and 700 nm intensity images were obtained. The raw data (620 nm PMT counts, 700 nm PMT counts, and stage position vs. time) was processed using a MATLAB script to form the 620 nm, 700 nm, and ratio images vs. stage position. The signal was averaged over each disc and normalized to the reference signal.

Conformal coating of orthopedic plate

The epoxy film containing the scintillator particles was prepared in the same manner as described above except that the film was coated onto the surface of a titanium animal orthopedic plate (2.7mm 4 Hole Limited Contact Ti Compression Plate, 38mm, part no. 308-04LC , Animal Orthopedics, Inc., Bremen, IN, USA) and allowed to cure (with sugar roughened surface) followed by dip coating the epoxy coated plate in 10% (w/w) PEG hydrogel solution containing a pH dye to create a pH sensitive smart orthopedic plate.

Coating Evaluation

Autoclave: The sensor-coated plate was autoclaved under the following condition:121°C, 1 atm, 15 min.

Load Test: The sensor-coated plate was fixed on a movable stage positioned under a rolling pin attached to a mechanical loading device (Mark-10, Mark-10 Corporation, Copiague, NY, USA). The stage was moved at a velocity of 1.5 mm/sec causing the rolling pin in contact with the sensor coated plate to roll over the plate while applying a variable load of up to 22 N.

Friction Test: The sensor-coated plate was fixed on a wooden block with two screws and rubbed with a piece of 2.5 cm thick chicken tissue. Both the plate and tissue were moistened with PBS and manually rubbed together 10 times. Pictures were taken before and after rubbing to look for any coating residue on the tissue.

Simulated Irrigation and Debridement: The sensor coated plate was tested in conditions simulating high pressure irrigation during irrigation and debridement by placing under a high flow of pressurized water with a flow rate of 300 mL/s and photographed before, during, and after treatment to look for potential delamination or wear.

Biofilm formation

The sensor coated plate was sterilized and initial pH of the whole plate was set to physiological pH by immersing in sterile PBS. *Staphylococcus aureus* biofilm was grown on one half of the sensor coated plate in TSA (tryptic soy agar) medium and incubate at 37 °C for 24 hours. The other half of the plate was kept moist by covering in agar containing PBS so that it maintained the physiological pH. The part of the plate covered in biofilm dropped in pH within 24 hours and was evident by the color change of the pH coating. The pH sensor coated plate covered with biofilm was imaged using XELCI.

RESULTS AND DISCUSSION

Epoxy and Hydrogel Coating

We developed a pH sensor read with XELCI to map the surface pH of the orthopedic devices in order to help detect and monitor implant associated infection.[5,15] In order to show the proof of concept and to characterize the sensor's luminescent signal through tissue, we previously used a 3D printed clip to attach the the sensor to the orthopedic plate. However, our ultimate goal is to develop the pH sensor into a conformal coating that can be used to cover the full surface of the orthopedic implant in order to map the surface pH of the whole implant. The sensor consists of two layers: a luminscent layer and a pH modulated absorbance / pH indicating layer. Figure 1 shows how the sensor and XELCI works: An implant (usually titanium alloy or stainless steel plate) coated with X-ray scintillator in a polymer matrix (luminescent layer) and this scintillator film is further coated with a film containing pH indicator dyes (pH indicating layer). The pH dye is selected such that the absorption of the pH dye overlaps the emission of the scintillator particles at a certain pH range so that the hydrogel coating containing the pH dye modulates the scintillator emission. For example, Figure S1 in supporting information shows the absorption spectra of the two pH dyes used in this study, Bromocresol green and Bromothymol blue. After implantation, the scintillator layer is excited point by point by a focused beam of X-rays and the resulting light emission diffuses through the tissue and is collected by a spectrometer or photodetector with filters to collect and analyze specific frequencies. The principle has been explained in detail in our previous publication.[15]

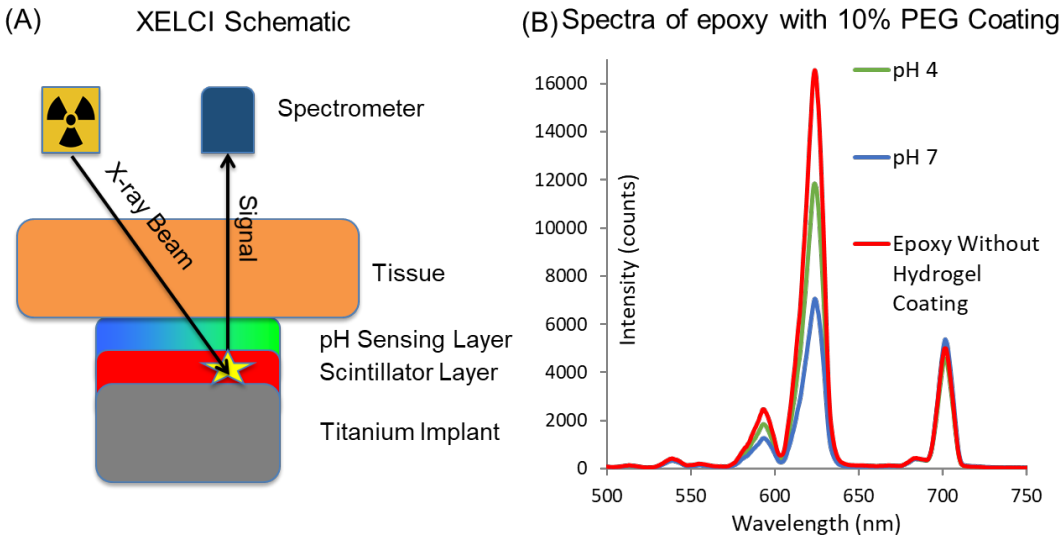

Figure 1: (A) Schematic of XELCI set up. (B) Spectra of 10% PEG Hydrogel coating with BCG pH dye on top of epoxy film containing Gd_2O_2S:Eu scintillator particles at pH 7 (blue), pH 4 (green), and without pH-indicator PEG hydrogel (red).

