CHAPTER 6: NON-INVASIVE MONITORING OF
WOUND HEALING USING OCT

Abstract: In this chapter we first describe the use of PSOCT imaging of tissues resected from Staphylococcus aureus infected and uninfected wounds, at different healing times, to assess the morphological changes and collagen remodeling at various stages of wound healing. Next we describe the use of a time domain real time (~ 8 frames/s) OCT set up for monitoring the healing of wounds non-invasively without sacrificing the animal. These measurements showed that compared to the uninfected wounds, the infected wounds had prominent edematic regions which leads to a significant delay in re-epithelization and collagen remodeling phases of wound healing. The OCT measurements were found to be consistent with the corresponding histological measurements demonstrating the potential of OCT for non-invasive monitoring of the progression of wound healing.

6.1 Introduction

Cutaneous wounds e.g., burns, chronic skin ulcers and surgical wounds are among the most common wounds in clinical medicine [113,114]. Wound healing is a complex and dynamic process that can be roughly divided into three phases – inflammation, proliferation and tissue remodeling. In the normal wound-healing process, after the inflammatory phase, re-epithelialization, thickening of the newly formed epidermis and remodeling of collagen fibers occur in a timely fashion [115]. However, in a chronic wound, these healing processes are disrupted at one or more points, resulting in a delay in
healing beyond the anticipated time. Bacterial infection is one of the main predisposing factors for the development of chronic wounds [116] and infected wounds could severely compromise the overall health of an individual, and may cause increased trauma and higher treatment costs [117]. Staphylococcus aureus is one of the most common human pathogens responsible for a variety of cutaneous and systemic infections including postoperative or injury wound infections [118] resulting in impairment of wound healing [119]. Clinical manifestations in infected wounds mainly arise due to the host response to the toxins secreted by bacteria [120].

In clinical practice, wound size, colour, odour, and drainage are used for gross evaluation of wound healing. These methods, however, do not provide structural information below the wound surface and can be very subjective [121]. Histology is the standard method for obtaining the structural details of the wound tissues [122] however biopsy is disruptive, may contaminate the wound, and also introduces a new wound, thereby prohibiting repeated assessment of the healing process in the same wound [123]. Other methods include measurements of the tensile strength and electrical impedance of the wound. Because of invasiveness, these methods are also not common in clinical practice [124]. To facilitate a timely decision for correct therapy, it is important to accurately monitor the morphological changes in the infected wounds using non-invasive tools. Several noninvasive methods have been investigated, which include high-resolution ultrasound, thermography, laser Doppler imaging, polarization imaging, fluorescence imaging [125], confocal microscopy, [126] and magnetic resonance imaging [127]. Although ultrasound imaging is used to examine wound healing, the depth resolution achievable (~75 µm) is insufficient to monitor fine structural changes like reepithelialization. Additionally, ultrasound requires matching media in direct contact with the wound surface, which may cause mechanical damage to wound tissue [126]. The morphological changes of wounds
can also be measured using OCT or using PSOCT which can also monitor changes in the ordering of collagen matrix. These structural and birefringence changes can be used to characterize the different phases of the wound healing and could provide a basis for non-invasive wound healing assessment.

The use of PS-OCT for quantitative evaluation of collagen denaturation induced by a thermal injury, for visualization of collagen fiber regeneration and for quantification of various drug effects during wound healing has been demonstrated [128]. While these previously mentioned studies have demonstrated the utility of PS-OCT for monitoring the normal wound healing process, the use of this technique for evaluating the morphological changes associated with bacteria infected wounds has not been investigated. Further, it has been demonstrated that bacterial toxins have a detrimental effect on wound tensile strength and have been found to decrease collagen deposition and cross-linking [129] that may show up as birefringence changes in PS-OCT imaging. We have therefore used PSOCT and real-time OCT imaging setups to characterize the different phases of healing of uninfected and S. aureus-infected superficial skin wounds under *ex-vivo* and *in-vivo* conditions respectively. The PSOCT setup was used to investigated the changes in collagen birefringence in the uninfected and S. aureus infected wounds while the real-time OCT imaging on samples were performed for studying the kinetics of healing of infected wounds under *in-vivo* conditions.

