In vitro Bioluminescence Used as a Method for Real-Time Inhibition Zone Testing for Antibiotic-Releasing Composites

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Authors' contributions

All authors took part in the design of the study. Author NA supervised the work and author NMM carried out the research work. The manuscript was written jointly by the authors NMM and NA. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: This study describes the potential of real-time bioluminescence imaging in evaluating the antibiotic efficiency of two cylinder-shaped bioabsorbable antibiotic-releasing composites by *in vitro* inhibition zone tests. The bacterial infections of bone tissue can cause extensive hard and soft tissue damage and decrease the efficiency of oral antibiotic therapy due to the poor blood circulation in the infected area. To overcome this problem, new, locally antibiotic-releasing biodegradable composites have been developed.
Study Design & Methodology: The two composites evaluated in this study were composed of poly(L-lactide-co-ε-caprolactone) matrix, β-tricalcium phosphate ceramic and either ciprofloxacin or rifampicin antibiotic. The composites were tested with genetically modified model pathogens of osteomyelitis (*Pseudomonas aeruginosa* and *Staphylococcus epidermidis*) *in vitro* in inhibition zone tests using a method of real-time bioluminescence.
Results: The first signs of the effect of the released ciprofloxacin or rifampicin became visible after four hours of incubation and were seen as changed bioluminescence around

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the composite pellet on a culture dish. Both of the composite types showed excellent effects against the sensor bacteria within the diffusion area. Bioluminescence measurements suggested that no survivor bacteria capable of evolving resistant strains were left inside the inhibition zones. The *S. epidermidis* bacterial strain was an inhibition sensor and *P. aeruginosa* was a stress sensor.

Conclusion: These results highlight the potential of the composite materials against the pathogens of osteomyelitis. The approach allows continuous visual inspection of the efficacy of the antibiotics against the bacteria.

1. INTRODUCTION

Osteomyelitis is a severe bacterial infection that can cause bone and soft tissue necrosis [1,2]. A major problem in the treatment of osteomyelitis is the wide spectrum of pathogens, i.e. staphylococcal, enterococcal, *Pseudomonas*and *Salmonella* species [1-4]. It is common that infected bone has degenerated blood circulation that greatly affects the efficacy of parenteral or intravascular antibiotics. To overcome this problem, local drug delivery systems have been developed to deliver the antibiotics directly to the infected tissue. Local antibiotic release, in the form of antibiotic-releasing implants, provides high local antibiotic concentrations and also reduces side effects [1,4]. Additionally, they can reduce the cost of drug therapy, increase the efficiency of drugs, and enhance patient compliance [4,5]. Osteomyelitis often requires surgical debridement of infected tissues that leaves a defect in the bone called a dead space. Currently, dead spaces are often treated with gentamycin-releasing poly(methyl methacrylate) beads that need to be removed later because they are not bioabsorbable and require bone grafting after bead removal [6]. This method of treatment is not optimal because it involves two surgeries. Bioabsorbable, osteconductive and antibiotic-releasing composites offer the possibility to treat osteomyelitis in one stage and to reduce both the risks for the patient and the costs. Such materials have been requested in the literature and the research is going on to achieve these goals [7].

In the development phase of antibiotic-releasing materials, screening the materials before preclinical testing is important to find the most promising materials. Often, the functionality of such materials is evaluated by inhibition zone testing. Typically, inhibition zones are measured using over-night-grown bacterial cultures, where the inhibition zone can be simply measured [8,9]. In this study, real-time bioluminescence imaging was used to investigate the potential and the antimicrobial activity of two bioabsorbable, antibiotic-releasing and cylinder-shaped composites in *in vitro* inhibition zone tests.

Bioluminescence imaging has been used successfully, for example, to monitor the antimicrobial efficacy of wound dressings *in vitro* [10], stem cell differentiation [11,12], vascularization [13,14], apoptosis [12], gene therapy [15,16], inflammation of tissue [17], implant-related bacterial infections [18,19] and in osteomyelitis animal model (mouse) [20]. The method, using whole bacteria cells as biosensors, offers several advantages: it is easy to use, it is non-invasive, and it has high throughput and low costs [12,21]. In the case of inhibition zone testing, bioluminescence imaging provides more information than the conventional method because information on the bacteria and their reactions can be followed in real time when exposed to the antibiotics [22]. Due to the light-emitting nature of bioluminescent bacteria, the number of emitted photons can be detected real-time and the

changes in light levels are proportional to the changes in the metabolism of the cells under study [10,23-27].

The osteoconductive, bioabsorbable, and antibiotic-releasing composites used in this study were developed for the treatment of osteomyelitis and their drug release and degradation properties *in vitro* were tested in our earlier studies [28,29]. A steady-state drug release period was observed after the initial burst in the beginning of the release [28,29]. Because the materials have been tested only in *in vitro* conditions, it is possible that the actual concentrations in tissue would be different due to differences in clearance from the tissue.

2. MATERIALS AND METHODS

2.1 Materials

Medical grade poly(L-lactide-co-ε-caprolactone) (PLCL) with the comonomer ratio of 70/30 and M_w of 246,000 g/mol was purchased from Purac Biomaterials (Gorinchem, the Netherlands). β-Tricalcium phosphate (β-TCP) (granule size < 38 μm) was purchased from Plasma Biotal Ltd. (Buxton, Derbyshire, United Kingdom). Rifampicin was purchased from Oriola (Espoo, Finland) and ciprofloxacin from Uquifa (Civac, Jiutepec-Morelos, Mexico). Sörensen phosphate buffer solution was prepared and used according to the ISO 15814 standard [30]. The chemicals used for the buffer solution (Na_2HPO_4 and KH_2PO_4) were purchased from J.T. Baker (Deventer, the Netherlands).

2.2 Processing of Composite Materials

Processing was done according to Ahola et al. [28,29]. In short: dried polymer (PLCL), β-TCP (50 wt-%) and antibiotics (either rifampicin or ciprofloxacin) were processed with a custom-built co-rotating twin-screw extruder in nitrogen atmosphere into rod-shaped billets, with a diameter of approximately 2.5 mm. Three different composites were processed: one composite had 8 wt-% of ciprofloxacin, one 8 wt-% of rifampicin and one with no antibiotic served as a control material. Cylindrical-shaped samples (length approx. 2.5 mm) were cut from the billets. Before inhibition zone testing, the samples were packed and gamma irradiated (28.7-34.0 kGy) for sterility.

2.3 Bacteria and Plasmids

Two engineered, non-pathogenic bacterial strains cloned with bacterial luciferase reporter genes were used as biosensor cells: *Pseudomonas aeruginosa* PAO-LAC carrying plasmid pUCP24GW and integrated mini-Tn7-Gm-GW-LUX carrying the PA0614 promoter [31] and *Staphylococcus epidermidis*ATCC-14990 carrying plasmid pAT19-lux-hlaP-frp. Minimum inhibitory concentrations (MIC) of ciprofloxacin against *P. aeruginosa* has been reported to be 0.1-1.2 μg/ml [32,33] and against *S. epidermidis* 0.1-0.8 μg/ml [34]. For rifampicin, the MIC reported against *S. epidermidis* is 0.015 μg/ml [35]. The values against *P. aeruginosa* vary greatly and values of 32-64 μg/ml [36] and 8-16 μg/ml [37] have been reported.