Previously, the luminescent layer was comprised of hydrophobic inert polymer (PDMS) enclosing the scintilllator partilcles while the pH indicating layer was made from a hydrophilic biocompatible PEG hydrogel containing a pH indicating dye. The pH indicating layer needs to be H+ permeable or hydrophilic to allow for the exchange of H^+ ions to indicate pH. Due to the contradictory nature of both layers, it is hard to polymerize the hydrophilic hydrogel on the hydrophobic PDMS. We tried various approaches to hold both layers together such as the use of silanes for surface functionalization of PDMS, trying various hydrogel compositions, different types of glues and 3D printed clips to physically press them together but to no avail. Bailey et al., reported an interesting preparation of a hybrid hydrogel from methacrylated star polydimethylsiloxane and diacrylated polyethylene glycol macromers using a solvent-induced phase separation (SIPS) that could be used as continuous gradient scaffolds for tissue engineering applcations but would yield non-uniform films if applied to our sensor bilayer structure due to macroporous nature of the hybrid hydrogel.[18,19] We also tried to incorporate the scintillaor particles in a PEG hydrogel matrix instead of PDMS to avoid the issues caused by the hydrophobic nature of PDMS used in the luminescent layer. This approach, however, is not feasible for the conformal coating of the orthopedic plates due to the swelling nature of hydrogel when exposed to humidity causing the hydrogel coating to peel off of the surface of the plate (Figure S2A in Supporting Information). The use of an epoxy based polymer for the luminescent layer combined with a roughened surface allows good adhesion of the pH indicating PEG hydrogel layer. The benefit of this approach is two folds; it not only serves as an anchor layer for the hydrogel but also adheres to the metal surface of orthopedic plates. To test if this approach would work for imaging, we obtained spectra of the epoxy based luminescent layer covered with the pH sensitive hydrogel layer. Figure 1B shows the emission of Gd_2O_2S:Eu microphosphor scintillators embedded in an epoxy matrix (red line) and the modulated scintillator emission when the epoxy layer is further coated with hydrogel containing bromocresol green dye at pH 4 (green line) and pH 7 (blue line).

We prepared small samples of the luminescent epoxy film and coated them with different concentrations of the PEG hydrogel containing the pH indicator. We tried coatings with different hydrogel compositions containing 10%, 50% and 80% (w/w) PEG. The original sensor consisted

of 80% PEG hydrogel but this concentration yielded thick non-uniform coatings that chips-off the surface of the epoxy layer. 10% PEG hydrogel formed the best coating when polymerized onto the epoxy layer. The epoxy layer was also roughened to create more surface area and to allow for better adhesion of the polymerized PEG hydrogel. Photographs of the different formulations tested including the surface roughening treatments and the concentrations of the PEG gels used are shown in the Supporting Information Figure S2. Granular sugar and coarse sea salt were used as the roughening agents since these can be easily dissolved away after the epoxy layer has cured producing a rough surface based on the imprints left by the sugar and salt crystals on the curing epoxy layer. Similar approach using salt leaching has been reported in literature to create a porous surface.[20] When exposed to humidity or even immersed in water, the PEG hydrogel coating remain stuck to the roughened areas of the epoxy for both the salt and sugar roughened surfaces. The pore size of the roughened surface depends on the size of the sugar or salt particle. Granular sugar produces a more uniformly roughened surface with smaller pore sizes. Due to the larger size of the coarse sea salt crystals, surface roughening was also dependant on the orientation of the salt crystals and produced an overall less uniformly roughened surface with larger non-uniform pore sizes. Based on these results, we proceeded with the 10% PEG hydrogel composition and sugar as the roughening agent to prepare the epoxy-PEG coatings for further testing.

These epoxy-PEG films retain the pH sensing properties as evident by the change in color of the film with a change in pH of the surrounding medium. Figure 2A shows a photograph of a sugar-roughened epoxy coated with 10% PEG hydrogel containing bromocresol dye. One half of this epoxy-PEG film was dipped in pH 7 buffer (blue in color) and the other half in pH 4 buffer (green color). The luminescence of the scintillator particles can be seen under the UV light, since at acidic pH, the pH dye does not absorb the red emission from the scintillator particles, the acidic half of the film dipped in pH 4 buffer appears bright red in color while the basic half dipped in pH 7 buffer shows a weak red luminescnce due to absorption of the red emission by the pH dye at higher pH. The epoxy-PEG film was plaaced on a piece of porcine tissue and imaged uncovered and then covered with another 6 mm piece of porcine tissue on the top using X-ray excited luminscent chemical imaging. We are measuring the pH modulated 620 nm emission and the pH-independent 700 nm light from the X-ray excited scintillator particles in the epoxy-PEG film. A ratio of these intensties is plotted to correct for any background and tissue attenuation.The sample showed a clear difference in light intensity where the hydrogel showed a change in pH for both with and without tissue as shown by the respective images in Figure 2B-C. The 600 nm emission showed a change in intensity with change in the surface pH of the sample, as expected. The image measured at 620 nm wavelength gives good indication of pH changes, and all images show good resolution to differentiate the two pH regions of the film with and without tissue. Since the 700 nm intensity is not absorbed by the pH dye at any pH, it is used to normalize the 620 intensity for changes caused by variations in the coating surface and attenuation by the tissue. This is done by taking a ratio of both intensities and the resulting ratiometric images are shown that look very similar for both with and without tissue.