### 6.2 Staphylococcus aureus bacterial culture

S. aureus MTCC No. 740 (equivalent to ATCC 9144) was obtained from the Institute of Microbial Technology, Chandigarh, India. Bacteria used for infection were grown overnight in Tryptic Soya broth (TSB, Himedia, Mumbai, India) under aerobic conditions at 37° C using a shaker incubator.
6.3 Wound creation and bacterial infection

Animal infection experiments were performed in accordance with institutional and national guidelines on animal care. Six- to 8-weeks-old female Swiss albino mice were used for all experiments. The mice were anesthetized by an intraperitoneal injection of Ketamine (80 mg/kg) and Xylazine (10 mg/kg). The tape-stripping wound model was generated according to the procedure described by Kugellberg et al. [130]. For tape stripping wounds, hair was removed from an area of ~ 2 cm² from the back of the mice using a depilatory cream after anesthetizing the animals. The skin was then cleansed with Betadine. For wound generation, the skin was stripped 12–15 times with Tensoplast (Johnson & Johnson, Mumbai, India). Following this procedure, the skin became visibly damaged and appeared red and glistening but no bleeding was observed. Microscopically, this procedure resulted in the partial removal of the epidermal layer. Infection was initiated by placing a 50 mL of stationary-phase culture of S. aureus suspension containing ~5 × 10⁸ cells on the tape-stripped skin. To confirm the establishment of wound infection, mice were euthanized at different time points and ~ 2 cm² wounds were excised. Wound tissue was divided into two parts. One part was used for determining bacterial counts by colony-forming units (CFU) and the other part was used for PSOCT and histological studies. The number of CFU in the wounds was assessed at different post-infection time points.

For CFU determination, the tissue was homogenized in 2 ml of phosphate-buffered saline (PBS) using a hand homogenizer. Homogenized samples were diluted in PBS and an appropriate volume of the diluted sample was plated on TSB agar plates to determine the CFU. The bacterial counts obtained were normalized with respect to the weight of the infected wound tissue. About 10⁸CFU/gm were recovered from the wound at 24 h
following the application of S. aureus (Figure 6.1). The number of CFU decreased by 1.5 log at 48 h. The bacterial count recorded (~ $10^6$ CFU/gm) at this time point indicated the successful colonization of bacteria in the wound [117]. Beyond 96 h, the bacterial counts were negligible.

![Graph showing post-infection time-dependent bacterial counts obtained from wounded skin tissue of mice.](image)

Figure 6.1: Post-infection time-dependent bacterial counts obtained from wounded skin tissue of mice. The error bar represents the standard deviation around the mean calculated from three different experiments. Data represented at each infection time point are obtained from three mice.

6.4 **Ex-vivo imaging of healing of wounds using PSOCT and Histology**

The PSOCT setup used for the ex-vivo imaging is described in chapter 2 of the thesis. Briefly, it measures the polarization components of the back scattered light from sample and calculates the intensity or reflectivity images and the birefringence images of tissue samples. For imaging, resected wounded skin was marked with an ink pen at regions of interest and placed on a linear translation stage.
In order to characterize the histopathology of the wound model, wounded skin close to the OCT imaged regions was excised on days 2, 4 and 10 of tape stripping and fixed in formalin (4%). The formalin-fixed biopsy specimens were processed using a standard histological procedure and stained with hematoxylin and eosin. The slides were examined under a microscope to determine tissue changes such as inflammation and reepithelization.

Figure 6.2: PSOCT image of the normal mice skin. Back scattered (a; image size: 1.5 mm × 3 mm), optical retardation (b; image size: 1.5 mm × 3 mm) OCT images of resected mice skin and the histology image (c) of the corresponding tissue. Scale bar: 200 µm.

Figure 6.2 (a) shows the backscattered intensity OCT images of normal mice skin. The upper layers in the skin correspond to the epidermis, which is approximately 50 ± 12 µm. The relatively higher scattering region of ~ 250 ± 12 µm below the epidermis corresponds to the dermis. The corresponding cumulative optical retardation (i.e. phase difference induced in the two orthogonally polarized light beams in the given thickness of the tissue) is shown in Figure 6.2 b. The transition from black to white bands in the PSOCT image of the dermal region represents the change in the accumulated phase retardation from 0 to π/2. This can be attributed to higher collagen density or organized collagen [128]. Figure 6.2 c shows the histology of the skin section imaged with OCT. The thicknesses of the epidermis and the dermis measured in this image are 30 ± 12 µm and
150 ± 15 µm, respectively. These values are lower (~ 1.5 times) than the values measured from the OCT images. This is attributed to the shrinkage of the tissue caused by histological processing [131].

Figure 6.3: Time-dependent structural changes in uninfected wound skin of mice. Left (a, d, g), middle (b, e, h) and right (c, f, i) panels represent backscattered intensity OCT images, PSOCT images and histological images, respectively. Top (a–c), middle (d–f) and lowermost (g–i) rows represent images of resected wounded skin sample imaged on days 2, 4 and 10 of wounding, respectively. OCT images; image size: 1.5 mm × 3 mm. Histology images; scale bar: 100 µm.