2.4 Bioluminescence Imaging

Bacteria were cultured on antibiotic L-agar plates (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L agar; 10 g/L gentamycin for *P. aeruginosa* and 5 g/L erythromycin for *S. epidermidis*) overnight at 30°C and suitable colonies were placed into 5 ml of liquid Luria-

Bertani medium (LB; 5 g/L yeast extract, 10 g/L tryptone, 5 g/l NaCl in 1L). The bacteria were cultured overnight at 30°C, shaken at 300 rpm, and suitable antibiotics were added. The level of luminescence of the cultures was measured by using a Plate Chameleon[TM]multilabel counter 1.001 (Hidex Ltd, Turku, Finland). At a volume of 200 µl, 1.1-2.3×10^6 counts for *S. epidermidis* and1.1-2 and 3×10^6 counts for *P. aeruginosa* were found to be optimal for subsequent steps.

Plates were prepared by casting two layers of LB-agar into a 6-well plate. Controls and antibiotic-containing composites were placed in the center of the bottom layer, one pellet per well. Bacteria culture of 350-500 µl per well was mixed with 1 ml of soft LB-agar (agar concentration 7.5 g/L) solution and cast on top. After solidification, the plate was taken to the imaging station of a Xenogen Vivo Vision IVIS[®] Lumina CCD camera (Caliper Life Sciences, USA). Images were taken every 20 minutes for 16 hours with an exposure time of 30 seconds. The images were analyzed using the Living Image[®] 3.1 program (Caliper Life Sciences, USA). The ciprofloxacin and rifampicin-releasing composite pellets were tested with both strains of bacteria after sterilization with gamma irradiation as well as after one and two weeks immersion in Sörensen phosphate buffer solution (pH 7.4) at 37°C.

After each 16-h measurement cycle, the outcome of bioluminescence was verified by visual inspection (halo around the antibiotic-releasing pellet showing no visual bacterial growth).

2.5 Interpretation of the Results

The results are presented as false color photos, where dark blue and purple were interpreted as dead bacteria with no bioluminescence. The dark blue color resulted from internal reflection from the high light levels in the stress zones. The red and yellow colors were interpreted to indicate a situation where the bacteria are in contact with sub-inhibitory concentrations of the antibiotics and produce a strong light emission that is presumably due to the nonspecific activation of central metabolic pathways [22]. These red and yellow zones are called stress zones (SZ). Green color was considered to be unaffected bacteria because this was the usual intensity in the control wells.

3. RESULTS

We have previously introduced the preliminary results of ciprofloxacin releasing composites [28] and rifampicin releasing composites[29] against *Pseudomonas aeruginosa* in connection with the *in vitro* drug release results. Here, we want to show the differences between the two bacteria *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. In addition, we want to show the results of the antibiotic releasing composites after 1 week immersion in Sörensen phosphate buffer solution (pH 7.4) at 37°C to simulate situation, when the composites have been implanted in tissue and the antibiotic release has been stabilized after the initial burst in the release. It is intended that the two composites will be used together in the local treatment of osteomyelitis in such a way that the surgeon treating the patient can decide the exact ratio of the antibiotics to be used.

The results of the bioluminescence imaging for the pellets tested directly after processing and sterilization are presented in Figs. 1-4 as part A. The light emission levels for the same samples are shown as photon counts in Figs. 1-4 as part B. The results of bioluminescence imaging for the pellets that were kept immersed in Sörensen phosphate buffer solution (pH 7.4) at 37°C for one week are presented in Appendix as Figs. 1-4 part A and the light

emission levels in Appendix as Figs. 1-4 part B. Because there were no major differences in the antibiotic activity between the samples that had been in *in vitro* conditions for one or two weeks, only the results for the samples that had been in *in vitro* conditions for one week are presented.

Both *S. epidermidis*and *P. aeruginosa* responded to ciprofloxacin released from the composite pellets that were tested directly after processing and sterilization (Figs. 1A and 2A, respectively). The yellow-red stress zone preceded the expanding inhibition zone that was seen as an increasing blue zone in the middle of the well. The decrease in the bioluminescence that suggested the death of bacteria near the composite material was clearly visible after 6 hours for *P. aeruginosa* and after 10 hours for *S. epidermidis*. At the time point of 12 hours, the inhibition zones had reached their maximum. After this, changes were mainly detected in the light emission levels, which can be seen in Figs. 1B and 2B as photon counts. In these graphs, high light emission, which represented the appearance of a SZ, was seen at the edges of the inhibition zones.

When the ciprofloxacin-releasing composites that had been immersed in Sörensen phosphate buffer solution (pH 7.4) at 37°C for a week were compared with those tested directly after sterilization, the stress zones (SZ) appeared earlier (Appendix Figs. 1 and 2). The SZs were, however, no larger in size, which indicated a longer presence of sub-inhibitory concentration areas. The initial burst, as seen in the results of the pellets tested directly after the sterilization step, was not present and the release of ciprofloxacin was steadier but yet strong enough to kill the bacteria. The ciprofloxacin release resulted in narrower inhibition zones that correlated well with the *in vitro* ciprofloxacin release study conducted earlier [28].