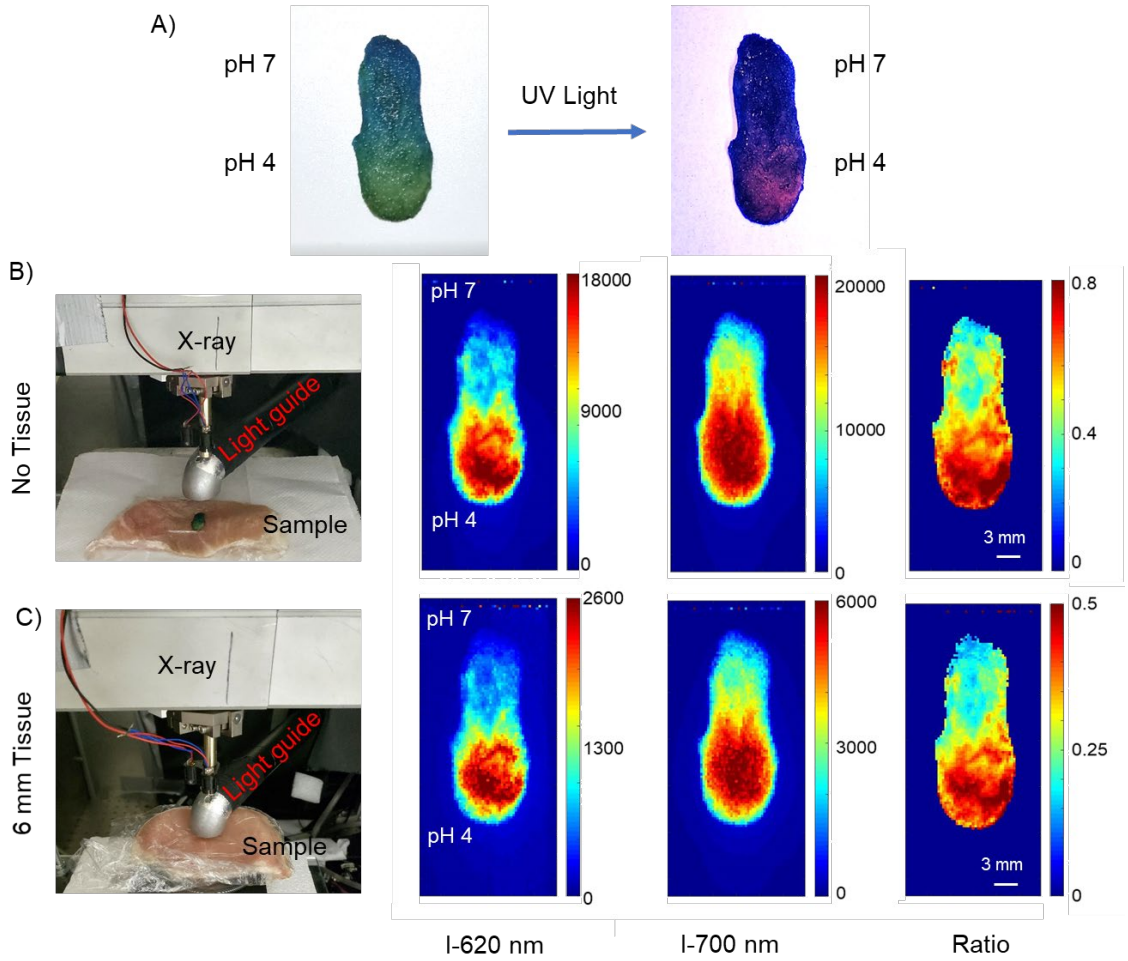

Figure 2: A) Epoxy coated with PEG hydrogel containing BCG pH dye and immersed in two different pH buffer solutions (green is pH 4, blue is pH 7) shown under room light and UV light. B) Setup photograph of (A) placed on top of tissue and XELCI images of sample at 600 nm and 700 nm intensities and ratio. C) Setup photograph of (A) sandwiched between two slices of tissue and XELCI images of sample at 600 nm and 700 nm intensities and ratio through 6 mm of porcine tissue.

Toxicity Study

To determine the effect of toxic leachates from epoxy, we conducted toxicity studies with both bacterial and mammalian cells. We did a preliminary study with different adhesives, bacteria grew on all of them and we selected the regular epoxy for further evaluation due to similar cell growth to control, was readily available and worked well due to quick setting time. The different sets of studies conducted were: (1) Toxicity study of different adhesives with bacterial cells, (2) Toxicity comparison of pre-leached and non-leached regular (quick set) epoxy samples with bacterial cells, (3) Toxicity comparison of pre-leached and non-leached regular (quick set) epoxy samples with mammalian cells, Results are shown in supporting information S3-S5. The bacterial cells (*S. aureus*) did not show significant toxicity caused by any of the types tested as shown in Figures S3 in the supporting information. Moreover, we did not observe a significant difference between the toxicity results of leached and non-leached epoxy resins on bacterial cells (Figure S4). The mammalian cells (fibroblasts L929) were proliferating in all wells despite morphological changes observed in some cells (Figure S5). Final cell concentration after 24 hours in all wells ranged between 2.7 – 3.0 x 10^4 per well. This is contrast to the results of the cytotoxicity study

done by Ejserholm et al. where they developed a flexible polymer based on epoxy and Off-Stoichiometry Thiol-Enes for neural implants. They observed that the polymer leached in water for a week did not show cytotoxicity to mouse L929 fibroblasts as tested by the Methyl Tetrazolium (MTT) assays whereas the non-leached polymer showed cytotoxicity.[21] However, we did not observe a significant difference in cytotoxicity results of leached and non-leached epoxy resins but this could be due to the type of resin used.

