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An implanted pH sensor read using radiography

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Abstract

A biomedical sensor is developed to measure local pH near orthopedic implants to detect and study implant-associated infection. The sensor is read using plain radiography, a technique which is noninvasive, inexpensive, ubiquitously available, and routinely used in diagnosis and follow-up. The sensor comprises a radiopaque tungsten indicator pin embedded within a chemically responsive hydrogel with a pH-dependent swelling. A stainless steel well holds this hydrogel and attaches to an orthopedic plate. The local pH is determined from the extent of hydrogel swelling by radiographically measuring the indicator position relative to the well. The sensor was calibrated in a series of standard pH buffers and tested during bacterial growth in culture. The sensor was robust: its response was negligibly affected by changes in temperature, ionic strength within the normal physiological range, or long-term incubation with reactive oxygen species generated from hydrogen peroxide and copper. Pooled data from several sensors fabricated at different times and tested in different conditions had a root-mean-square deviation from a pH electrode reading of 0.24 pH units. Radiographic measurements were also performed in cadaveric tissue with the

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Authors Contributions:

JNA, CJB, JDD, and MA conceived ideas for this project and JNA supervised the overall project. MA designed and coordinated experiments. MA fabricated the hydrogel films and carried out characterizations. SBB, YR and MA carried out bacterial culture experiments under TRJ's guidance. Surgeon CJB performed the implant surgery on the cadaver tibia. MA, PWM, YR, and CJB tested the sensor on the cadaver. MA, PWM, TBP, and JNA helped write and edit the manuscript. All authors participated in discussions and revised the manuscript.

Associated Content:

All data supporting the findings of this study are available within the paper and the Electronic Supporting Information.

All experimental methods were approved by the Clemson University Institutional Biosafety Committee (IBC) and followed the standard guidelines and regulations. Human cadaveric specimens were obtained via the Hawkins Foundation (Greenville SC) from Restore Life USA (Elizabethton, TN), a nonprofit donation program, and donors had consented their body to be used for medical education and research in accordance with the Uniform Anatomical Gift Act (UAGA).

Competing Interests: JNA, JDD, and CJB are inventors on a related pending Patent Cooperation Treaty (PCT) application (serial: 62/204,111, with a filing date of August 12, 2015) assigned to Clemson University and now licensed to a company, Aravis BioTech, LLC., that they co-own.

sensor attached to an implanted orthopedic plate fixed to a tibia. Pin position readings varied by 100 μm between observers surveying the same radiographs, corresponding to 0.065 pH units precision in the range pH 4 – 8. The sensor was designed to augment standard radiographs of tissue, bony anatomy, and hardware by also indicating local chemical concentrations.

Abstract Graphical

The constructed biosensor enhances the capability of traditional plain film radiography, enabling the noninvasive measurement of postoperative infection indicating chemical concentration such as pH on the implant surfaces.

Introduction:

X-ray projection imaging (plain radiography) is an indispensable tool for evaluating patients in clinical settings.¹ It is ideal for imaging anatomy including bones and calcifications, the lungs, vascular and the gastrointestinal tract using exogenous contrast agents, and implanted medical hardware.² However, plain radiography does not traditionally provide biochemical information, such as local pH, which is important for understanding, diagnosing, and monitoring numerous conditions and diseases. Local pH changes are implicated in infection and inflammation, tumor physiology and progression, compromised blood supply in compartment syndrome, ischemia, and necrosis.^{3–5} Herein we describe the first implantable sensors that measure local pH using plain radiography.

Our initial sensor application was to measure pH on the surface of implanted orthopedic hardware to detect implant-associated infection.^{6–8} In the United States, approximately 5–10% of the 2 million fracture fixation surgeries performed annually progress to infection, which constitutes an enormous economic impact on patients, physicians and the healthcare system at large.^{8–12} For instance, a study of the operative complications of ankle fractures found that both professional and technical expenses were substantially higher in patients presenting with infection, with median total charges of \$128,122 compared to \$49,983 for controls.¹⁰ Similarly, in patients with periprosthetic joint infection (PJI), the mean annual cost was \$116,383, compared to \$28,249 for controls.^{11,12} Furthermore, implant infection increases morbidity and mortality, reduces productivity, and decreases health-related quality of life.^{8–12} While systemic antibiotics kill individual (planktonic) bacteria and alleviate symptoms, the bacteria colonizing implants can form densely packed communities of bacteria in extracellular polymer films (biofilms) which are highly resistant to antibiotics and form a nidus for infection once antibiotic therapy ceases.^{13,14} If bacterial colonization could be detected early, antibiotic treatment or surgical irrigation and debridement are often successful.^{7,8} However, once a mature biofilm has been established, it is extremely difficult

to eradicate with conventional antimicrobial therapies, and two-stage revision surgery involving implant removal and exchange is typically required.^{13,14}