Both bacteria also responded to rifampicin release (Figs. 3 and 4), but the inhibition zones produced were much smaller than the zones produced by ciprofloxacin (Figs. 1 and 2). Due to the larger molecular size of rifampicin compared with ciprofloxacin, the diffusion in agar is probably slower and limited when compared with the diffusion of ciprofloxacin and, as a result, the inhibition zone was smaller. The first signs of decreased bioluminescence could already be seen after 4 hours for both of the studied bacteria. In the case of *S. epidermidis*, the formation of inhibition zones began as an appearance of red and yellow SZs around the antibiotic composites, but such behavior was not seen in the case of *P. aeruginosa*. The light emission levels of *S. epidermidis* and *P. aeruginosa* are shown in Figs. 3B and 4B, respectively.
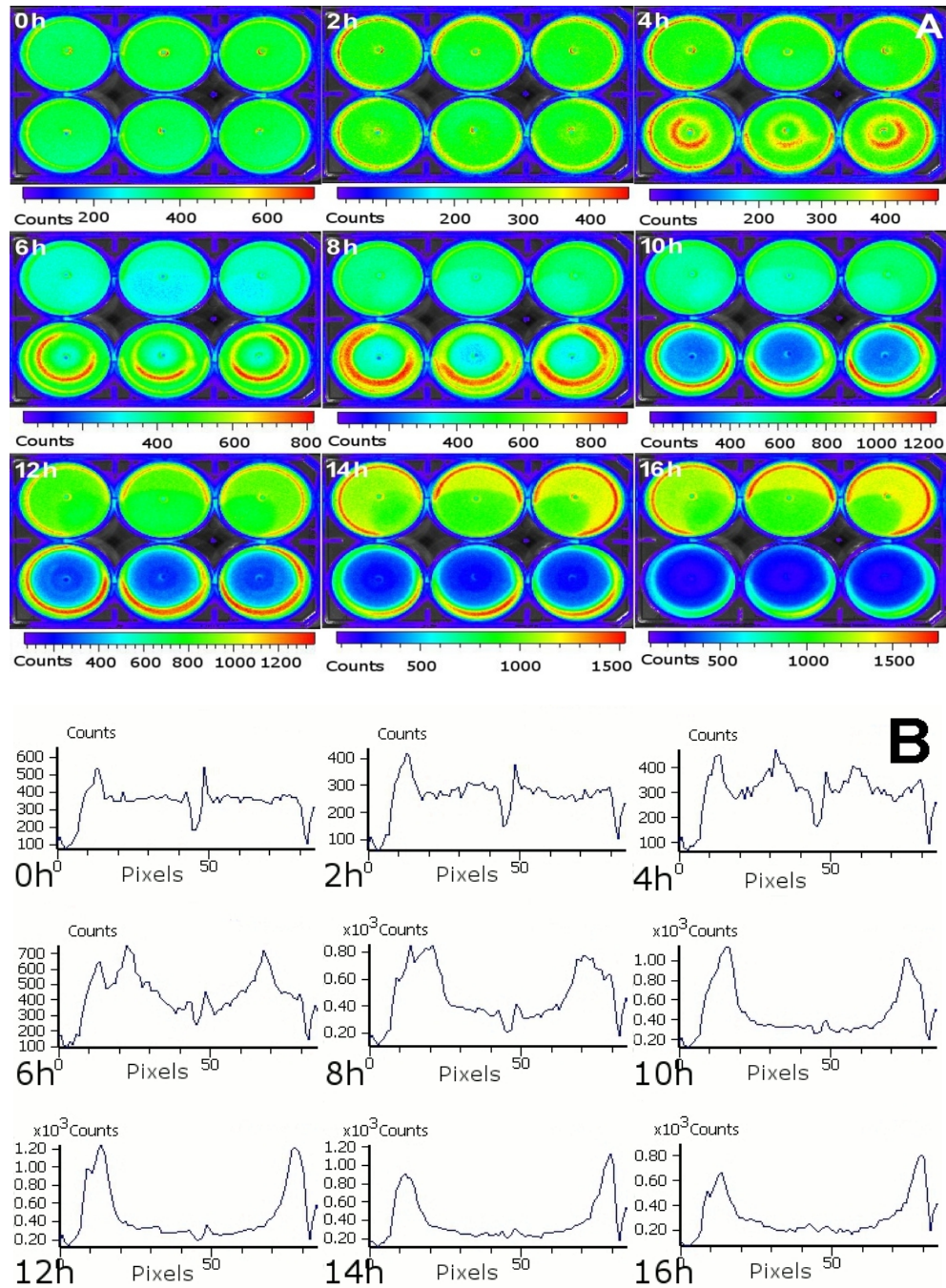
Fig. 1.(A) The development of the inhibitory zones was seen as growing blue areas in the middle of the wells of cultured S. epidermidis and ciprofloxacin composite pellets (in triplicate on the lower row). Pellets without antibiotic were used as controls (in triplicate on the top row). The plate was photographed at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. The diameter of one well is 35 mm. (B) Light intensity levels as photon counts of S. epidermidis exposed to one ciprofloxacin releasing composite pellet in the middle of the well. The results are presented at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. Note the different scales of the y-axes.
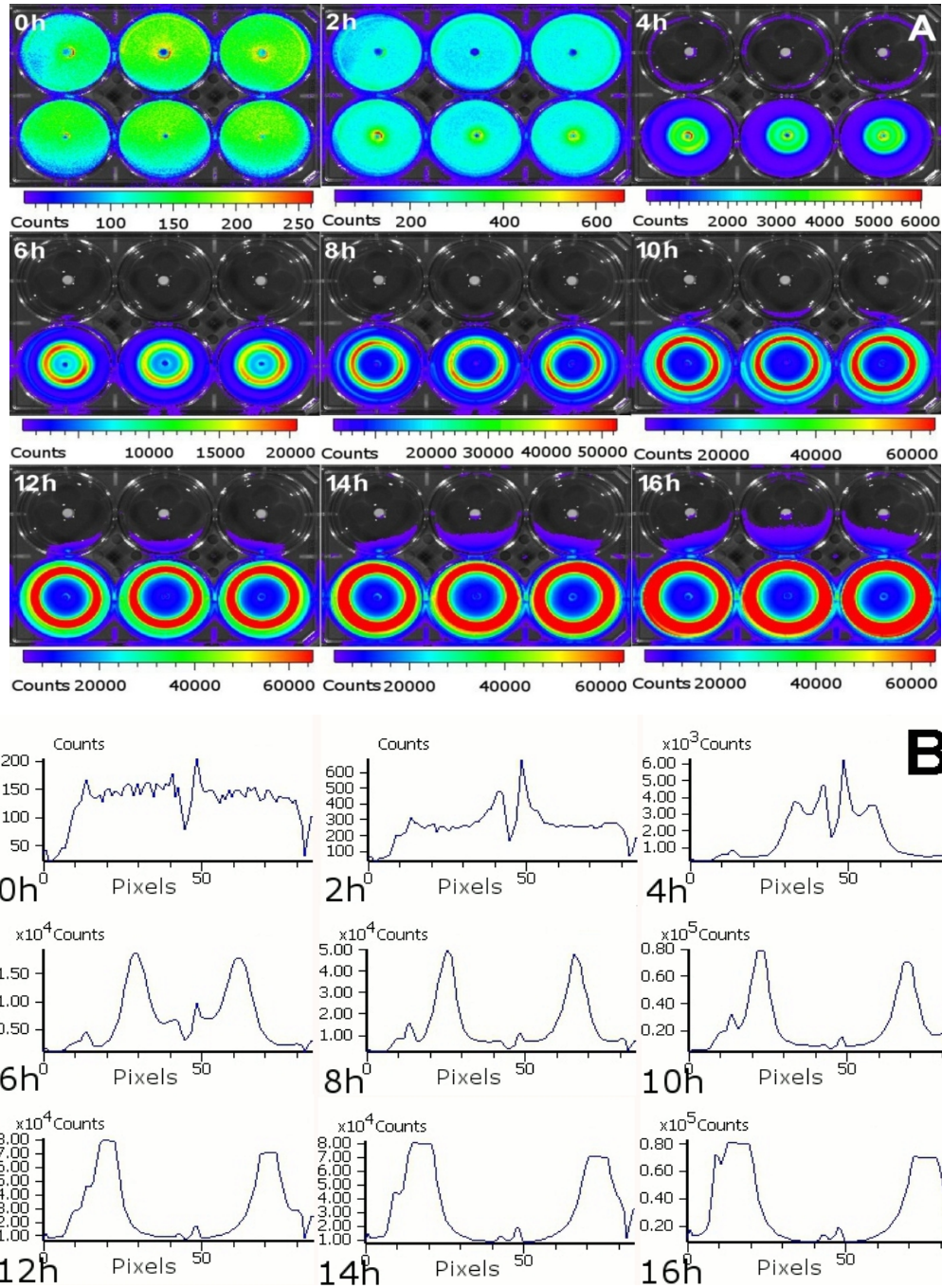