The results of the three toxicity studies performed by our group indicate a lack of toxicity and are consistent with the toxicity studies done on other epoxy-based resins that are widely used in the medical applications such as endodontic sealers.[22] Bagheri et al., report a Carbon Fiber-Flax-Epoxy composite as a potential candidate for orthopedic implants to replace the metal plates used for bone fractures and confirmed the biocompatibility of the material *in vitro*. They also found the material to not affect the osteogenesis process negatively.[23] Eventually, we plan to move on to a medical grade epoxy to conformally coat the orthopedic plates but this lack of toxicity even for the non-medical grade epoxy is an important and necessary step for establishing biocompatibility of the coating.

Calibration Curve

pH calibration curves for epoxy-PEG coatings containing bromocresol green (BCG), and bromothymol blue (BTB) were acquired through tissue using XELCI imaging of the sensor discs in pH buffer solutions (Figure 3). A reference disc with just epoxy layer and no pH-indicator coating was used to account for the pH-independent attenuation caused by tissue or variation in the film thickness. The sample and reference discs are shown in the photo (Figure 3A & 3E) with the respective ratiometric images through two thicknesses of tissue (Figure 3B-C & 3F-G). Reconstructed images of the 620 nm and 700 nm raw signal intensities through porcine tissue are shown in the Supporting Information Figure S6. For both, the ratiometric and raw signal intensities, the reference discs appear brighter than the hydrogel coated discs. The 620 and 700 nm raw signal intensities decrease when passing through thicker (11 mm vs 6 mm) tissue due to attenuation and scattering by the tissue. Sample discs that are at acidic pH also appear brighter (depending on the pH range of the dye used) than those at higher pH due to increased absorption of the scintillator emission by the pH dye at higher pH, this is especially evident for the 620 nm images as the pH dyes absorb the 620 nm emission while the 700 nm emission remains unaffected by the pH indicating dyes. The 700 nm images are used to correct for any attenuation or variations caused due to tissue and sample preparation in the resulting ratiometric images obtained by dividing the pH modulated 620 nm signal by the pH independent 700 nm signal. Figure 3C and 3F shows the plotted normalized ratios for pH 3,4,5,6,7, 8 and the reference disc through 6 mm and 11 mm thick tissue. The normalized plots for both tissue thicknesses for each dye show good overlap. Plots of the 620 nm and 700 nm signal intensities through porcine tissue for both Epoxy-PEG-BCG and Epoxy-PEG-BTB coatings are available in the Supporting Information Figures S7 and S8. For the BCG containing discs, the effective range of sensor is between pH 3 and 5. This can also be seen from the color response at different pH (Figure 3A), the discs appear yellow, green and blue at pH 3, 4 and 5 respectively, and above pH 5, all discs look blue. In order to make the pH sensing range to be applicable to physiological pH, epoxy-PEG films were prepared using a different pH dye, bromothymol blue (BTB). This extends the pH sensing range from pH 5 to 8 making it more biologically relevant. Figure 3E shows the prepared discs with BTB in the respective buffer solutions; the discs appear yellow in color for pH 3, 4 and 5 changing to green at pH 6, bluish-green at pH 7 and blue at pH 8. These color changes associated with pH can be seen in the MATLAB constructed ratiometric images (Figure 3F-G) and the plotted ratios of the underlying signal

intensities (Figure 3H) even through 6 and 11 mm of porcine tissue. These color changes are impossible to see through the tissue either by the eye or plain radiography. However, XELCI enables us to see these color changes clearly through thick tissue.

