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Original Article

Ultrasound Triggered Drug Release from Affinity-Based β-Cyclodextrin Polymers for Infection Control

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Abstract—This work demonstrates a slow, sustained drug delivery system that provides on-demand delivery bursts through the application of pulsed therapeutic ultrasound (TUS). Insoluble β-cyclodextrin-polymer (pCD) disks were loaded with a saturated antibiotic solution of rifampicin (RIF) and used for drug delivery studies. To obtain on-demand release from the implants, TUS was applied at an intensity of 1.8 W/cm^2. The therapeutic efficacy of the combination treatment was assessed in bacterial culture via an *in vitro Staphylococcus aureus* bioluminescence assay. The results demonstrated that the application of pulsed TUS at 3 MHz and 1.8 W/cm^2 to pCD implants leads to a significantly higher short-term burst in the drug release rate compared to samples not treated with TUS. The addition of TUS increased the drug release by 100% within 4 days. The pCD disk + RIF stimulated with TUS showed a comparatively higher bacterial eradication with CFU/mL of 4.277E + 09, and 8.00E + 08 at 1 and 24 h compared with control treated bacteria at 1.48E + 10. Overall, these results suggest that the addition of pulsed TUS could be an effective technology to noninvasively expedite antibiotic release on demand at desired intervals.

Keywords—Surgical infection, Therapeutic ultrasound, Affinity-based drug release, Cyclodextrin polymer, Rifampicin.

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INTRODUCTION

Despite regulations in place to ensure sterilization of operation room environments, as well as the use of antimicrobial prophylaxis[3,19] there is still a significant risk of post-surgical infections for both doctors and patients.[12] Implant-associated bacterial infections can be introduced by improper cleaning of skin during implantation. Infections may also arise due to improper handling methods during prosthetic implantation which results in abnormal bacterial populations in the wound area. Other risk factors arise from the patients' individual preoperative conditions such as: diabetes mellitus, obesity, malnutrition, progressive age, rheumatoid arthritis, suboptimal antibiotic prophylaxis, smoking and iatrogenic immunosuppression,[30] elevating the risk and challenge of infection. Regardless of their origins, all postoperative infections have the potential to be catastrophic.

One example, orthopedic implant infection, presents a crucial complication that arises in the majority of patients presenting with infection. These infections, if not properly addressed, can eventually result in the development of osteomyelitis and eventually implant failure due to chronic infection. Staphylococcal species have the highest infection rate following these procedures[6,8,10] with *S. aureus* being the most commonly associated with implant infections, followed by *S. epidermidis*.[33] To correct for these complications, revision surgeries often become necessary.[4,26] In addition, the probability of infection arising after an infection-associated revision surgery can be as much as twice that of the probability in either the primary knee

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or hip implant surgery.[25] Once infected, bacteria can form a biofilm, making it very difficult to remove them, since they have multiple mechanisms making them less sensitive to systemic antibiotics.[7] In conjunction with this resistance, infections in orthopedic implants are also challenging due to slow immune and inflammatory defense responses. This is due to the lack of vascularization present in the tissues located at the implant surface.

Between 2012 and 2015, American Joint Replacement Registry (AJRR) reported that in the United States, 169,060 hip arthroplasty procedures were performed, 17,180 of which resulted in revision surgery. The same study reported 258,121 knee arthroplasty procedures were performed, 22,403 of which required revision surgery. Consequently, this increased the revision surgical burden in patients by 10.2 and 8.7% for hip and knee respectively.[22,32] The economic burden for this revision surgery due to prosthetic joint infection has increased every year since 2007 and is projected to continue to increase for the foreseeable future. The annual cost for these procedures in the U.S. alone is over $500 million and is anticipated to reach $1.62 billion by 2030.[23]

The existing methods to overcome post-surgical infections include pre-soaking of surgical gauze grafts and other equipment used in surgery with antibiotics and irrigation with other antibiotic-containing solutions.[13,31] Other common practices include direct application of antibiotics such as vancomycin in a powder form to reduce surgical site infection (SSI) ratio during the tibia plateau, spinal deformity and fracture fixation.[28] The efficacy of these methods is limited however, as they have very narrow therapeutic windows as the antibiotics are rapidly washed out by the system. Presently, several approaches have been taken to respond to these extremely prolific infections. These methods include the development of implants coated with polymeric gels for sustained drug delivery at the surgical site.[34] More recently it has been demonstrated that affinity-based cyclodextrin-polymer (pCD) delivery systems have promising potential in the more effective inhibition of infections at surgical sites.[14] Cyclodextrins (CDs) are cyclic oligosaccharides with the D-glucopyranose units making the inner cavity slightly lipophilic and outer surface hydrophilic. This morphology allows for CDs to form reversible complexes with small molecule drugs, and allows the polymer (pCD) to present affinity binding characteristics.[5] The advantage these pCDs have over other hydrogels is that the drug release from conventional hydrogels is governed strictly by diffusion.[24] Whereas, affinity-based CDs have an additional, predictable and tunable association kinetics with the loaded drugs, and thus work by delaying the release of the guest antibiotic beyond that of diffusion alone.[21]