Fig. 2.(A) The development of the inhibitory zone was seen as growing blue areas in the middle of the wells of cultured P. aeruginosa and ciprofloxacin composite pellets (in triplicate on the lower row). Pellets without antibiotic were used as controls (in triplicate on the top row). The plate was photographed at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. The diameter of one well is 35 mm. (B) Light intensity levels as photon counts of P. aeruginosa exposed to one ciprofloxacin releasing composite pellet in the middle of the well. The results are presented at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. Note the different scales of the y-axes.
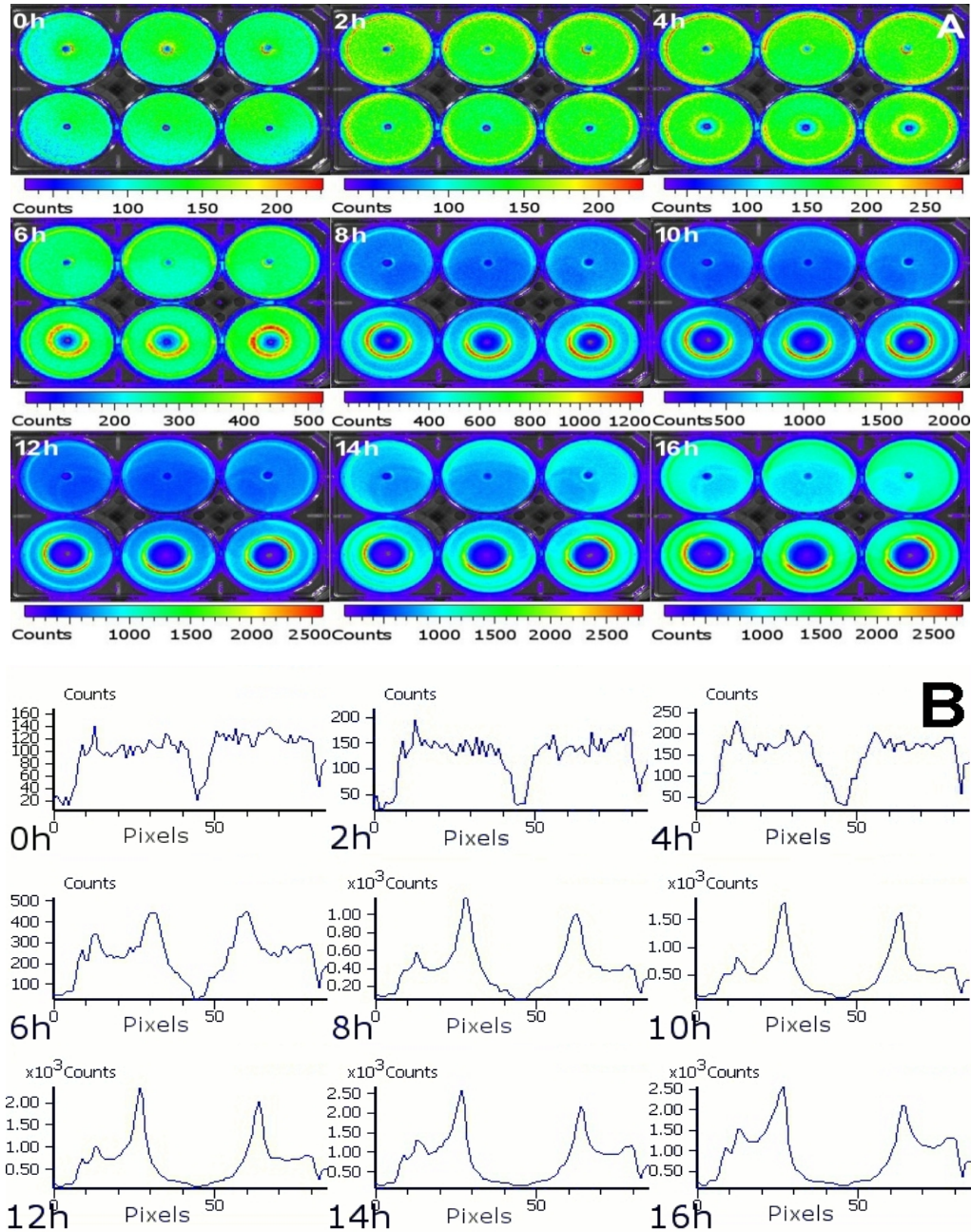
Fig. 3.(A) The development of the inhibitory zones was seen as growing blue areas in the middle of the wells of cultured S. epidermidis and rifampicin composite pellets (in triplicate on the lower row). Pellets without antibiotics were used as controls (in triplicate on the top row). The plate was photographed at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. The diameter of one well is 35 mm.(B) Light intensity levels as photon counts of S. epidermidis exposed to one rifampicin releasing composite pellet in the middle of the well. The results are presented at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. Note the different scales of the y-axes.
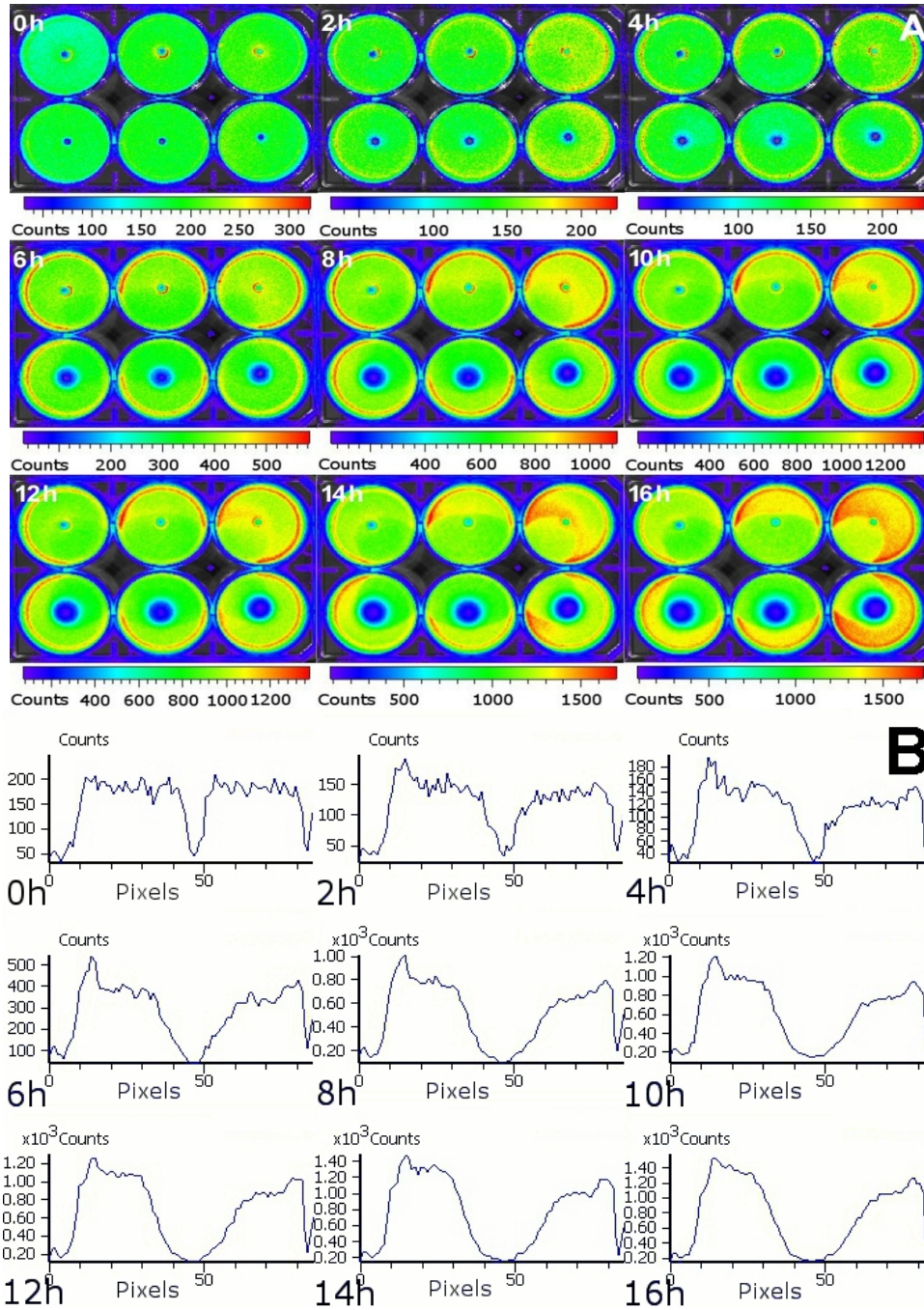
Fig. 4.(A) The development of the inhibitory zone was seen as growing blue areas in the middle of the wells of cultures P. aeruginosa and rifampicin composite pellets (in triplicate on the lower row). Pellets without antibiotic were used as controls (in triplicate on the top row). The plate was photographed at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. The diameter of one well is 35 mm. (B) Light intensity levels as photon counts of P. aeruginosa exposed to one rifampicin releasing composite pellet in the middle of the well. The results are presented at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. Note the different scales of the y-axes.