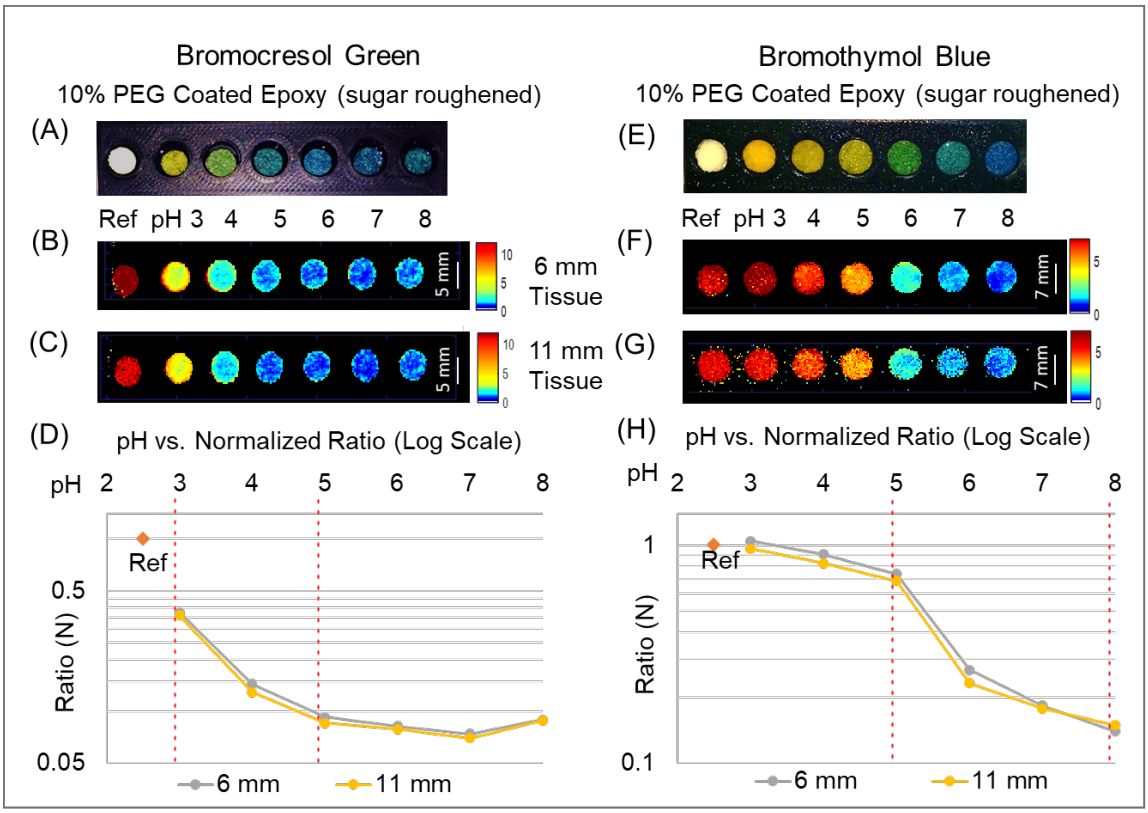

Figure 3: pH calibration curve using two dyes: Bromocresol green (range pH 3-5) and Bromothymol blue (range pH 5-8). Left (Bromocresol green): (A) Photograph of sugar roughened epoxy discs coated with 10% PEG hydrogel containing bromocresol green dye. (B) XELCI ratiometric images of (A) through 6 mm and (C) through 11 mm of porcine tissue. pH sensor coated epoxy discs were placed in pH 3 – 8 buffers and a non-coated epoxy reference was also imaged for each case. (D) Plots of ratio normalized to reference at pH 3 – 8 for 6 mm and 11 mm tissue. Reference is plotted arbitrarily as pH 2.5. Right (Bromothymol blue): (E) Photograph of sugar roughened epoxy discs coated with 10% PEG hydrogel containing bromothymol blue dye. (F) XELCI ratiometric images of (A) through 6 mm and (G) 11 mm of porcine tissue. pH sensor coated epoxy discs were placed in pH 3 – 8 buffers and a non-coated epoxy reference was also imaged for each case. (H) Plots of ratio normalized to reference at pH 3 – 8 for 6 mm and 11 mm tissue.

Conformal coating of orthopedic plate

Finding the new epoxy-PEG coating generated decent XELCI images, a sample was made with the same coating on a titanium orthopedic implant. Figure 4 shows the different stages of the coating process. The pH sensitive coated plate was then tested in two buffers of pH 4 and pH 7 to show a pH response. Figure S2a shows a titanium orthopedic plate that was coated with the PEG hydrogel alone without the epoxy layer. The hydrogel coating peels off the implant plate as soon as it is exposed to humidity or fluids due to swelling of the hydrogel. The use of epoxy as an anchor layer for the hydrogel overcomes this issue and make the coating more robust and easier to handle.

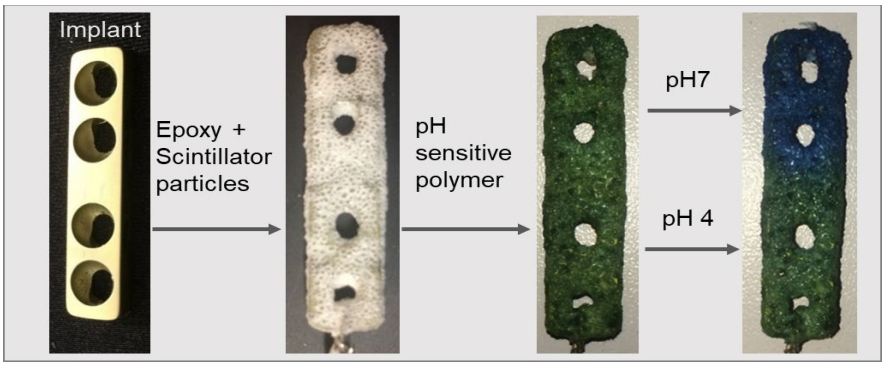

Figure 4: Preparation schematic for conformal coating of the whole implant plate with pH sensitive coating.