In other settings, the application of pulsed therapeutic ultrasound (TUS) at a range of frequencies from kHZ to 3 MHz and low intensities typically < 3 W/cm^2[35] has been widely shown to elicit biological effects in tissues and provide external control over drug release over time, effectively allowing for the on-demand and localized delivery of drugs.[11,18] The combination of ultrasound application with antibiotics entrapped by CD polymer implants has the potential to enhance both the permeability and uptake of the drug within the tissue following on-demand triggered release.[1,16] However, no work has previously investigated the application of TUS on macro-scale pCD delivery devices in this context. Prior studies have examined the effects of focused ultrasound- heating system on a cyclodextrin-based nanocarrier with the rise in temperature up to 43 °C (± 2 °C STDEV) to increase in cellular uptake of doxorubicin by 9.6 factor.[15]

In this work we examined whether the addition of TUS at relatively low intensity to avoid heating, can effectively increase the release of the antibiotic rifampicin (RIF) from cross-linked β-CD polymer implants. We hypothesized that the triggered drug release will instead be stimulated via a primarily mechanical effect, allowing the on-demand triggered burst release over time.

MATERIALS AND METHODS

Materials

A lightly epichlorohydrin-cross linked β-CD pre-polymer (β-CD, 2-15 kDa, average 10 CDs per chain) was purchased from CycloLab Ltd (Hungary). N, N-Dimethylformamide (DFM, extradry) was purchased from Applied Biosystems (FosterCity, CA). 1,6-Diisocyanatohexane (HDI) (Aldrich, St. Louis, MO), and rifampicin (RIF) from Research Products International (Mt. Prospect, IL) were used as received. Therapeutic ultrasound unit (Sonicator 740X therapeutic ultrasound system), thermocouple probe with k calibration 36-gauge wire (Evolution Sensors and Controls, LLC) were used. All the other reagents, solvent, and chemicals used in the research were purchased from fisher Scientific in the highest grade available.

Synthesis of Polymerized β-Cyclodextrin

For the synthesis pCDs, 1 g of pCD pre-polymer was placed in a beaker and heated for 24 h at 70 °C. Then the pCD's were transferred to a 20 mL sample vial and added to a 3 mL dry DMF and dissolved at

room temperature by using a stir plate without heating. After this, 288 μL (10:32) of HDI crosslinker was added dropwise and homogeneously mixed using vertex for 2 min. Finally, the resulting polymers were transferred to a 6 cm Teflon dish which was Parafilmed closed and kept in a fume hood for 4–5 days. From these sheets, pCD disks with an 8 mm diameter were punched using a surgical disk punch.

Affinity Drug Loading in β-Cyclodextrin-Polymer Disk

The pCD disks were pre-weighed and loaded with 0.05 g of RIF dissolved in 1.2 mL of DMF. The disks were then placed in the vertical shaker for 72 h at room temperature. They were then removed from the loading sample and dried in a fume hood for 24 h. The total amount of drug-loaded in the disks was calculated using the formula below.

$$\text{Wt. of drug laded} = \text{wt. CDs before drug loading} - \text{wt. CDs after loading drug}$$

The percentage release from each disk was measured using the formula below.

$$\%\ \text{Drug (release)} = \frac{\text{wt. } D \text{ released}}{\text{wt. total } D \text{ loaded}} \times 100\%$$

Each pC disk consists of a network of crosslinked cyclodextrin molecules which have a hydrophobic pocket, and hydrophilic outer shell, allowing pCD to act as a host-guest complex and in our experiment the drug binds in inner core of each CD. Prior publications have compared affinity to non-affinity materials for release of antibiotics and other drugs (e.g. NMR, DSC, DMA, TGA, XRD, and FTIR).[9]

Therapeutic Ultrasound Treatment

Implant disks were exposed to the TUS using a commercial therapeutic ultrasound unit (Sonicator 740X therapeutic ultrasound Mettler Electronics Corp., Anaheim, CA) with a duty cycle of 20%, 1.8 W/cm^2 intensity, and a frequency of 3 MHz with a 5 min application time. We have used similar parameters previously in studies focusing on ultrasound-mediated drug delivery with in situ forming implants.[20] The footprint of the transducer fit into individual wells of a 12-well cell culture plate, to normalize sonication and reduce the well size to a point where the implants would remain within the ultrasound beam throughout the sonication process. The transducer was immobilized 5 mm above the implant surface.