In the case of the rifampicin-releasing samples kept immersed in Sörensen phosphate buffer solution (pH 7.4) at 37°C for one week, the initial burst was not present, as was also the case with ciprofloxacin. There was also no significant change in light emission levels (Appendix Figs. 3B and 4B), which indicated no clear inhibition.

In Fig. 5, the growth of the area of the inhibition zones is shown as a function of time. The graphs indicate the size of the inhibition zone that one composite pellet, releasing either ciprofloxacin or rifampicin, is capable of producing. The inhibition zone areas are presented without and with stress zones (5 A and C without stress zones and 5 B and D with stress zones). The graphs also show the differences in the effects between the different bacterial species. With ciprofloxacin (Figure 5A), the inhibition zone of *S. epidermidis* was around 9.0 cm^2, but with *P. aeruginosa* the area was only half the size. When the ciprofloxacin-releasing composites had been immersed in the Sörensen phosphate buffer solution at 37°C for a week, the zone area decreased to around 8.0 cm^2 for *S. epidermidis* and to 2.0 cm^2 for *P. aeruginosa*. With *S. epidermidis*, the inhibition zone of rifampicin was around 2.3 cm^2 (Fig.5C), and only 0.4 cm^2 after immersion in Sörensen phosphate buffer solution at 37°C. For *P. aeruginosa*, the values were 1.5 and 0.2 cm^2, respectively. When the stress zones were included in the inhibition zones (Figs. 5B and 5D), the areas differed only slightly for *S. epidermidis*. With *P. aeruginosa* the stress zones made a larger difference, which indicated larger stress zones for *P. aeruginosa*.
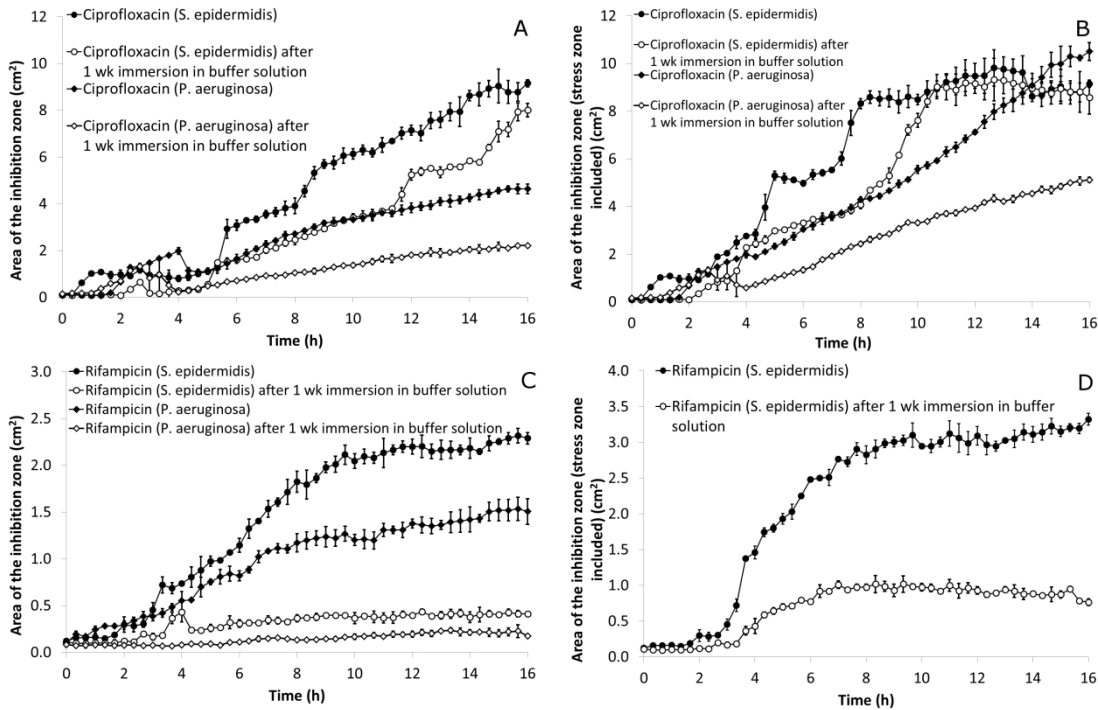

Fig. 5. Measured area of the bioluminescence inhibitory zones with (A) ciprofloxacin, (B) ciprofloxacin with stress zones included in the inhibitory zones, (C) rifampicin and (D) rifampicin with stress zones included in the inhibitory zones (n=3). Results using pellets that had been immersed in Sörensen phosphate buffer solution (pH 7.4) at 37°C for one week prior to testing are shown as well.

4. DISCUSSION

The aim of this study was to demonstrate the potential of bioluminescence in inhibition zone tests and to show that bioluminescence provides more information about the effects of antibiotics on bacteria than the conventional over-night-grown inhibition zone measurements. The possibility for real-time observation of the evolving inhibition zones and so-called stress zones provides an insight in to how the antibiotics affect the bacteria.

The antibiotic-releasing composites studied here were cylindrical in shape and were composed of bioabsorbable poly(L-lactide-co-ε-caprolactone) (PLCL) as the polymer matrix, 50 wt-% β-tricalciumphosphate (β-TCP), and either 8 wt-% of ciprofloxacin or rifampicin. It is intended that the composites will be used together in the local treatment of osteomyelitis in such a way that the surgeon treating the patient can decide the exact ratio of the antibiotics to be used. The main advantage of this kind of composite implant is that removal after treatment is not required due to the bioabsorbability of the composites. β-TCP acts as an osteoconductive material and is capable of attracting osteoblasts. This can both facilitate controlled drug release and enhance bone repair and regeneration [4,38-41].

The antibiotics used in this study, rifampicin and ciprofloxacin, have been widely used because they are known to form an effective, synergistic combination even against bacterial strains that are not susceptible to, for example, β-lactams or tetracyclines. A quinolone-rifampicin treatment is safe and well tolerated by patients [2,4,42,43]. Ciprofloxacin is used as the main antibiotic because it is an active fluoroquinolone that inhibits DNA gyrase and stops DNA replication [22,44]. Rifampicin supports ciprofloxacin by inhibiting the emerging resistant strains. It also penetrates biofilm well [45,46], which is an advantage because osteomyelitis is often caused by bacteria forming biofilms. Some bacteria are more resistant to antibiotics than others, a good example being the MRSA strain of *S. aureus*[22]. The use of two or more antibiotics that have a synergistic action is frequently employed [22,47] because this seems to prevent or inhibit the development of resistance.