Coating Evaluation

To check the robustness of the epoxy-hydrogel coating, the coated plate was subject to several tests. These include autoclaving the coated plate, testing it under a mechanical load and simulation of high-pressure irrigation during irrigation and debridement. The epoxy based pH sensor coated plate was found to be able to withstand the temperature and pressure required to autoclave and does not peel off under high flow of water (flow rate of 300 mL/s) or crack under the tested mechanical load. The friction test was performed to simulate the rubbing of the plate against the soft tissue when implanted in the body. For the friction test, the plate and tissue were moistened with PBS before rubbing and we did not see any residue or debris from the coating on the tissue after rubbing. Similarly, we did not observe any delamination of the coating under a high flow of water simulating irrigation and debridement. (Images of the plate taken before and during or after the tests shown in supporting information, Figure S9).

pH Mapping of Biofilm

Two orthopedic plates were coated with the same epoxy-PEG coating but one containing the BCG dye and another with the BTB dye (Figure 5A&B). These plates were then dipped in two pH buffers to create a pH difference on one half of the plate and XELCI images obtained without any covering and then covered by a piece of 1 cm porcine tissue. The 620 nm images in Figure 5 show the reduction in signal intensity at high pH while a brighter signal was measured for the half exposed to low pH, these signal intensities are indicative of the pH change. The 700 nm images remain unaffected by pH and show the variations in the coating as well the attenuation caused by the tissue. Taking a ratio of 620 nm/700 nm gives a pH map of the surface and the pH difference between the two halves of the plate can be clearly seen in ratio images for both with and without tissue even though the raw intensities are much attenuated for the images through tissue as compared to the respective images without tissue.

After successfully imaging the pH difference on the surface of the coated orthopedic plates (Figure 5A&B), we wanted to image the pH changes caused by the formation of a biofilm on the plate surface to mimic the case of implant-associated infections. For this purpose, a biofilm was grown from *S. aureus*, the predominant strain found associated with implant associated infections, on one half of each of the epoxy-PEG coated plates, one containing the BCG pH dye (pH range 3-5) and other containing the BTB pH dye (pH range 5-8). The other halves of the plates were covered with agar containing sterile PBS to simulate physiological pH. After growing the biofilm for 24 hours, the pH dropped and became acidic in the half covered by the biofilm. This pH change can be seen in the Figure

5D where the part of the plate covered in biofilm appears green while the part covered in PBS is blue. Referring to the calibration curve for the BTB dye, green color indicates a pH of about 6 and blue between pH 7 and 8. This change in pH, however, is not visible in Figure 5C where both regions of the plate, with and without biofilm appear blue in color. The plate coating in Figure 5C with BCG dye does not respond appreciably to changes in pH above 5 and therefore was not able to detect the pH change produced by the biofilm. Selecting the pH dye to get the maximum response in the desired pH range is the key for detecting pH changes. Another approach can be combining both the pH dyes in the same coating to create a larger pH sensing range to suit the application. The low pH produced by the biofilm on the epoxy-PEG-BTB coated plate can also be clearly seen in the respective XELCI images in Figure 5D. The intensity decreases with increasing tissue thickness, for example, the 700 nm images in Figure 5D, maximum intensity for no tissue was 2,000 counts/ms compared to 190 counts/ms through 1 cm tissue for the same sample. The spatial resolution was 450 µm at the edge of the of the plate covered with biofilm when imaged without tissue and about 540 µm when covered with 1 cm of tissue indicating minimal scattering of the low energy X-ray beam while still providing good imaging resolution even when the sample was imaged through 1 cm thick porcine tissue and part of the plate covered by the biofilm can be distinctly seen in the images. This demonstrates that the coating and imaging technique can map pH changes indicative of infection through tissue.

