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Near-infrared fluorescence imaging as an alternative to bioluminescent bacteria to monitor biomaterial-associated infections

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ABSTRACT

Biomaterial-associated infection is one of the most common complications related to the implantation of any biomedical device. Several in vivo imaging platforms have emerged as powerful diagnostic tools to longitudinally monitor biomaterial-associated infections in small animal models. In this study, we directly compared two imaging approaches: bacteria engineered to produce luciferase to generate bioluminescence and reactive oxygen species (ROS) imaging of the inflammatory response associated with the infected implant. We performed longitudinal imaging of bioluminescence associated with bacteria strains expressing plasmid-integrated luciferase driven by different promoters or a strain with the luciferase gene integrated into the chromosome. These luminescent strains provided an adequate signal for acute (0–4 days) monitoring of the infection, but the bioluminescence signal decreased over time and leveled off at 7 days post-implantation. This loss in the bioluminescence signal was attributed to changes in the metabolic activity of the bacteria. In contrast, near-infrared fluorescence imaging of ROS associated with inflammation to the implant provided sensitive and dose-dependent signals of biomaterial-associated bacteria. ROS imaging exhibited higher sensitivity than the bioluminescence imaging and was independent of the bacteria strain. Near-infrared fluorescence imaging of inflammatory responses represents a powerful alternative to bioluminescence imaging for monitoring biomaterial-associated bacterial infections.

1. Introduction

Device-related bacterial infections are a growing healthcare problem [1–3], accounting for more than 50% of the 2,000,000 annual hospital-acquired infections associated with indwelling devices and implants in the USA [2]. *Staphylococcus aureus* is one of the most common pathogens associated with these cases. Bacterial colonization and biofilm development can lead to both malfunction of the device and systemic infection, since biofilms are complex cooperative communities, and such biofilm bacteria are nearly impervious to antimicrobial therapy or host defense mechanisms [4,5]. In most cases, the affected devices must be removed to eliminate the infection, given the fact that currently there are no drugs that specifically target bacteria in biofilms [6–8].

A requirement to efficiently treat implant-associated infections are in vivo monitoring approaches that allow better understanding and control of biofilm formation, together with novel methods for targeting efficient drug candidates [9]. Optical imaging of bacterial infections in vivo using engineered bioluminescent bacterial strains is a widely used approach for spatial and temporal assessment of the infection [10]. This method is based on bioluminescent bacteria expressing a luciferase-based reporter system that emits light that can be monitored longitudinally and nondestructively in the same animal.

In view of the fact that biomaterial-associated infections modulate the inflammatory response to the biomaterial, changes in inflammatory markers may be used to improve monitoring of an ongoing infection [11]. In particular, reactive oxygen species (ROS) form part of the oxygen-dependent bactericidal mechanisms that phagocytic cells employ [12]. Near-infrared fluorescence (NIRF) imaging probes, such as hydrocyanines, allow real-time fluorescence imaging and ROS detection in the vicinity of an implant [13]. Moreover, NIRF imaging is an excellent noninvasive method for whole-body scanning that can determine the extent of the infectious disease throughout the body, especially in

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clinically challenging cases involving trauma, infection and compromised tissue beds.

Herein, we directly compared two imaging approaches of implant-associated infection: bacteria engineered to produce luciferase to generate bioluminescence and ROS imaging of the inflammatory response associated with the infected implant. These approaches were correlated to bacterial counts before and after 7 days of implantation (Fig. 1).

2. Materials and methods

2.1. Disk fabrication

Poly(3-hydroxyoctanoate-*co*-hydroxyhexanoate), PHOHHx, was kindly provided by Bioplolis S.L. The monomer composition of PHOHHx was determined by gas chromatography–mass spectrometry as previously described [14] and consisted in 8.5% 3-hydroxyhexanoate (OH-C6) and 91.5% 3-hydroxyoctanoate (OH-C8). An optimized downstream processing was applied to eliminate endotoxins as previously described [15]. Briefly, 1 g of PHOHHx was dissolved in 100 ml of chloroform at 40 °C under vigorous stirring; the suspension was pressure-filtered and the polymer was precipitated by the addition of non-solvent methanol. Finally, the polymer was dried under vacuum at 40 °C for 48 h. This procedure was repeated two times to obtain PHOHHx with endotoxin units (EU) <20 EU g^{-1}, in compliance with the endotoxin requirements for biomedical applications as stipulated by the US Food and Drug Administration [16]. The endotoxin content was measured using a Limulus amebocyte lysate (LAL)-test (Pyrogent Plus Single Test Kit, Lonza) and the endotoxin content was determined to be <15 EU g^{-1}.

Poly(ethylene terephthalate) (PET) disks (6 mm diameter) were coated with PHOHHx by solvent-casting. PHOHHx dissolved in chloroform (2% w/v) was applied over sterile, endotoxin-free PET disks (kindly supplied by ACCIONA, Barcelona, Spain), in a dust-free atmosphere. The coatings were allowed to dry for 72 h at room temperature and the resulting PHOHHx-coated disks (referred to hereafter as PHOHHx disks) were sterilized with ethylene oxide at 40 °C.