Pulsed TUS was applied to a 2 mL solution of PBS containing pCD disks in 8 of 12 wells of the 12-well plates, as shown in Fig. 1. The middle row remained empty to reduce off target effects of the sonication. With these parameters, the change in temperature was measured with a thermocouple probe with type-k calibration 36-gauge wire (evolution sensors and controls, LLC) placed in the PBS solution before and after the US application. The samples saw an average 3 ± 0.5 °C increase in a room temperature environment from 22 ± 0.5 °C to a final temperature of 26 ± 0.5 °C.

Drug Release Study

To measure the amount of RIF released in the presence or absence of TUS, pCD disks loaded with affinity based drug RIF were placed in a 12 well plate, with one disk in each compartment and leaving the middle row empty. A 2 mL solution of fresh phosphate buffered saline (PBS) was then added. The plate was kept in an incubated shaker at 37 °C and 100 rpm. Samples were collected every 24 h for both groups. The TUS with above mentioned parameters was applied every 24 h. Samples were collected before and after the application in the TUS. The remainder of the solution present was replaced with fresh PBS at each sampling. RIF release from the pCD hydrogel disks was measured by UV–Vis spectroscopy (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek Inst.). Periodic aliquots of solution were analyzed over a period of time for samples that received TUS and were compared to a control that received no US. The wavelength of maximum absorbance was (λ_{max}) 473 nm. The drug concentration present in the sample was determined using this data.

Bioluminescence Assay

A bioluminescence assay of Xen 36 *Staphylococcus aureus* (Caliper Life Sciences, Hopkinton, MA) testing delivery from the pCD disks was carried out to analyze the real-time *in vitro* effect of drug release with and without the application of the TUS. Here, 10 μL of concentrated Xen 36 *S. aureus* preserved in − 80 °C was mixed in multiple test-tube filled with 10 mL of culture medium which was then placed in incubator 37 °C over night. To determine the initial number of bacteria in tube, 200 μL concentrated bacteria from tube was place in 96 well plate (n = 6) for each tube sample and calculated using UV–Vis bioluminescence spectroscopy. Those tubes which had the similar concentration of bacteria were used in the bioluminescence assay. Bacteria solutions were then placed in a 12-well plate and were treated with pCD disks with or without the application of TUS, as described above.