The antibiotics used in the treatment of osteomyelitis must be active against a broad spectrum of common disease-causing pathogens [4]. The treatment must also ensure that no bacteria survive. If the duration of the treatment is too short or the antibiotic reaches the infection site in sub-inhibitory concentrations, the remaining bacteria can cause clinical failure and potentially develop resistance [1-3,48]. Often, two antibiotics are used together because of their synergistic effect against the causative bacteria. The benefits of this kind of treatment are accelerated bacterial count decline, a wider antibacterial spectrum and reduction in the risk of resistant strain development to one of the antibiotics [49].

In the bioluminescence measurements conducted in this study, it was shown that both ciprofloxacin and rifampicin caused very strong, non-specific activation of bioluminescence in both of the model bacteria. The same kinds of reactions have been previously reported several times in solution-based assays in the high-throughput assays of antimicrobial agents in microtitreplate format [23,50,51]. Even though ciprofloxacin-releasing composite pellets were shown to be more effective against bacteria than rifampicin-releasing ones, it was noticed that rifampicin composites reached the level of total eradication earlier. This was seen, when using rifampicin-releasing composites, as a faster decrease in luminescence in the middle of inhibition zones (Figs. 3B and 4B). The fast response was most probably due to the burst in the release that is caused when the composite materials first release the antibiotic molecules on or near the surface as they are exposed to fluids. Here, rifampicin caused a stronger burst than ciprofloxacin, as was seen in the *in vitro* drug release test

conducted earlier [28,29]. The burst effect is a well-known phenomenon in controlled drug delivery and is sometimes considered to be unwanted [52]. In the case of antibiotics, however, reaching drug concentrations over the minimum inhibition concentration quickly is crucial because antibiotic resistance is a serious problem worldwide. In this sense, a moderate burst in the beginning of the release is useful. After the burst, the drug may be released in a continuous manner that can last for weeks [4,28].

The formation of stress zones was more visible for the ciprofloxacin-releasing composites. Within these zones, the concentration of antibiotic was considered to be too low to kill bacteria, but still able to cause the up-regulation of certain promoters. As a part of this regulation, the bacteria were presumably partially shutting down some of their metabolic pathways and thus liberated more energy for the bioluminescence reaction.

S. epidermidis is a metabolic inhibition sensor and *P. aeruginosa* is a stress sensor. *P. aeruginosa* is constructed to respond to the threat caused by gyrase inhibitors such as ciprofloxacin: up-regulation of luminescence genes only results if expression of the *gyrA* gene is reduced and gyrase is inhibited by ciprofloxacin [31]. The shut-down of light emission, especially in control wells (Fig. 2), may occur when the bacteria have reached the stationary phase and the quantity of active gyrase is not reduced by inhibition [31]. Therefore, the promoter in front of the luciferase reporter genes is efficiently shut down in non-stressing conditions and results in a dark background over the control pellets. Another explanation may be that the light emission levels of the wells affected by ciprofloxacin were very high and because of the internal recalibration system of the equipment, the low background light emission was under the detection limit. Thus, it may have seemed that there was no light emission. The blue light that was seen in the control wells in Fig. 2 was probably only a reflection of the strong light emission produced by the bacteria in contact with ciprofloxacin. Because *P. aeruginosa* is a stress and inhibition sensor, the light emission was up-regulated as ciprofloxacin diffusion proceeded and turned off as bacteria died. This was seen as the inhibition zone expanded within stress zone progression. Rifampicin being a transcriptional inhibitor did not produce a similar stress effect with *P. aeruginosa*.

Because sub-inhibitory concentration levels of quinolones are enough to promote an SOS response that regulates several promoters, sub-inhibitory concentration levels can lead to a possible increased tolerance to DNA damage [44]. As a result, an inadequate antibiotic release of the composites can cause the development of resistant bacterial strains. Most promoters regulate virulence genes and survival and some promoters are also sensitive to low concentrations of rifampicin [53]. It is, therefore, vital to decrease the formation of low concentration release barriers in order to lower the mutation rate of these promoters.

Because the bioluminescence method uses light-emitting bacterial cultures from which the range of the antibiotic release and the level of inhibition inside the zones formed can be estimated and measured, the method can be used to screen the potency of antibiotic-releasing systems before preclinical studies. Direct correlation between the light production and viable light emitting bacteria has been reported several times and with several different bacteria [10,23-27]. If toxic substances such as antibiotics kill the sensor bacteria, the decrease in light emission correlates directly to the number of viable bacteria [24-26]. Thus, bioluminescence seems to be a very suitable method for assessing the antimicrobial effects of antibiotic-releasing materials.

By imaging the antibiotic release utilizing bioluminescence and a CCD camera in real-time, it is easy to demonstrate whether the antibiotic-releasing capacity of the material is high

enough to eradicate the bacteria without any potential survivors developing antibiotic resistance. The antibiotic release from a small, ciprofloxacin-containing pellet seems to be sufficient to kill all the bacteria inside the inhibition zones. The inhibition zones formed by rifampicin-releasing material may, however, pose the risk of resistant strain formation if used alone. Therefore, the materials must be used together in order to support each other and to decrease the risk of resistance. In this study, we investigated the antibiotics separately in order to highlight the differences more clearly. According to the results, the composite materials have good potential to release antibiotics in concentrations high enough to eradicate bacteria from tissues infected by pathogens of osteomyelitis.

5. CONCLUSION

The *in vitro* inhibition zone test using real-time bioluminescence was demonstrated to be an efficient tool to observe the effects of the released antibiotics on the model pathogens of osteomyelitis. The method provides more information on the effects against the bacteria than the conventional over-night-grown bacterial cultures. Additionally, the potential of the new antibiotic-releasing biodegradable composites were shown to be adequate on model pathogens. However, it has to be kept in mind that the results presented here were obtained using single antibiotic-releasing composite pellets. In real clinical cases, several of these kinds of composite pellets would be used together. The therapeutic value of the release of ciprofloxacin and rifampicin from composites needs to be demonstrated carefully in order to validate the potential of new antibiotic-releasing composites in efficient antibiotic release. Studying the composite material with bioluminescence inhibition tests helped to monitor the efficiency of antibiotic release.

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COMPETING INTERESTS

Authors have declared that no competing interest exists.