2.2. Bacterial strains, media and growth conditions

The bacterial strains used throughout this study were *S. aureus* subsp. *aureus* ATCC 12600, its two derivate luminescent strains *S. aureus* (pAmiBlaz) and *S. aureus* (pAmiSPA) and the *S. aureus* Xen29 strain containing *lux* operon stably inserted into the chromosome (Caliper Life Sciences, PerkinElmer Company). All bacterial strains were pre-cultured in trypticase soy agar (TSA) plates and incubated at 37 °C for 24 h. The appropriate selection antibiotics, chloramphenicol (10 μg ml^{-1}) or kanamycin (200 μg ml^{-1}), were added when indicated. Trypticase soy broth (TSB, Difco) was used as the growth medium to culture all bacterial pathogens.

2.3. Construction of S. aureus luminescent strains

Bioluminescent *S. aureus* strains were generated by transforming ATCC 12600 strain with a modified *Photorhabdus luminescens luxCDABE* (*lux*) gene cluster using the pAmiBlaz or pAmiSPA plasmids. To construct the vectors, *blaZ* (β-lactamase) or *spa2* (protein A) promoters were inserted into promoterless-*lux* cloning vector pAmilux [17] to yield pAmiBlaz or pAmiSPA plasmid, respectively. Vectors were introduced into the cells by electroporation, as previously described [18]. Transformants were selected on TSA plates containing chloramphenicol (10 μg ml^{-1}). Successful transformation was confirmed by bioluminescent colonies screening using an IVIS Lumina bioimaging system (Xenogen).

Expression of *lux* operon in pAmiBlaz vector was driven by the *BlaZ* promoter, whereas in pAmiSPA the *lux* operon was controlled by the protein A promoter. These two promoters were used with the aim of generating different expression patterns. The *BlaZ* promoter was used for constitutive expression of luciferase. In contrast, the *S. aureus* protein A is involved in the development of biofilm-associated infections [19]; therefore the *spa* promoter drives the expression of luciferase during biofilm development. *S. aureus* Xen29 (Caliper, PerkinElmer) is a commonly used, commercially available strain containing luciferase construct stably integrated into the chromosome.

2.4. Preparation of hydro-indocyanine green

Hydro-indocyanine green (H-ICG) was synthesized from indocyanine green dye (ICG) (Acros Organics) by reduction with sodium borohydride as described [13]. Briefly, 2 mg of ICG was dissolved in 4 ml of methanol and reduced with 2–3 mg of sodium borohydride (Aldrich). The solvent was removed by stirring reaction mix for 5 min under reduced pressure. The dye was nitrogen capped and stored overnight at −20 °C.

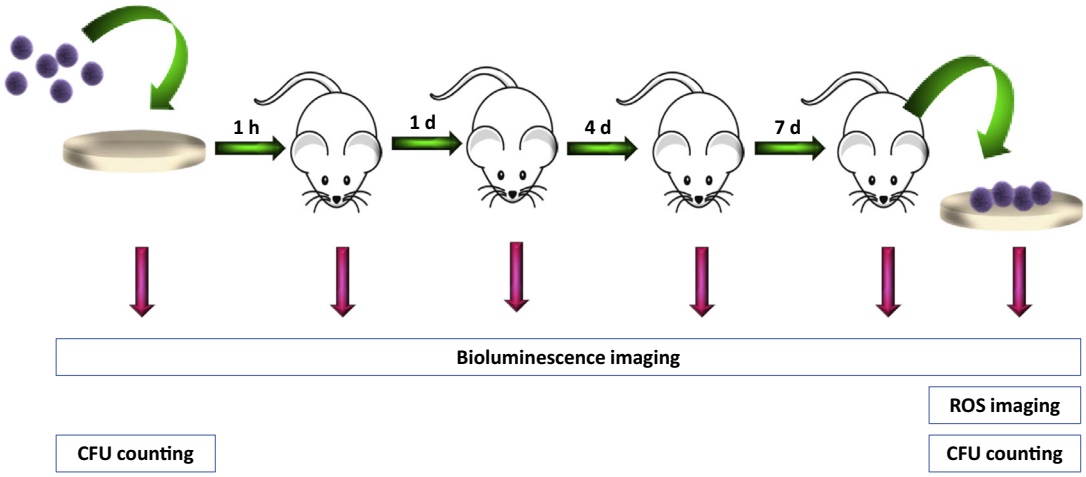

Fig. 1. Experimental timeline for comparison of bioimaging approaches of biomaterial-associated infection. PHOHHx disks were pre-colonized with engineered bioluminescent bacteria. Following counting bacteria and bioluminescence imaging, disks were implanted subcutaneously. Bioluminescence was measured at 0, 1, 4, 7 days. After ROS imaging at day 7, disks were retrieved and analyzed for bacterial counts and immunostaining.