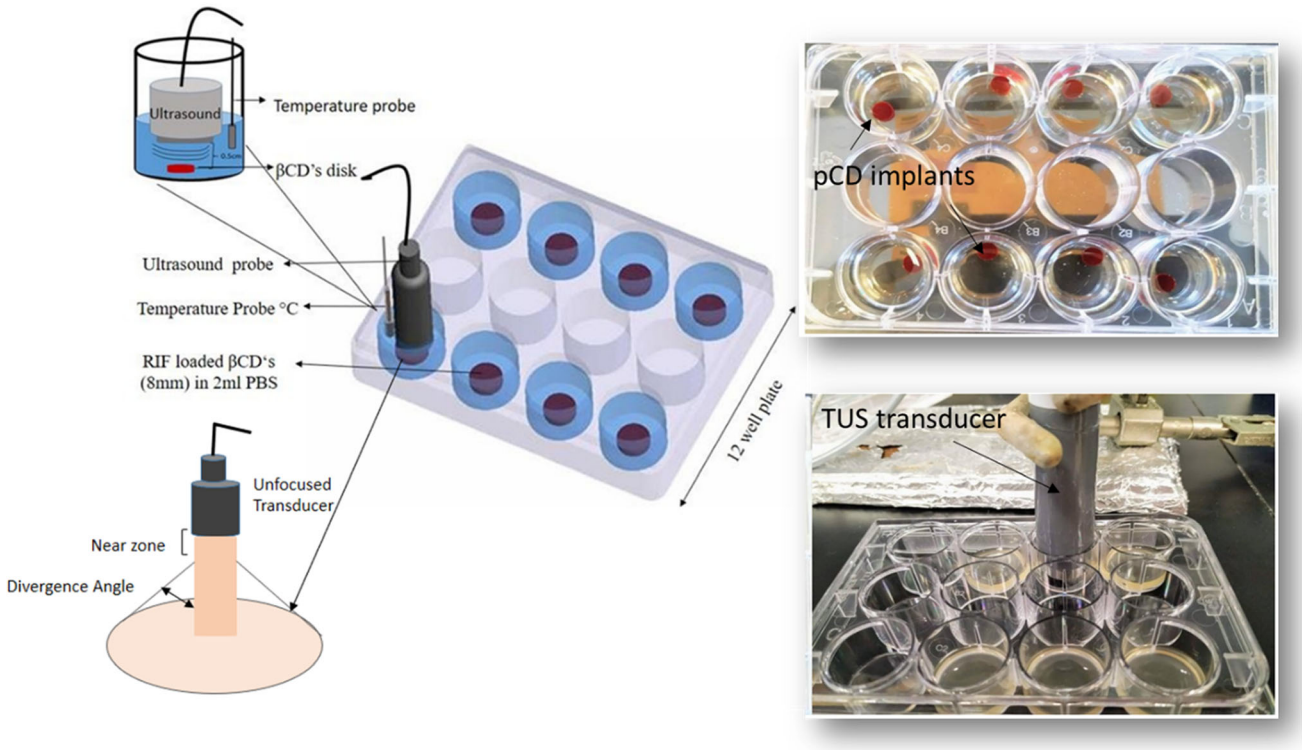

FIGURE 1. Schematic and images of experimental setup for TUS application to RIF loaded pCD disk in a single well of a 12-well tissue culture plate.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy of pCD disk encapsulating RIF with and without TUS treatment were obtained using high resolution dual-focused ion beam devices used in SEM mode. For the examination RIF loaded pCD disks were treated with TUS and air dried for 24 h and the samples were attached to a brass stub using double-sided tape and gold coating (thickness 10 nm) in vacuum by a sputter coater. The images were taken at 15 kV and 150 × and 800 × magnification.

Statistical Analysis

Experiments were performed at least in triplicate, and all data were analyzed using GraphPad Prism 7 software. Results plotted are indicative of the average cumulative amount released at each time point. Error bars represent the standard mean error.

RESULTS

Comparison Between Affinity-Based Drug Release With and Without the Application of TUS

Implant disks which were exposed to TUS treatment daily showed increased RIF release compared to samples not exposed to TUS. The RIF release with the application of TUS was on average 30–50% higher with TUS treatment than without, as shown in Fig. 2.

We also examined the ability of TUS to trigger periodic release at different time points after the initiation of the release study. Here the sample was treated with TUS separately on either day 0, 1, 2, 3 and 4 (Fig. 3) and were compared to implants without TUS exposure. The results again demonstrate that adding TUS contributes to on-demand triggered drug delivery. The effects were most pronounced on days 3 and 4, where the amount of RIF was increased on average 50% compared to control implants. However, there was an unexpectedly high release of RIF compared with other sample. The large difference in release may have been due to extra drug molecules not fully associated with the CD pockets, which released more easily due to lower affinity. The higher drug release could also be due to the variable pCD disk size.

Identifying the Effect of TUS on RIF Release Rate

To determine whether the increase in drug release from pCD disks after the application is an instantaneous or a delayed effect, pCD disks were loaded with RIF and then exposed to TUS immediately and samples taken at 15, 30, 45 min. At 60 min, one group