Figure 6.3 shows the representative backscattered intensity (a, d, g), birefringence (b, e, h) and the corresponding histological images of uninfected wounded skin on days, 2, 4 and 10. On day 2, a bright scattering discontinuous layer appeared at the top that corresponded with the early crust (Figure 6.3; a–c). The back-scattered intensity in the epidermis and dermis region of the wound tissue (Figure 6.3; a) was lower and more nonuniform than that of normal skin, which indicated loosening of the tissue matrix due to
tissue inflammation [132]. In addition, several signal-free regions were also observed in these images (arrows), which may be due to the development of edema. These scattering changes were comparable with the histological images (Figure 6.3; c). Further, in contrast to normal tissue, scattering changes corresponding to adipocytes and the muscle layers below the dermis were clearly observed. This indicated an increase in imaging depth. In the corresponding PS-OCT image, accumulated phase retardation decreased with respect to that of the normal skin, indicating randomization in collagen (Figure 6.3; b). On day 4, the backscattered intensity of the top layer appeared brighter and continuous as compared with day 2, indicating the presence of a continuous crust layer (Figure 6.3; d). Inflammation at this time point was much lower as compared with that of day 2. In addition, a new scattering layer appeared below the edematous regions of the dermis corresponding to a new epithelial layer formation as observed in the histology image (Figure 6.3; f). The appearance of a high scattering layer below the newly formed epithelial layer suggested the formation of granulation tissue [132]. The PS-OCT image at this time point showed an increase in retardation with respect to that of day 2, indicating an increase in tissue compactness (Figure 6.3; e, f). With an increase in the post wounding time (day 10), the intensity of back-scattered light increased. In the OCT images, the granulation tissue appeared more uniform compared with that of the earlier time points, and the dermis epidermis junction (DEJ) was clearly visible (Figure 6.3; g). A relatively higher retardation observed on day 10 (Figure 6.3; h) indicated the recovery of collagen morphology. The histology image showed an almost reconstituted dermis and some budding hair follicles (Figure 6.3; i).
Figure 6.4: Postinfection time-dependent structural changes in infected wound skin of mice. Left (a, d, g), middle (b, e, h) and right (c, f, i) panels represent backscattered intensity OCT images, PS-OCT images and histological images, respectively. Top (a–c), middle (d–f) and lowermost (g–i) rows represent images of resected infected skin sample imaged on days 2, 4 and 10 of wounding, respectively. Image size: 1.5 mm × 3 mm.

Figure 6.4a shows the backscattered images of an infected wound on day 2. Compared with the uninfected wound, the back-scattered intensity from the dermis regions of infected wound tissue was significantly lower. Inflammatory, edematous regions in the dermis below the crust were clearly observed in these images (Figure 6.4 a, b). These observations were comparable with the histology images (Figure 6.4 c). On day 4 (Figure 6.4 d), the severity of edema and inflammation did not show a significant change compared with that of day 2. However, in comparison with the uninfected counterpart, the severity was higher. Further, unlike in the uninfected wound, reepithelialization and granulation tissue were not observed at this time point. Compared with the uninfected
wound, nonuniform scattering and many signal-free regions (Figure 6.4 g) were still evident on day 10 in the infected wound skin, and phase retardation images also showed a disordered collagen morphology. However, the new epithelium started to appear at this time point. These morphological features were comparable with the histology features (Figure 6.4 i) that showed the presence of an incompletely reconstituted dermis.

![Graph showing phase retardation values for normal, uninfected, and infected wounds](image)

Figure 6.5: Measured mean phase retardation of normal (N), wound skin without (UI) and with infection (I) measures along the skin depth on day 10 of wound creation. Individual columns represent the mean value per sample measured from six different images taken from two different experiments. The scale bars represent standard deviation around mean. The statistical significance was determined using oneway ANOVA (*Po 0.05).

In Figure 6.5, a comparison of the phase retardation values of normal, uninfected and infected wounds on day 10 is shown. A significant reduction in phase retardation was observed in both infected and uninfected wounds, with infected wounds showing a lower value (~45%). Student’s t-test was used to determine the significance of the difference between two means. One-way ANOVA was used to compare the significance of the
difference in the phase retardation values of different groups. The level of significance was set at $P < 0.05$.

### 6.5 *In-vivo* monitoring of wound healing

![Image of skin and wound healing stages](image)

Figure 6.6: Kinetics of healing of uninfected and infected wound imaged using real-time OCT. Top panel (a): image of normal skin. Image size: $1.5 \text{ mm} \times 3 \text{ mm}$. The images show, compared with uninfected infected wounds, a delay in different phases of wound healing.

Wound-healing in infected and uninfected mice under *in-vivo* conditions were also investigated using a real-time OCT imaging setup, the details of which are presented in
chapter 3. In each group (uninfected and infected), a single mouse was used for monitoring the morphological changes during wound healing. Before OCT imaging, the mice were anesthetized and placed on the XY stage. Figure 6.6 shows the back-scattered intensity (in-vivo) images of uninfected (left panel) and infected mice (right panel) skin on days 2 (b, c), 4 (d, e) and 10 (f, g). The discontinuous scattering observed from the new epithelium of uninfected mice on day 4 becomes uniform on the 10\textsuperscript{th} day. The back-scattered intensity images of normal skin, uninfected and infected wounds monitored using real-time OCT were comparable to that of resected normal and wounded skin imaged using the PSOCT set up.