Unfortunately, early detection and monitoring of implant-associated infection is challenging. Traditional biochemical analyses of infection diagnosis rely on systemic inflammatory markers including C-reactive protein (CRP) levels, erythrocyte sedimentation rate (ESR), and leukocyte counts.^{15,16} In addition, antimicrobial proteins and pro-inflammatory cytokines such as human β -defensins, procalcitonin, and various interleukins are associated with infection.¹⁵ However, blood draws are required, sensitivity is reduced with antibiotic use, and the measures are not specific to a given implant or infection source. When a joint infection is suspected, physicians often aspirate synovial fluid (arthrocentesis) and analyze it for white blood cell count, pH or lactic acid, glucose, CRP, and other assays.¹⁶ However, arthrocentesis requires training, is painful, and carries a small risk of introducing bacteria from the skin.^{15,16} Although ultimately the presence and effects of implant-associated infection may be evident radiographically via bony erosion, implant loosening, and/or sinus tracts (with such bone loss in part driven by low pH), these features are rarely present at early stages and are difficult to quantify objectively to track infection. Bone scintigraphy and positron emission tomography (PET) can be used to detect local infection and inflammation, but require radiolabeled diagnostic agents and expensive imaging equipment.^{8,17} These techniques are typically used as confirmatory tests given a high initial index of suspicion. In short, identifying the presence of bacteria on implants remains a pressing medical need.

Recent studies have found that tissues and other environment near an implant surface with bacterial colonization is acidic in the range of pH 4 – 7 depending on location and other various factors.^{6,18–20} These acidic regions play a critical role in biofilm resistance to antimicrobials because low pH affects antibiotic chemistry, host innate immune response, and bacterial metabolism.^{21,22} Measuring and tracking local pH changes *in vivo* could thus potentially be used as a proxy for infection. The challenge then becomes analyzing pH levels noninvasively. PET can indirectly estimate tissue pH from the accumulation of ¹¹C–dimethylloxazolidinedione in acidic regions.²³ However, the technique is not surface specific and lacks both resolution for this application. Magnetic resonance imaging (MRI) can also determine pH using ³¹P or ¹³C-carbonate contrast agents, although the resolution and sensitivity are similarly poor.²³ Further, MRI is expensive, cannot be used in the presence of ferric implants, and is susceptible to the effects of artifacts from the screws, plates, or other metallic devices at the site of bony injury.¹⁷ At initial stages (first 3 weeks) of implant infection bacteria are often localized near the hardware surfaces, which increases the difficulty of detection using PET, MRI, computed tomography (CT), ultrasonography and other imaging modalities or with microscopy or microelectrodes.^{8,17,19}

In summary, the literature indicates that the currently available diagnostics lack sufficient sensitivity, specificity, and simplicity for early and effective noninvasive detection of microenvironmental changes around implant surfaces.^{8,17,19,23} Numerous detection methods have been reported to measure pH in tissue during infection using PET, MRI, upconversion luminescence, and electrochemical implants for chronic wounds,^{8,17,19,23,24} however, to the best of our knowledge, there have been no articles describing an implanted chemical sensor that can be read with radiography. Below, we describe a passive implantable radiographic

sensor to measure local pH near implanted medical devices using pH-dependent hydrogel swelling to control the position of a radiopaque pin with respect to a fixed scale. We previously reported on a mechanical sensor with similar pin-and-scale readout to measure orthopedic implant bending under load to track fracture healing.²⁵ The sensor described here provides complementary local chemical information relevant for monitoring implant-associated infections.

Experimental Section:

This section briefly summarizes the synthesis and characterization techniques; a detailed description is provided in Electronic Supporting Information (ESI).