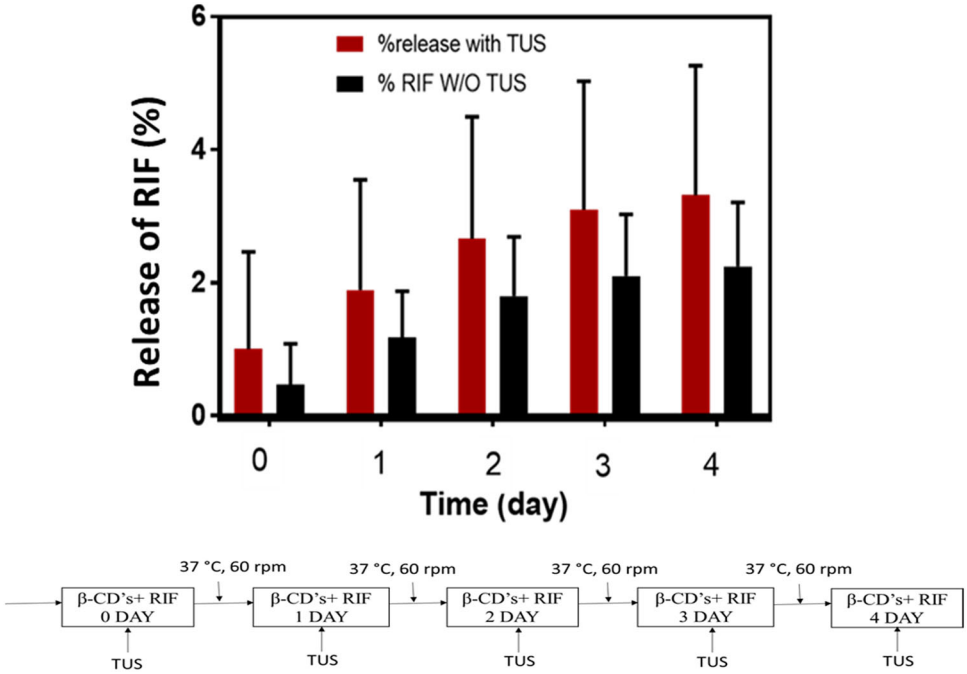

FIGURE 2. Average cumulative percent drug delivered over the course of 4 days from pCD with (red) or without (black) the application of TUS.

received an additional TUS treatment with immediate sampling following TUS exposure. As shown in Fig. 4, an immediate increase in RIF release is triggered by the TUS application. The elevated release is only seen immediately after TUS exposure. This was confirmed by re-exposing one set of implants to TUS after 60 min, which again resulted in a significant increase in RIF release. We conclude from this experiment that TUS has an acute effect on drug release from the pCD disks.

Bioluminescence Assay Using Xen 36 Staphylococcus aureus

A bioluminescence assay of Xen 36 *Staphylococcus aureus* was carried out for: pCD disks containing RIF with or without TUS ($n = 3$), as shown in Fig. 5. For each of the sample 2 mL of *Staphylococcus aureus* solution which had the concentration of bacteria 10^{10} was placed in each compartment of 12 well culture plate with RIF loaded pCD disks and kept in incubator 37 °C for 1 and 24 h. The samples were then taken out of incubator and treated with and without TUS. 200 μL of sample solution in placed in 96 well plate ($n = 6$) and result was calculated using UV–Vis bioluminescence spectroscopy. Bacteria samples treated with the combination of RIF loaded pCD disks and TUS express a greater bacterial eradication with CFU/mL of 4.27E + 09 and 8.00E + 08 for 1 and 24 h, which is 1-log and 2-log difference respectively compared with concentrated bacteria which is 1.48E + 10.

Similarly, non-RIF containing pCD disks were tested with or without the application of the TUS, as shown in Fig. 6a; this experiment was carried out to identify if the unloaded pCD's in the presence of the TUS can eradicate bacteria. Lastly, TUS alone in the absence of the pCD's + RIF, was tested (Fig. 6b); which tested whether TUS alone has effect on bacteria. This experiment suggests that, pCD's + RIF with TUS has a higher eradication rate, while non-loaded pCD's with TUS, and TUS alone do not contribute to bacterial eradication.

Scanning Electron Microscopy of β-CD's Loaded with RIF

The cross-section and surface morphology of pCD's loaded with RIF with and without the treatment of TUS were observed under different SEM magnifications 150 × (20 μm) and 800 × (100 μm) as shown in Fig. 7. pCD disks were observed to express a spherical shape and to have spherical particles evenly distributed throughout them. Figs. 7a, 7b, and 7c, shows cross-section and surface morphology of pCD's + RIF, while Figs. 7d, 7e, and 7f represents cross-section and surface morphology of pCD's + RIF + TUS. The implant morphology in both groups appears similar, suggesting that TUS exposure does not lead to structural changes of the pCD implant disks.