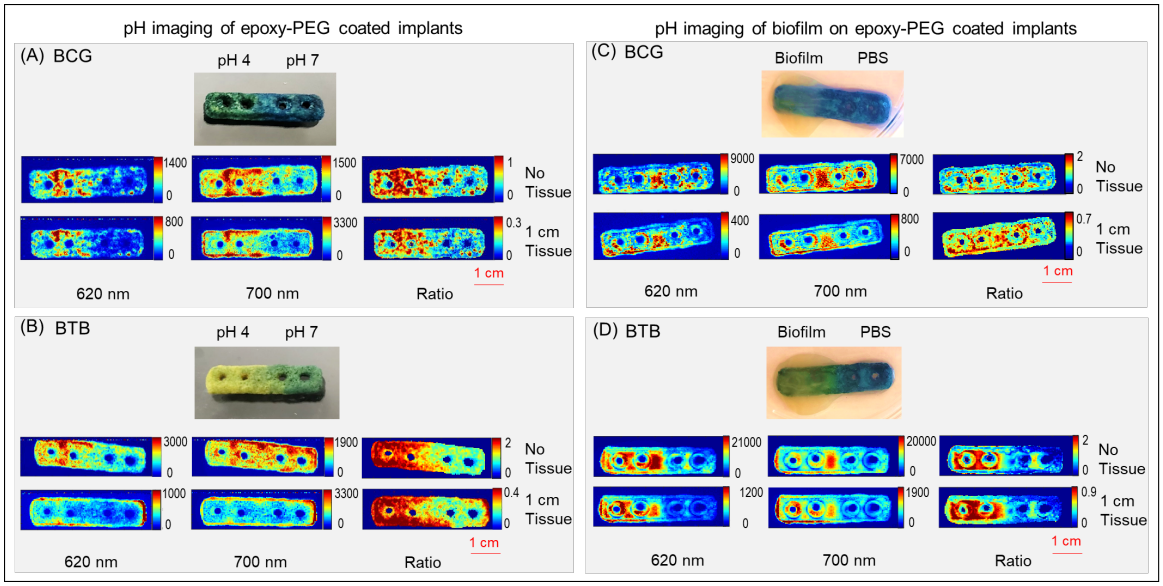

Figure 5: pH imaging of epoxy-PEG coated veterinary orthopedic plates. A) Photograph of a plate coated with the epoxy-PEG polymer containing Bromocresol green (BCG) pH dye with one half of the plate dipped in pH 4 buffer (green) and the other half dipped in pH 7 buffer (blue). XELCI images of the plate at 620 nm, 700 nm and ratio of both without tissue and through 1 cm of porcine tissue. B) Photograph of a plate coated with the epoxy-PEG polymer containing Bromothymol blue (BTB) pH dye with one half of the plate dipped in pH 4 buffer (yellow) and the other half dipped in pH 7 buffer (green). XELCI images of the plate at 620 nm, 700 nm and ratio of both without tissue and through 1 cm of porcine tissue. C) Photographs of an epoxy-PEG-BCG coated plate 24 hours after growing a biofilm on the left half and keeping the right half in agar containing PBS. XELCI images (at 620 nm, 700 nm and ratio of both) of the plate after 24 hours of biofilm growth with no tissue and covered with 1 cm of porcine tissue. D) Photographs of an epoxy-PEG-BTB coated plate 24 hours after growing a biofilm on the left half (green) and keeping the right half (blue) in agar containing PBS. XELCI images (at 620 nm, 700 nm and ratio of both) of the plate after 24 hours of biofilm growth with no tissue and covered with 1 cm of porcine tissue. Note: Color-bars indicate intensity in counts per 10 milliseconds.

CONCLUSIONS

We developed a conformal coating for XELCI imaging of local pH and applied it for pH imaging on orthopedic plates. The use of the epoxy-PEG coating prevented the sensor from peeling off the plate surface and improved the robustness and handling under irrigation and friction conditions. We generated pH calibration curves and demonstrated pH imaging through tissue on the sensor-coated orthopedic plates during formation of a biofilm and formulated a calibration curve. Biocompatibility tests of epoxy on mammalian cells and bacterial cells did not show significant toxicity. Different pH dyes have different response ranges (e.g., the PEG hydrogel with BCG responded best between pH 3-5 while BTB responded best between 5-8); in future, a combination of pH dyes can be used for applications requiring sensing over an extended pH. Additionally, the optical absorption approach could be modified utilizing other chemical indicators or a mixture of sensors at different locations. In future, further testing will be done using medical grade epoxy to allow for imaging implant surfaces in vivo to monitor pH changes that could indicate possible biofilm formation. Early diagnosis of bacterial growth could help to effectively treat infection and reduce the number of implant removals and replacement surgeries each year. Finally, while we have focused on orthopedic plates, we expect coating will have utility for other implants and the stand-alone films could be used as exogenous sensors to map local pH in other acidosis conditions (e.g., tumors, inflammation, ischemia, etc.).

ACKNOWLEDGEMENTS

The authors would like to thank Clemson University, especially COMSET and Clemson SC BioCRAFT. Funding for CJ was provided by an NSF REU grant, under DMR-1062873; the XELCI setup was initially developed with funds from NSF CAREER CHE 12255535, and later by NIH NIAMS R01 AR070305-01. UU was supported by the above NIH R01 grant and a Fulbright Fellowship.

REFERENCES

(1) Zalavras, C. G.; Christensen, T.; Rigopoulos, N.; Holtom, P.; Patzakis, M. J. Infection Following Operative Treatment of Ankle Fractures. *Clin. Orthop.* **2009**, *467* (7), 1715–1720. https://doi.org/10.1007/s11999-009-0743-8.

(2) Mody, R. M.; Zapor, M.; Hartzell, J. D.; Robben, P. M.; Waterman, P.; Wood-Morris, R.; Trotta, R.; Andersen, R. C.; Wortmann, G. Infectious Complications of Damage Control Orthopedics in War Trauma. *J. Trauma* **2009**, *67* (4), 758–761. https://doi.org/10.1097/TA.0b013e3181af6aa6.

(3) Vertes, A.; Hitchins, V.; Phillips, K. S. Analytical Challenges of Microbial Biofilms on Medical Devices. *Anal. Chem.* **2012**, *84* (9), 3858–3866. https://doi.org/10.1021/ac2029997.

(4) Bryers, J. D. Medical Biofilms. *Biotechnol. Bioeng.* **2008**, *100* (1), 1–18. https://doi.org/10.1002/bit.21838.

(5) Wang, F.; Raval, Y.; Tzeng, T.-R. J.; Anker, J. N. X-Ray Excited Luminescence Chemical Imaging of Bacterial Growth on Surfaces Implanted in Tissue. *Adv.*