Hydrogel disk fabrication

A pH sensing hydrogel was synthesized via photopolymerization, as shown schematically in Figure 1. The following monomers were combined and dissolved in N, N- dimethyl formamide (DMF) solvent: 10 wt% acrylic acid, 5 wt% n-octyl acrylate (n-OA) 1 wt% along with poly(ethylene glycol) diacrylate, average M_n 700 (PEGDA700) as a chemical crosslinker. A UV initiator, 2-oxoglutaric acid (OGAc) (0.1 wt%) was added to this solution to polymerize and crosslink the monomers under 365 nm UV irradiation for 6 hr. The reaction was carried out in a reaction vessel consisting of two flat glass plates separated by a 500 μ m silicone spacer in a nitrogen-filled glovebox. The resulting poly(AAc-co-n-OA) hydrogel film was immersed in 70% ethanol to remove the residual monomers, DMF and hydrate the hydrogel. The hydrogel was washed daily for at least 5 days to ensure removal of unreacted monomers and initiators in the hydrogel film and transferred to pH 7.4 phosphate buffered saline (PBS). A metallic circular hole punch was used to cut the hydrogel film into disks. A radiopaque tungsten rod 0.26 mm in diameter and 4 mm was then attached to the hydrogel disks to allow the pH-dependent disk diameter to be measured via radiography.

Hydrogel sensor size vs. pH calibration curve

To generate a calibration curve, a hydrogel disk (initially 13 mm in diameter in pH 7.4 PBS) was fully immersed in a series of pH buffers ranging from 2 to 11 at room temperature (25 °C) and its diameter measured photographically using the ImageJ software package. To exchange buffer, the disk was removed from its buffer, rinsed three times with the new buffer, and left in 25 mL of the same new buffer for > 6 hours, sufficient time to establish equilibrium and stabilize the disk's diameter. The hydrogel diameter was also measured at 40 °C and compared to the room-temperature data (Figure S2 in ESI).

Response in a growing bacteria culture suspension

The hydrogel sensor was removed from the PBS storage buffer and sterilized by rinsing with 70 % ethanol and irradiating with UV light (254 nm, 125 μ W/cm²) in a biosafety cabinet for 60 minutes. The hydrogel was immersed in sterile PBS and then gently transferred to a culture plate containing 2.5 mL sterile tryptic soy buffer (TSB). Next, 100 μ L of *S. aureus* (approximately 10³ cells) was added to the TSB, shaken softly, and incubated at 37 °C. Every hour thereafter, the hydrogel was photographed to measure its diameter (d_t), and the bacterial culture pH was measured for 14 hr. A pocket pH electrode (model:H135 minilab,

HACH, Loveland, CO) was used to measure cultures and control media pH. A similar hydrogel disk maintained in sterile conditions acted as a negative control.

X-ray imaging of sensor on a plated cadaveric tibia

A cadaveric tibia was obtained via the Hawkins Foundation (Greenville SC) from Restore Life USA (Elizabethton TN), a nonprofit donation program, and the donor had consented to use their body for medical education and research in accordance with the Uniform Anatomic Gift Act. A surgeon attached an orthopedic plate to the tibia, and then mounted the pH sensing device to the plate. The pH sensing device was a stainless steel well with a hydrogel disk secured to the well using a pin press-fitted to the base of the well. The disk diameter was determined by radiographically measuring the position of the radiopaque tungsten rod attached to the disk edge. Measurements were acquired approximately every five minutes, while the pH buffer was cycled from pH 7.4 PBS to pH 4.0 buffer and back. Each time the buffer was changed, the hydrogel was rinsed with the buffer solution three times. In a subsequent experiment with the same cadaveric tibia, after the hydrogel diameter had stabilized, it was rinsed another three times with the same buffer to ensure complete exchange.

Results and Discussion:

pH-responsive hydrogels

Hydrogels are water-swollen biomaterials which can be made to expand or contract in response to various stimuli.²⁶ They are used in chemically responsive drug delivery, artificial muscles, and chemical sensors based on cantilevers, photonic band gaps, and magnetoelastic or electrical readout.^{27–29} A pH-sensing implantable device requires a biocompatible polymer material that responds within the physiologic range of pH about 4 – 7.5. A polyacrylic acid (polyAAc)-based hydrogel was selected for its biocompatibility and pH-responsive properties.^{29–32} PolyAAc coatings have been previously studied for use in preventing corrosion on titanium and other metallic implants.³³ PolyAAc is also commonly used in various household and personal care products including diapers, toothpaste, mascara, aftershave, and hand sanitizer.³² The detailed hydrogel synthesis protocol is described in ESI experimental section and shown schematically in Figure 1. The carboxylic acid (–COOH) groups in the polyAAc chains of the network are neutral at low pH and become negatively charged carboxylates (–COO[–]) when deprotonated at high pH. This increased ionization in more alkaline environments causes the hydrogel to swell due to a combination of increased electrostatic repulsion between bound charges on the polymer chains and increased osmotic pressure.³⁴