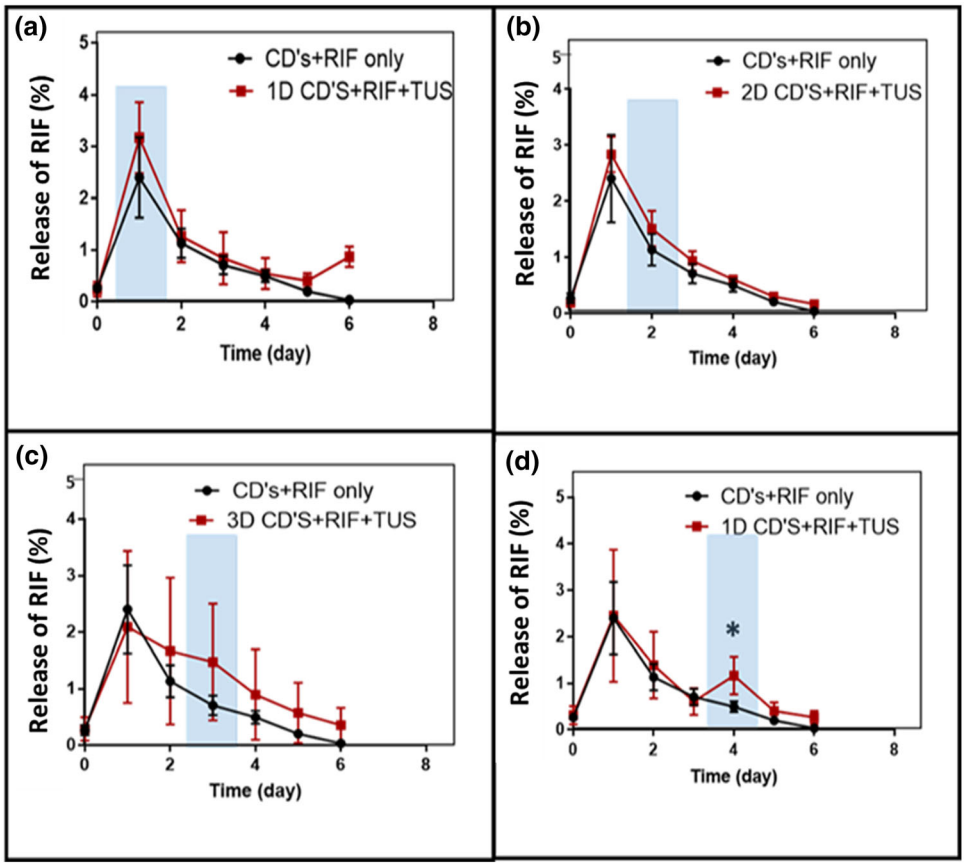

FIGURE 3. Average release of RIF from pCD's with and without the application of TUS for 7 days. The blue bar represents the day the sample was treated with TUS. (a) TUS was applied only on day 1, (b) TUS was applied only on day 2, (c) TUS was applied only on day 3, (D): TUS was applied only on day 4.

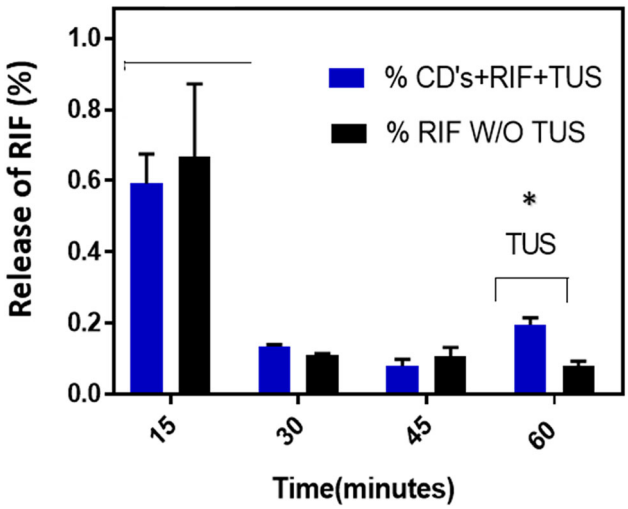

FIGURE 4. Short-term effects of TUS application on pCD implant disks. TUS was applied to both the control group and samples were collected every 15 min. TUS exposure results in an immediate increase in RIF release, which rapidly subsides at 30 and 45 min. Upon re-exposure at 60 min, an increase is seen in the TUS group but not in the control. *A significant difference (p = 0.013) was observed