6.6 Discussion

In this study, PS-OCT and real-time OCT imaging were used to characterize the different phases of healing of uninfected and S. aureus-infected superficial skin wounds under ex-vivo and in-vivo conditions. The changes observed in the OCT images of wounded skin on day 2, such as an increase in imaging depth compared with normal skin and the presence of signal-free regions (Figure 6.3; a–c) corresponding to edema, indicate an early inflammatory response of the wound tissue. The inflammatory phase is the first step in the wound-healing process comprising both a vascular and a cellular response [133]. The vascular response involves local vasodilatation, blood and fluid extravasations into the extra vascular space, and blockage of lymphatic drainage leading to the development of edema. The cellular response of inflammation, which normally starts within 24–48 h of wound, is characterized by infiltration of neutrophils and other immune cells. In the OCT images, the non-uniform dermal back scattering (Figure 6.3; a) observed on day 2 is an indication of the presence of inflammatory cells [132]. The migration of immune cells is
accompanied by the production of matrix-degrading hydrolytic enzymes, which initiate the
degradation of the provisional matrix [134]. This may have contributed to the low and
random birefringence observed on day 2 (Figure 6.3; b). The crust layer was also visible in
the OCT images at this time point. These observations are in agreement with the wound-
healing-associated morphological changes in mice reported using ultra-high-resolution
OCT [128]. In the OCT images, the presence of a noticeable bright scattering layer below
the crust layer of wound on day 4 (Figure 6.3; d) indicated the formation of a new
epithelial layer [135]. This corresponded with the proliferative phase of wound healing.
During this phase, the epidermis is restored following a cutaneous injury. In addition,
below the new epithelial layer, a relatively uniform high scattering layer was observed.
This indicated the onset of granulation on the fourth day after wound creation in
uninfected wounds (Figure 6.3, d) during which new collagen is synthesized [115]. On day
4, the increase in birefringence, tissue compactness and decrease in imaging depth (Figure
6.3, d) also correlated with the formation of granulation tissue. More intense
backscattering and ordered birefringence observed on day 10 in the wounded tissue than
on previous days suggests restoration of collagen corresponding to the remodeling phase
of wound healing. It has been reported that 2–3 days after wound infliction, collagen is
deposited [136]. This is randomly oriented initially and becomes reoriented in an orderly
fashion during the late granulation stage [133]. In some regions, higher birefringence than
that of normal skin was also observed, suggesting an increase in collagen synthesis.
Previous studies have shown that the collagen content increases during early wound repair,
peaks around 2–3 weeks after injury and returns to the preinjury morphology over a longer
period [115]. Concurrent with these dermal changes, on day 10, the reepithelialization was
almost complete and was in agreement with the histology (Figure 6.3; i). The results
presented in this study showed that the structural changes associated with the healing of
infected wounds were different as compared with the uninfected wounded tissue. Unlike the uninfected wounds, infected wound tissue showed persistence of inflammation and edema on day 10 even when the bacterial count was negligible after day 4. It may also be noted that in the uninfected wound, the reepithelialization and granulation started around day 4, but in the infected wound, both the processes were delayed almost by a week. The lower phase retardation value (Figure 6.5) and the higher imaging depth observed on day 10 in the infected wound in comparison with that of the uninfected counterpart are suggestive of a degraded collagen matrix and a delay in the collagen remodelling phase. Our real-time OCT images (Figure 6.6) also showed kinetics of wound healing under in-vivo conditions that had features like edema and reepithelialization similar to that observed in resected tissue using PS-OCT. Our OCT results also showed that even in the epidermal wounds generated by tape stripping, the collagen morphology was disturbed, which was repaired during the healing process. The appearance of epidermal invaginations observed in the epithelial layer of wounded skin during the healing process in the histology images confirms the renewal of hair follicle growth. This observation shows that the dermis and the dermal appendages were recycled during the repair of epidermal wound healing. These results are in agreement with the epidermal wound healing reported by Sugata et al. [137], wherein they have shown the involvement of dermal papilla in the restoration of the epidermal layer using confocal microscopy.

### 6.7 Summary

PSOCT imaging was used to monitor the differences in the morphological and birefringence changes in resected tissues from bacteria-infected and uninfected wounds. The observed results have been validated by histological studies. A real-time OCT was
also used to monitor the progress of healing during infection in a single animal. This imaging technique can provide a rapid assessment of morphological changes, thereby facilitating timely treatment planning.