A stainless-steel well was fabricated to hold the hydrogel and buffer solution (Figure 2). This well and hydrogel sensor was then mounted to an orthopedic plate to measure pH near the plate surface. An array of holes was machined in the floor of the well (and sealed with paraffin wax) to serve as a radiolucent scale against which the position of the radiopaque indicator pin (attached to the gel) could be read. Changing the solution in the well from pH 7.4 to 4.0 buffer caused the hydrogel to contract by approximately 50%, corresponding to

indicator pin movement from hole 12 to hole 7 (Figure 2). This demonstrated that pH changes could be read radiographically using the indicator position.

Calibration and response rate

To establish a pH calibration curve, the hydrogel was immersed in a series of standard pH buffers and its diameter measured photographically after equilibrating (> 6 hours) (Figure 3). The calibration curve was fitted to the Henderson-Hasselbalch equation with the degree of swelling assumed to be proportional to the fraction of negatively charged (deprotonated) carboxyl groups, α .^{35,36} For a pure polyAAc hydrogel, the pK_a was found to be 4.19 (Figure S1 in ESI) with a dynamic range of pH 3–5, similar to the previously reported system.³⁷ To perform appropriately as a bio-sensor however, the pK_a of the hydrogel should closely match the physiological pH range found within infected and normal tissue, which is between about pH 4.5 – 7.4.^{6,19,18} Hence, a hydrophobic monomer, n-octyl acrylate (n-OA), was added to the hydrogel synthesis solution to stabilize the collapsed form of the hydrogel, shifting the swelling transition to more alkaline pH as well as broadening the transition.³¹ In addition, PEGDA700 was introduced as a cross-linker, which increased the maximum expansion ratio.³⁸ The resulting calibration curve fit well to a modified Henderson-Hasselbalch equation,³⁶ with a prefactor n added to account for the distribution of dissociation constants (Equations 1 and 2).

$$pH = pK_a + n \log \frac{\alpha}{1 - \alpha} \quad \text{Equation 1}$$

$$d_e = \frac{d_{\max} \times 10^{(pH - pK_a)/n} + d_{\min}}{1 + 10^{(pH - pK_a)/n}} \quad \text{Equation 2}$$

The least squares regression parameters were $pK_a = 5.50$ and $n = 2.36$. Thus, adding n-OA monomer and PEGDA700 cross-linker increased the average pK_a by 1.3 units and broadened the distribution by a factor of about 2.4. This modified the sensor's dynamic range to between about pH 4 and 8, encompassing the pH range typically seen in infected and healthy tissues.

Next, we investigated how temperature, ionic strength, and more complex protein solutions affect hydrogel response. The initial hydrogel calibration was performed in 100 mM standard pH buffers at 25 °C; the calibration experiment was repeated at 40 °C in more complex tryptic soy broth (TSB) with similar ionic strength to interstitial fluids. The TSB results aligned well with the initial calibration curve (Figure S2). Next, the effect of variation in salt concentrations (within the physiological range) were explored since large changes in ionic strength are known to affect hydrogel swelling by altering the osmotic pressure within the hydrogel and screening fixed charges on the polymer chains.³⁵ The sensor was immersed in TSB buffer with various NaCl concentrations, and the pH was adjusted by adding 0.1 N HCl dropwise. *In vivo* sodium blood levels are tightly regulated (normally 140 ± 5 mM).

Concentrations below 120 mM or above 160 mM can cause seizures, coma, and even death.³⁹ Although large changes in NaCl concentration (e.g., 17 mM) significantly affected the hydrogel disk diameter, variation between 120 mM and 160 mM had only a small affect (~0.2 pH units), suggesting that solute concentrations within the physiologically normal range would negligibly impact the pH calibration curve (Figure S3).