Healthc. Mater. **2015**, *4* (6), 903–910. https://doi.org/10.1002/adhm.201400685.
(6) Chen, H.; Rogalski, M. M.; Anker, J. N. Advances in Functional X-Ray Imaging Techniques and Contrast Agents. *Phys. Chem. Chem. Phys.* **2012**, *14* (39), 13469–13486. https://doi.org/10.1039/C2CP41858D.
(7) Wang, X.; Li, Y.; Tong, H. H. Y.; Yuan, P.; Wong, K.-L.; Yang, Y. A Noninvasive Precise Treatment Strategy for Implant-Related Infections Based on X-Ray-Induced Luminescent/Photodynamic Therapeutic Multilayered Device Surface Materials. *J. Lumin.* **2020**, *222*, 117108. https://doi.org/10.1016/j.jlumin.2020.117108.
(8) Zhang, W.; Zhu, D.; Lun, M.; Li, C. Collimated Superfine X-Ray Beam Based x-Ray Luminescence Computed Tomography. *J. X-Ray Sci. Technol.* **2017**, *25* (6), 945–957. https://doi.org/10.3233/XST-17265.
(9) Zhang, Y.; Lun, M. C.; Li, C.; Zhou, Z. Method for Improving the Spatial Resolution of Narrow X-Ray Beam-Based x-Ray Luminescence Computed Tomography Imaging. *J. Biomed. Opt.* **2019**, *24* (8), 1–11. https://doi.org/10.1117/1.JBO.24.8.086002.
(10) Gao, P.; Pu, H.; Rong, J.; Zhang, W.; Liu, T.; Liu, W.; Zhang, Y.; Lu, H. Resolving Adjacent Nanophosphors of Different Concentrations by Excitation-Based Cone-Beam X-Ray Luminescence Tomography. *Biomed. Opt. Express* **2017**, *8* (9), 3952. https://doi.org/10.1364/BOE.8.003952.
(11) Zhang, W.; Lun, M. C.; Nguyen, A. A.-T.; Li, C. X-Ray Luminescence Computed Tomography Using a Focused x-Ray Beam. *J. Biomed. Opt.* **2017**, *22* (11), 116004. https://doi.org/10.1117/1.JBO.22.11.116004.
(12) Lun, M. C.; Cong, W.; Arifuzzaman, M.; Ranasinghe, M.; Bhattacharya, S.; Anker, J.; Wang, G.; Li, C. X-Ray Luminescence Imaging for Small Animals. In *Optics and Ionizing Radiation*; International Society for Optics and Photonics, 2020; Vol. 11224, p 112240F. https://doi.org/10.1117/12.2544601.
(13) Lun, M. C.; Li, C. X-Ray Luminescence Imaging of Water, Air, and Tissue Phantoms. In *Multimodal Biomedical Imaging XIII*; International Society for Optics and Photonics, 2018; Vol. 10487, p 104870K. https://doi.org/10.1117/12.2287023.
(14) Wang, F.; Raval, Y.; Chen, H.; Tzeng, T.-R. J.; DesJardins, J. D.; Anker, J. N. Development of Luminescent PH Sensor Films for Monitoring Bacterial Growth through Tissue. *Adv. Healthc. Mater.* **2014**, *3* (2), 197–204. https://doi.org/10.1002/adhm.201300101.
(15) Uzair, U.; Benza, D.; Behrend, C. J.; Anker, J. N. Noninvasively Imaging PH at the Surface of Implanted Orthopedic Devices with X-Ray Excited Luminescence Chemical Imaging. *ACS Sens.* **2019**, *4* (9), 2367–2374. https://doi.org/10.1021/acssensors.9b00962.
(16) Jingbao Lian, X. S.; Jingbao Lian, X. S. Preparation of Gd_2O_2S:Pr Scintillation Ceramics by Pressureless Reaction Sintering Method. *J. Mater. Sci. Technol.* **2009**, *25* (02), 254–258.
(17) Kim, H.-W.; Koh, Y.-H.; Li, L.-H.; Lee, S.; Kim, H.-E. Hydroxyapatite Coating on Titanium Substrate with Titania Buffer Layer Processed by Sol–Gel Method.

Biomaterials **2004**, *25* (13), 2533–2538. https://doi.org/10.1016/j.biomaterials.2003.09.041.
(18) Bailey, B. M.; Nail, L. N.; Grunlan, M. A. Continuous Gradient Scaffolds for Rapid Screening of Cell–Material Interactions and Interfacial Tissue Regeneration. *Acta Biomater.* **2013**, *9* (9), 8254–8261. https://doi.org/10.1016/j.actbio.2013.05.012.
(19) Bailey, B. M.; Fei, R.; Munoz-Pinto, D.; Hahn, M. S.; Grunlan, M. A. PDMSstar–PEG Hydrogels Prepared via Solvent-Induced Phase Separation (SIPS) and Their Potential Utility as Tissue Engineering Scaffolds. *Acta Biomater.* **2012**, *8* (12), 4324–4333. https://doi.org/10.1016/j.actbio.2012.07.034.
(20) Chiu, Y.-C.; Larson, J. C.; Isom, A.; Brey, E. M. Generation of Porous Poly(Ethylene Glycol) Hydrogels by Salt Leaching. *Tissue Eng. Part C Methods* **2009**, *16* (5), 905–912. https://doi.org/10.1089/ten.tec.2009.0646.
(21) Ejserholm, F.; Stegmayr, J.; Bauer, P.; Johansson, F.; Wallman, L.; Bengtsson, M.; Oredsson, S. Biocompatibility of a Polymer Based on Off-Stoichiometry Thiol-Enes + Epoxy (OSTE+) for Neural Implants. *Biomater. Res.* **2015**, *19* (1), 19. https://doi.org/10.1186/s40824-015-0041-3.
(22) Cintra, L. T. A.; Benetti, F.; de Azevedo Queiroz, Í. O.; Ferreira, L. L.; Massunari, L.; Bueno, C. R. E.; de Oliveira, S. H. P.; Gomes-Filho, J. E. Evaluation of the Cytotoxicity and Biocompatibility of New Resin Epoxy–Based Endodontic Sealer Containing Calcium Hydroxide. *J. Endod.* **2017**, *43* (12), 2088–2092. https://doi.org/10.1016/j.joen.2017.07.016.
(23) Bagheri, Z. S.; Giles, E.; El Sawi, I.; Amleh, A.; Schemitsch, E. H.; Zdero, R.; Bougherara, H. Osteogenesis and Cytotoxicity of a New Carbon Fiber/Flax/Epoxy Composite Material for Bone Fracture Plate Applications. *Mater. Sci. Eng. C* **2015**, *46*, 435–442. https://doi.org/10.1016/j.msec.2014.10.042.