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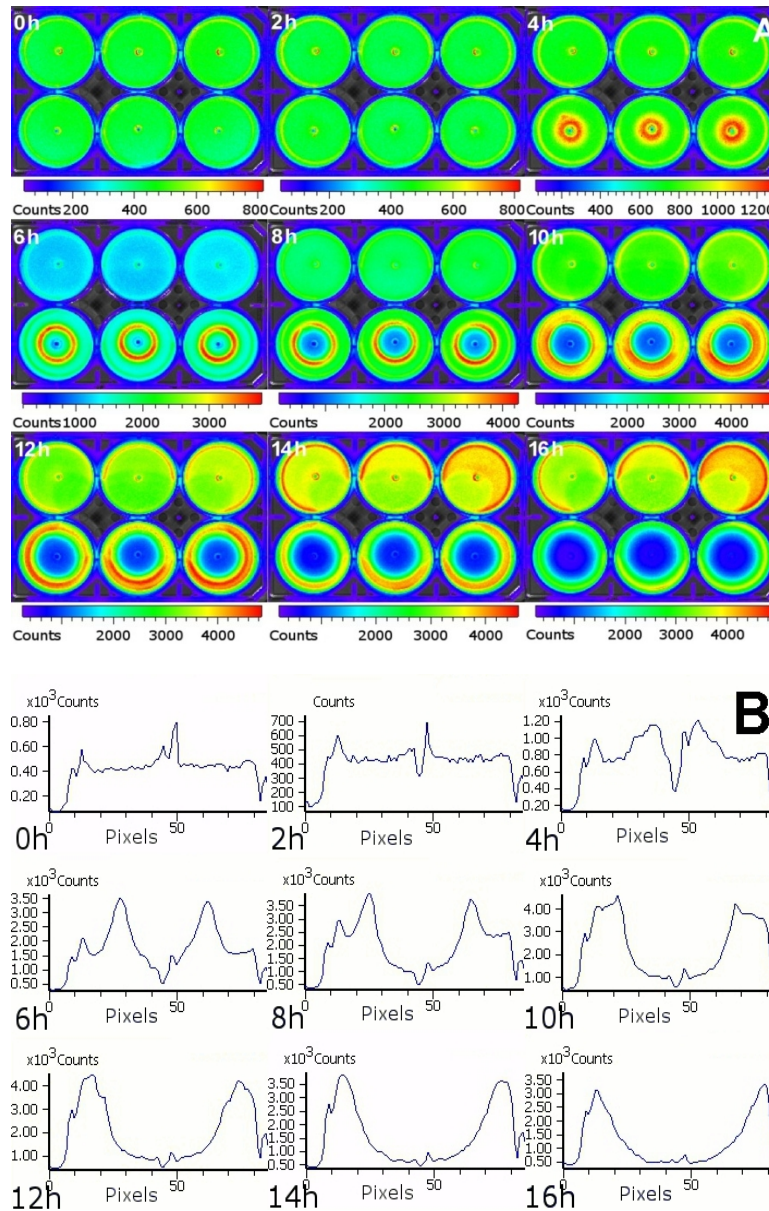
APPENDIX

Figure. 1. (A) The development of the inhibitory zones was seen as growing blue areas in the middle of the wells of cultured S. epidermidis and ciprofloxacin composite pellets, which had been immersed in Sörensen phosphate buffer solution (pH 7.4) at 37°C for one week (in triplicate on the lower row). Pellets without antibiotic were used as controls (in triplicate on the top row). The plate was photographed at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. The diameter of one well is 35 mm. (B) Light intensity levels as photon counts of S. epidermidis exposed to one ciprofloxacin releasing composite pellet in the middle of the well. The pellet had been immersed in Sörensen phosphate buffer solution (pH 7.4) at 37°C for one week before the experiment. The results are presented at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. Note the different scales of the y-axes.
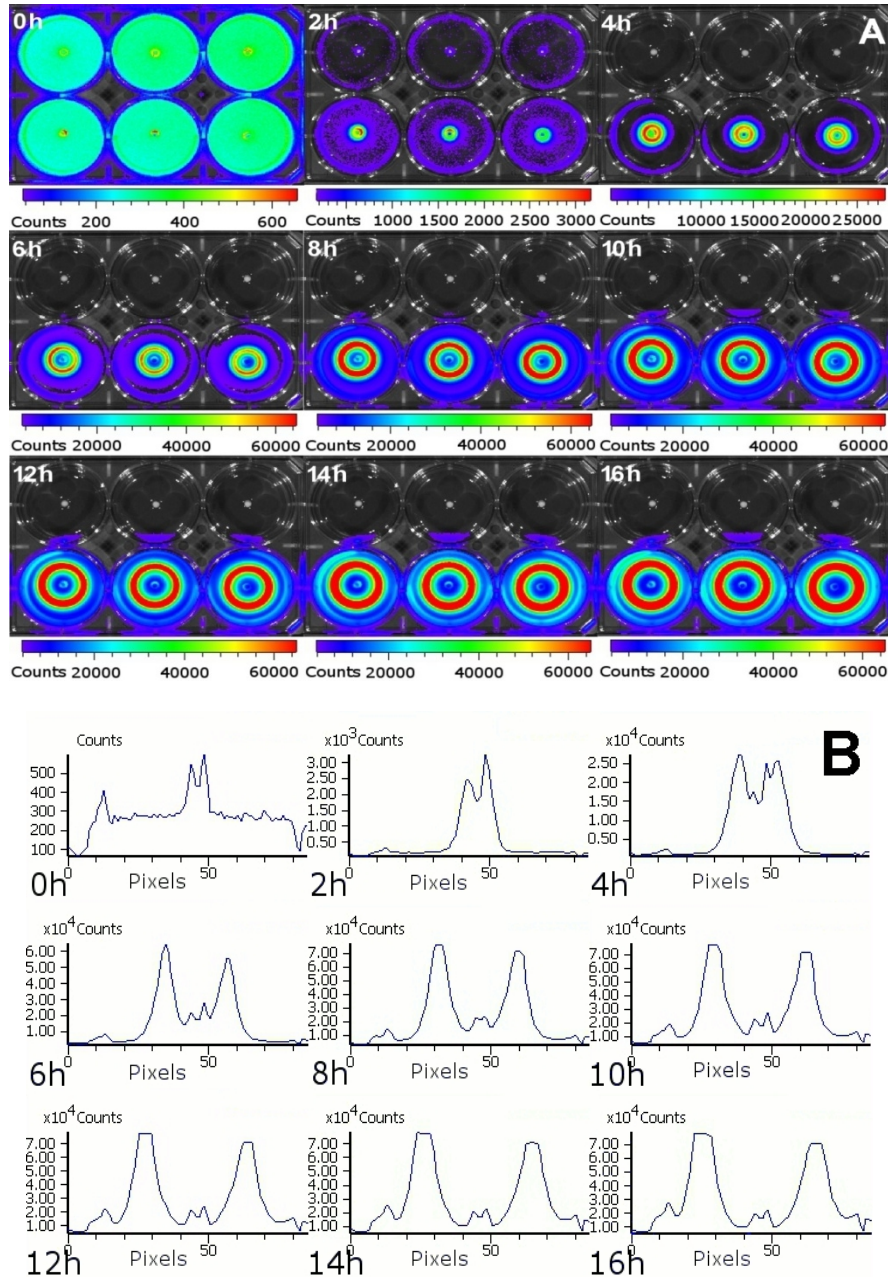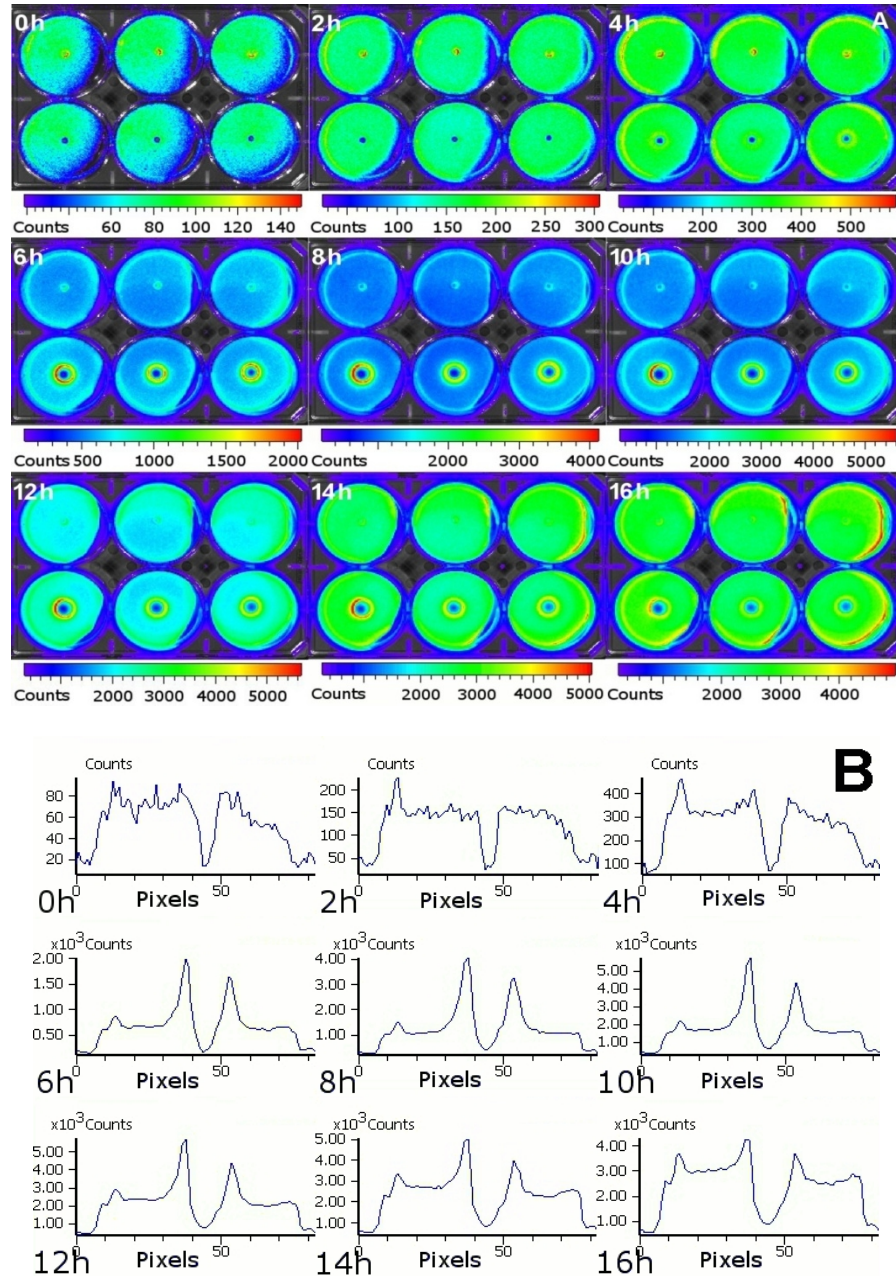
Figure 2.(A) The development of the inhibitory zones was seen as growing blue areas in the middle of the wells of cultured P. aeruginosa and ciprofloxacin composite pellets, which had been immersed in Sörensen phosphate buffer solution (pH 7.4) at 37°C for one week (in triplicate on the lower row). Pellets without antibiotic were used as controls (in triplicate on the top row). The plate was photographed at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. The diameter of one well is 35 mm. (B) Light intensity levels as photon counts of P. aeruginosa exposed to one ciprofloxacin releasing composite pellet in the middle of the well. The pellet had been immersed in Sörensen phosphate buffer solution (pH 7.4) at 37°C for one week before the experiment. The results are presented at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. Note the different scales of the y-axes.