To assess the sensor response time and reversibility, the hydrogel was exposed alternately to pH 4.0 and pH 7.4 buffers every 100 minutes and the hydrogel diameter was measured after equilibration (Figures 3b and ESI S4). Since the sensor action requires proton diffusion into the hydrogel, the response is slower for thicker hydrogels, and this theoretically scales with the square of the minimum dimension of the hydrogel.⁴⁰ Sensors have been reported with sub-second response times using thin hydrogels in quartz crystal balance setups.²⁹ In our case, the overall swelling time constant for the hydrogel film decreased when the initial hydrogel thickness in PBS was reduced from $h_i \approx 1.0$ mm (approximately 25 minutes response time) to $h_i \approx 0.7$ mm (about 18 minutes response time), see Table ST1 in ESI. For our application, the expected physiological pH changes induced by bacteria and the ensuing inflammatory response usually occurs over the course of hours or days,⁴¹ thus the 1.0 mm thick hydrogel was deemed to respond rapidly enough and was mechanically robust for handling.

The sensor response was fully reversible (Figure 3b). Initially, the hydrogel disk was submerged in pH 7.4 buffer and its equilibrium diameter, d_e in pH 7.4, was approximately 13 mm. When the hydrogel was submerged in pH 4.0 buffer and enough time passed for equilibrium to be reestablished, its diameter was approximately 6.5 mm. When the sensor was again immersed in pH 7.4 buffer, its final diameter was restored to 13 mm. The entire process was repeated several times with the same results.

Response in bacterial culture

The hydrogel response was recorded over time in a *S. aureus* suspension cultivated in TSB at 37 °C. The hydrogel diameter was measured photographically (Figure S5 in ESI) every hour for 14–15 hours, while a pH electrode simultaneously measured the medium acidity (Figure 4). Acidification results from bacterial metabolic byproducts including carbonic and lactic acid.^{23,41} In our experiment, the culture pH decreased sharply 4 hours after inoculation, reaching steady state after approximately 10 hours. The initial TSB media pH was 7.2, and the initial hydrogel diameter was approximately 13 mm; fourteen hours later, the pH decreased to 4.8 – 4.9, and the hydrogel diameter (d_t) decreased about 40% to 8.5 mm (Figures 4a and 4b). We also repeated the experiment with a lower inoculated cell concentration and an additional three controls. The shape of the pH/time and diameter/time curve was essentially the same, although with a ~90-minute delay in acidification. Next, the diameter was plotted against microelectrode pH, and this curve matched well with the calibration curve from the original (set 1) standard buffers (Figure 4c). The pooled root-mean-square (RMS) difference between pH electrode readings and the pH estimated from hydrogel diameter was 0.21 pH units (5 different gels/experiments with a total of 73 data points). Similarly, for the pooled data including an additional 4 experiments performed with different batches at different temperature (ESI Figure S2), NaCl concentration (ESI Figure

S3), and standard pH buffers validation (set 2) by using gels left in PBS buffer for 6 months prior to use (Figure 4c) the RMS difference from the original calibration was 0.24 pH units (total 9 experiments, 94 data points in between pH 4 and 9). Finally, the hydrogel was rinsed in PBS and incubated in fresh TSB (pH 7.2) returning to its initial diameter (~13 mm). These results indicated that complex media with various solute concentrations and changes in temperature within the physiological spectrum did not significantly affect the hydrogel's pH response and that the sensor could monitor acidosis from bacterial growth in culture.

The sensors are expected to be less prone to drift from surface changes and long-term incubation than many electrochemical sensors since the sensing mechanism is based on equilibrium hydrogel swelling volume rather than current and voltage generated from concentration gradients and redox reactions.⁴⁴ To assess sensor stability and drift in a fixed buffer at physiological pH, we removed 5 hydrogel disks from the TSB solution, washed, and placed in PBS buffer for 13 days. The solution pH was measured by pH electrode to be 7.33 on day 1 and 7.34 on day 13. The hydrogel sensors (n = 5) displayed negligible and statistically insignificant size changes over this period, < 0.2 % mm in diameter (corresponding to 0.01 pH units, see Figure S6 in ESI). We also observed essentially identical sensor response after storage in PBS buffer for 6 months (second set of standard buffers in Figure 4c).