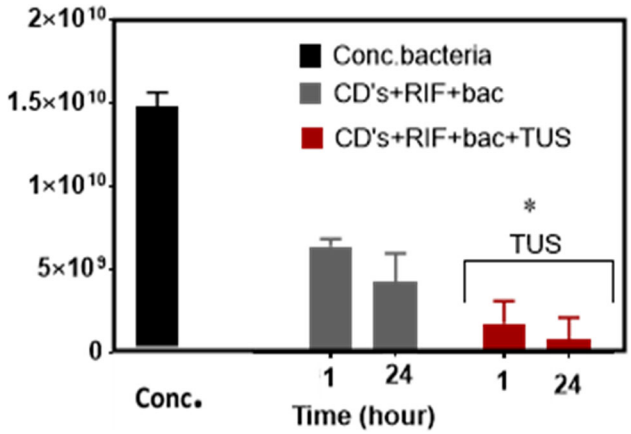

FIGURE 5. Luminescence assay of Xen 36 *S. aureus* with the pCD's + RIF with and without the TUS treatment analyzed after 1 and 24 h. (* represents statistically significant difference)

DISCUSSION

In this study we examined, for the first time, the effects of low intensity, pulsed therapeutic ultrasound on antibiotic release from β-CD polymer implants. Results show that the application of ultrasound led to

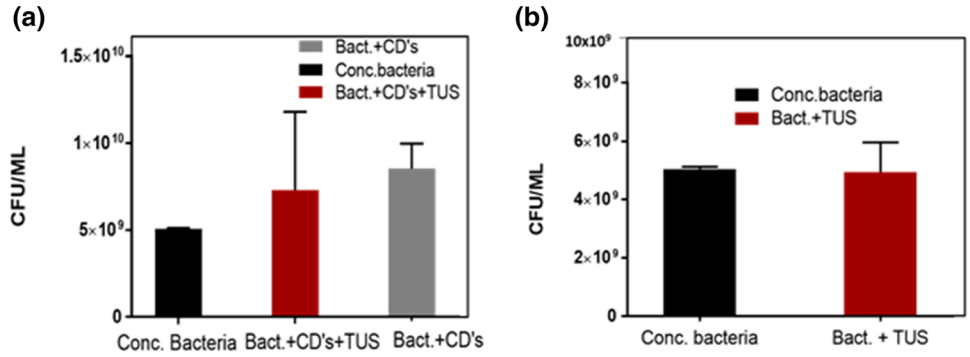

FIGURE 6. (a) *S. aureus* treated with non-loaded pCD's with and without the TUS treatment. This demonstrates that TUS alone and pCD's without RIF have no significant effect on bacterial eradication. (b) *S. aureus* were treated solely with TUS in the absence of RIF and pCD's. Treating the sample for over an hour with the application of TUS, showed there was no decrease in CFU/mL of bacteria.