Figure 3.(A) The development of the inhibitory zones was seen as growing blue areas in the middle of the wells of cultured S. epidermidis and rifampicin composite pellets, which had been immersed in Sörensen phosphate buffer solution (pH 7.4) at 37°C for one week (in triplicate on the lower row). Pellets without antibiotic were used as controls (in triplicate on the top row). The plate was photographed at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. The diameter of one well is 35 mm. (B) Light intensity levels as photon counts of S. epidermidis exposed to one rifampicin releasing composite pellet in the middle of the well. The pellet had been immersed in Sörensen phosphate buffer solution (pH 7.4) at 37°C for one week before the experiment. The results are presented at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. Note the different scales of the y-axes.
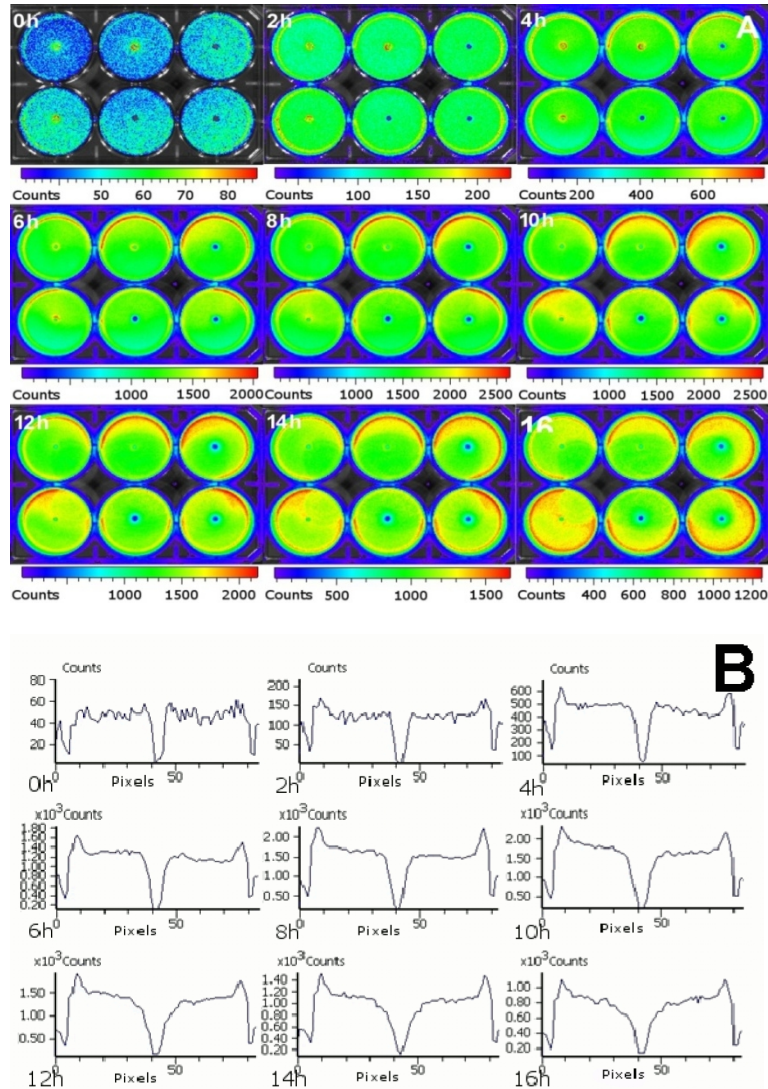
Figure 4.(A) The development of the inhibitory zones was seen as growing blue areas in the middle of the wells of cultured P. aeruginosa and rifampicin composite pellets, which had been immersed in Sörensen phosphate buffer solution (pH 7.4) at 37°C for one week (in triplicate on the lower row). Pellets without antibiotic were used as controls (in triplicate on the top row). The plate was photographed at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. The diameter of one well is 35 mm. (B) Light intensity levels as photon counts of P. aeruginosa exposed to one rifampicin releasing composite pellet in the middle of the well. The pellet had been immersed in Sörensen phosphate buffer solution (pH 7.4) at 37°C for one week before the experiment. The results are presented at time points of 0, 2, 4, 6, 8, 10, 12, 14, and 16 hours. Note the different scales of the y-axes.