To determine whether the sensor would be robust in chemically harsh biological environments, the hydrogels were incubated for 10 days at 37 °C in either sterile TSB media (control), *S. aureus* culture in TSB, or TSB media containing 1 mM dissolved CuSO₄ and 50 mM H₂O₂ as reactive oxygen species (ROS) generator. No hydrogel degradation was observed in any of these samples, and post-incubation sensor response was indistinguishable from the calibration fit curve calculated from standard buffers at 25 °C (Figure S7 in ESI). Similar polyacrylic acid materials have been used for to coat titanium implants³³ and electrodes implanted in rat cortex⁴² without *in vivo* degradation, thus we expect that these materials or a variant would be useful for *in vivo* application. In addition, the sensor can be modified to improve selectivity (e.g., carbon dioxide-sensors have been made by coating pH sensors with chemically inert gas permeable membranes such as PDMS)⁴³. In future, after suitable device miniaturization, we will study *in vivo* response in small animal models.

Response on a cadaver tibia and comparison with *in vitro* experiments

To explore how the sensor would perform with human anatomy, the hydrogel was mounted to an orthopedic plate fixed to a cadaveric human tibia and imaged with a mobile C-arm X-ray unit. The pH-dependent hydrogel diameter was determined radiographically by measuring the tungsten pin position relative to the radiolucent reference holes in the base of the well (Figure 5). Although the sensor was designed to be mounted over an unused screw hole (the hole nearest the fracture site is usually left unfilled), if necessary the pin position could be measured along the side of the plate or even through the 4.5 mm stainless steel tibia fracture plate using a higher X-ray tube voltage (110 kV) (Figure S8).

The pin position changed as the immersion buffer was alternated between pH 7.4 and 4.0 (Figure 5). In Figure 5d, the pin position was measured using a coarse gradation (hole number on the radiolucent scale) while Figure 5e shows position more precisely analyzed

using the Measure tool on NIH ImageJ software (National Institutes of Health, Bethesda, MD). Both plots demonstrate a reversible response with a timescale similar to the *in vitro* experiments.

Although the response curve was similar in both the *in vitro* and *ex vivo* experiments, surprisingly the indicator pin did not move as much in the cadaver study as in the *in vitro* study. When switching between pH 7.4 and 4.0 buffers, the pin moved about 2.8 mm in the cadaver study (Figure 5) versus a 6.7 mm hydrogel diameter change *in vitro* (Figure 3). Part of this discrepancy arose because the hydrogel was attached to the well with another pin such that only about $\frac{3}{4}$ of the hydrogel diameter lay between the attaching and indicating pins. Correcting for this effect would bring the cadaver experiment response to 3.7 mm (still low compared to the *in vitro* response). Although the same buffers were used, a key difference between the experiments was that a large petri dish was used for the *in vitro* experiments while a relatively small fluid well held the hydrogel and buffer in the cadaver study. Specifically, the swollen hydrogel (at pH 7.4) occupied about a third of the buffer volume within the well in the cadaver study, compared to only 0.6% of the buffer volume in the petri dish. The hydrogel requires about 30 minutes to respond (Figures 3b and 5), thus, changing the buffer and rinsing immediately with new buffer would have exchanged fluid in the well and hydrogel periphery, but not within its core.

We hypothesized that after 100 minutes the sensors had equilibrated with the immersion solution and were properly reporting local pH in both experiments, but in the cadaver studies with limited external buffer volume this local pH was elevated because the acrylic acid groups and solution within the hydrogel had partially neutralized the added buffer. To test this hypothesis, a similar experiment was conducted using a Sawbones® composite bone mimic and older veterinary X-ray system. This time, the final pH was measured after the hydrogel had reached equilibrium to determine the actual pH change, additional rinses performed to ensure equilibration, (Figure S9 in ESI) and the sensor response to the actual pH change was observed. The hydrogel began in PBS buffer that measured pH 7.2 after incubation, with the pin positioned at hole 11, identical to the cadaver experiment. After switching to pH 4.0 buffer (rinsing the hydrogel three successive times and waiting 100 minutes for equilibration), the hydrogel contracted, moving the pin to just above hole 4, similar to the cadaver experiment (just above hole 5). At this point, the pH in the well was found to be approximately 4.7 rather than 4.0, confirming our hypothesis that the local pH was elevated compared to the buffer. The hydrogel was then rinsed with pH 4.0 buffer three additional times, and the hydrogel contracted further, moving the pin by an additional two holes (1 mm); the indicator finally rested in a position consistent with the calibration curve at pH 4.0. After replacing the acidic buffer with slightly basic pH 7.4 buffer, the indicator pin returned to position 11, and the pH measured 7.2; rinsing three additional times with the same buffer increased the pH to 7.4 and moved the pin by one more hole (0.5 mm). The observed contraction in the Sawbones® experiment without additional equilibration (pH 4.7 to 7.2) was reasonably consistent with the cadaver results (3.25 mm and 2.8 mm, respectively), with small differences probably arising from variation in the rinsing process. The total pin motion from an equilibrated pH 4.0 to 7.4 was 10 holes (5 mm), consistent with the swelling observed in the petri dish (after accounting for the fact that $\frac{3}{4}$ of the hydrogel was between the fastening and indicating pins). Overall, these results demonstrated