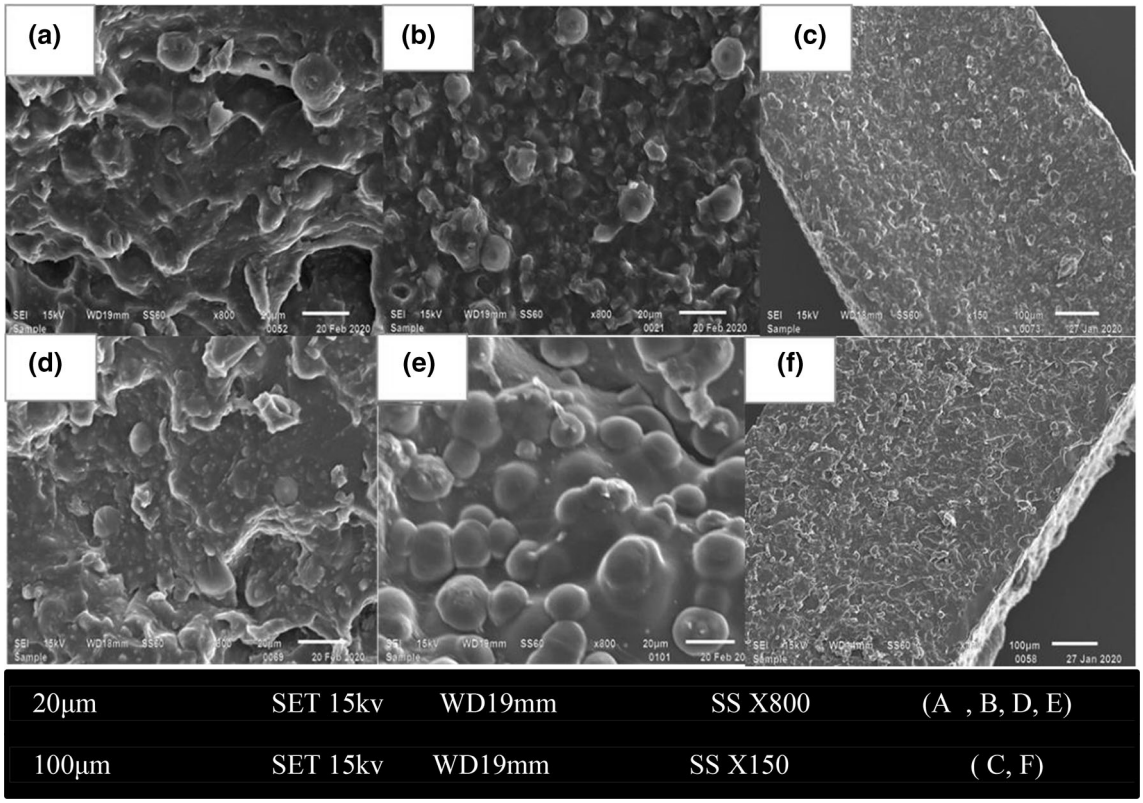

FIGURE 7. Scanning electron microscopy (SEM) images of (a) cross-section of pCD's + RIF (800X), (b) surface morphology of pCD's + RIF (800X), (c) Cross section of pCD's + RIF (150X), (d) cross-section of pCD's + RIF + TUS (800X), (e) Surface of pCD's + RIF + TUS (800X), (f) cross section of pCD's + RIF + TUS (150X).

a consistent increase in drug release compared with the samples which did not receive ultrasound application. Pulsed TUS application creates thermal and mechanical molecular instability in the system, reducing the effect of affinity between drug and polymer, allowing the drug to migrate more easily out of CD pockets, and thereafter diffuse out of the polymer similar to normal (non-affinity) diffusion polymers. The drug release could be triggered on demand at various time-points after the initiation of the drug release study (up to four days), and no morphological changes were noted in the implant microstructure. The mechanism of action of the increased RIF release is not yet established, but is likely due to domination of mechanical bioeffects

leading to the effective dislodging of RIF from β-CD pockets causing faster mobility of drug molecules to the outer implant surface. Previously published work used focused ultrasound at higher energy deposition parameters was used to elicit release from CD-based nanocarriers. In these experiments, the temperature increase was a net of 6 °C (43 °C ± 2 °C from 37 °C), and the authors concluded that a combination of thermal and mechanical effects resulted in the increased drug release. The effect of FUS was greater than the effect of hyperthermia alone. The observed 3 °C temperature change in our study is less likely to contribute to the increased drug release, especially since the surface area to volume ratio of the disks was much lower than that of the nanocarriers.

A bacterial luminescence assay with Xen 36 *S. aureus* showed that TUS applied to pCD loaded with RIF is more effective than the passive release alone, leading to a significantly higher bacterial eradication at 1 and 24 h. Bacteria samples treated solely with TUS, showed ultrasound alone gives no significant difference in CFU/mL count.

Prior work has examined bacterial treatments solely with ultrasound. For example, Sesal and Kekeç[27] used pulsed ultrasound to eradicate *S. aureus* and *Escherichia coli*, however, ultrasound alone was shown to be unsuitable for the elimination of *S. aureus*. Alneami *et al*.[2] used ultrasound stimulation (pulsed and continuous waves) *in vitro* showing bactericidal ability on *Pseudomonas aeruginosa*. Work has also shown that a combination of microbubbles with ultrasound may be an effective strategy for treating stubborn urinary tract infections.[17] However, to the best of our knowledge, no study has previously examined the combination of affinity-based polymers loaded with antibiotics and ultrasound as a treatment for infection. Prior work has examined drug release from individual cyclodextrin pockets, using kHz range ultrasound. The authors there concluded that a thermal effect (increase of temperature to 40 °C) was the primary driver of drug release.[15] In this study we successfully prepared the pCD polymer disks that are loaded with rifampicin. Past work from our lab and others shows that β-CD polymers can be combined with various antibiotics, such as vancomycin, rifampicin, novobiocin, and show slower, more sustained drug delivery than that from conventional diffusion-based polymers.[29] Future studies will observe the effects of varied TUS parameters such as the DC, frequency, intensity and duration of application on the drug release rates from this system. Additionally, the effect of introducing a thermal change will be determined in order to produce the optimal release and distribution profile from these hydrogel devices.

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