that the hydrogel responded appropriately to local pH equilibrium conditions. Results also suggested that the hydrogel should be immersed in a buffer volume many times larger than the swollen hydrogel volume to prevent the gel from affecting the measurement. However, this would be less concerning *in vivo* where the system is large and the hydrogel is continually immersed. In addition, although the cadaver and Sawbones® experiments were performed with different X-ray equipment, the reference scale made the results directly comparable.

Results from the cadaver and Sawbones® experiments demonstrated that small pin displacements could be measured radiographically, providing high pH-resolution within the considered physiologic range. A survey was conducted to investigate interobserver variation in determining pin position. Images from the cadaver experiment were randomized and sent to eight graduate students who received instructions on how to count the pin position but no further training. The observers were asked to estimate the indicator position relative to the internal scale. The intraclass correlation coefficient (ICC) was found to be 0.993 (95% CI: 0.983–0.998). The mean standard deviation (MSD) for each reading was found to be 0.19 holes (range 0.09–0.31), corresponding to 100 μm . The interobserver agreement could probably be further improved with practice and by adding labels to the positions to reduce counting errors. The overall displacement of the indicator pin moving from pH 4.0 to 7.4 was approximately ten holes after equilibration in the Sawbones® model, corresponding to three holes or 1.5 mm/pH unit. Therefore, the MSD in the indicator position corresponded to a standard deviation of approximately 0.065 pH units (0.19 holes $\times$ 3.4 pH/10 holes). In addition to uncertainty introduced when reading the radiographs, pH accuracy could also be limited by calibration fitting, variations in the hydrogel fabrication, aberrancy in machining the internal scale positions, and attachment of the indicator components. Nonetheless, given all data considered within the range of physiologic solute concentrations (Figure 4c, and Figures S2, S3, and S5 in ESI), using multiple sensors at different times and various conditions, the root-mean-square deviation between the pooled experimental pH values and standard buffer calibration curve was 0.24 pH units.

Limitations

While plain radiography is attractive because it is widely used in diagnostic evaluation and patient follow-up, the approach has limitations in how and when measurements can be taken and imposes practical limitations on sensor design. First, plain radiography requires an X-ray source, which generally limits measurements to discrete time points and makes continuous measurement unfeasible in live patients. Second, practical considerations limit the sensor dimensions: the length should generally be at least 1 mm so that swelling of nearly 10% is easily visible as 100 μm displacement (although smaller sizes may be possible with centroid fitting, moving the X-ray source to enlarge the image, and rotational or moiré pattern schemes may help highlight smaller changes). Similarly, the indicator should generally be at least 100 μm thick so that it is clearly visible against the background using typical hospital X-ray machines (especially when it is placed near thick bone and orthopedic hardware). Third, a scale is needed to measure the sensor position; if the scale is not in the same plane as the pin, parallax error will shift the apparent position of the pin by an amount $z \times \tan(\theta)$, where z is the distance from the plane of the pin and θ is the

displacement angle. Generally, radiographs can be taken within 20° of normal, causing at most 72 μm if $z = 200 \mu\text{m}$. Fourth, a physical barrier should protect the sensor from direct application of force. We have found that a thin stainless-steel mesh can protect the sample without significantly affecting the images and other biocompatible metals or fabric materials could also potentially be used. Fifth, risk of cancer from ionizing radiation must be balanced against benefit, especially in pregnant patients and children or where many measurements would be taken. Radiographs are already routinely acquired as part of standard patient work-up, and the chemical measurement could be made from those images with no additional X-ray exposure. If radiographs are needed specifically to measure local pH, the additional effective full body dose for imaging an extremity would be around 3 μSv ⁴⁵ which would cause an increased lifetime risk of cancer death of 0.15 patients per million, assuming the linear no-threshold dose dependence and average patient age.⁴⁶ This could be compared to significant and imminent morbidity and mortality from implant-associated infections, especially in immune-compromised patients. In principle, similar sensors could also be read using medical imaging modalities that do not use ionizing radiation such as ultrasound, MRI, or magnetic particle imaging (MPI), and future work could explore these options. Nonetheless, plain radiography is the standard of care for orthopedic follow-up and many diagnoses and will continue to be for the foreseeable future. In general, some form of imaging is indispensably required to follow patients *in vivo*. Fundamentally, our sensor adds functional information to imaging that is already being acquired.

Outlook

The constructed sensor attaches to orthopedic hardware and provides a simple passive pH readout using conventional X-ray imaging that is routinely acquired for assessing bone healing or pathology. The presented device does not represent a marketable implant, rather a proof-of-concept for the first X-ray readable biochemical sensor. While the prototype device was too large for some applications (e.g., small plates or within screws), it fits well on a tibial fixation plate and was able to measure pH in a cadaveric tibial model. The additional surface area introduced by the sensor is small compared to the surface area of the plate, and unlikely to appreciably increase risk of infection. Future work will involve developing other analyte-responsive hydrogels, combining chemical sensing with drug eluting devices, and reduction of device dimensions so that multiple sensors could be applied simultaneously at several positions along the implanted hardware. Device miniaturization will also enable conceptions such as using injectable sensors to track bioanalytes change in soft tissues. Ultimately, this mechanized hydrogel approach could be used as a general tool for many biomedical applications including noninvasive examinations of bacterial growth *in vivo*, the expansion and inflammatory response of tumors or other lesions, and the diffusion and activity of neurotransmitters, thus functionalizing radiography.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

A) Polymerization of monomers and cross-linker in presence of UV initiator in inert nitrogen glovebox. **B)** The resulting hydrogel film is cut with a hole punch and stored in PBS.

Figure 2:

In basic solution (Left), the carboxylic acid groups in the hydrogel are mostly deprotonated and the hydrogel is swollen; in an acidic solution (Right), the polymer anionic carboxylic acid groups protonate and the hydrogel contracts. This pH-dependent swelling regulates the position of a radiopaque indicator pin attached to the hydrogel. pH is determined by measuring pin position relative to a radiopaque scale (holes machined in the base of the well that holds the hydrogel and attaches to an orthopedic plate).

Figure 3:

A) Equilibrium hydrogel diameter in standard pH buffers, pH 2 to 11. Inset photos show the hydrogel at pH 5 and 9, respectively. Regression is made to a modified Henderson-Hasselbalch equation (Equation 2). **B)** Cyclic hydrogel deswelling (contraction) in pH 4.0 buffer and swelling (expansion) in pH 7.4 buffer. Line shows exponential fit with time constant, τ .

Figure 4:

A) pH time profile in a *Staph. aureus* culture measured with electrochemical pH electrode; **B)** Corresponding changes in hydrogel diameter with time. **C)** Hydrogel diameter vs. pH for hydrogels in *Staph. aureus* cultures and standard pH buffers; the regression curve is a fit to original (set 1) standard pH buffers, also shown in Figure 3a. Set 2 standards were a repeat with sensors stored in PBS buffer for 6 months prior to use. In the figure legends, s1, s2, and c0 represents 2 cultures and 1 control performed at the same time; b1, b2, b3 and c1, c2, c3 represent 3 cultures and 3 controls performed together at a later time with lower initial bacterial concentration.

Figure 5:

A) Photo of the sensor on a plated cadaver tibia. The hydrogel disk (in pH $\approx$ 7.4 buffer) contains a radiopaque indicator pin (arrow). **B)** Radiographs showing pin and scale before and after pH 4.0 buffer addition, respectively (time elapsed since the experiment began is indicated). **C)** Radiographs before and after pH 7.4 buffer addition. **D)** Indicator pin position versus time elapsed as determined by eye (hole-number scale). **E)** Indicator pin position as more precisely measured using ImageJ software. The horizontal and vertical scale bars in the radiographs are 20 mm and 5 mm, respectively.