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Raman spectroscopy of bone composition during healing of subcritical calvarial defects

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Abstract: Subcritical calvarial defects heal spontaneously and optical methods can study the healing without mechanically perturbing the bone. In this study, 1mm defects were created on the skulls (in vivo) of Sprague-Dawley rats (n = 14). After 7 (n = 7) and 14 days (n = 7) of healing, the subjects were sacrificed and additional defects were similarly created (control). Raman spectroscopy (785nm) was performed at the two time points and defect types. Spectra were quantified by the mineral/matrix ratio, carbonate/phosphate ratio and crystallinity. Mineral/matrix of in vivo defects is lower than that of controls by ~34% after 7 days and ~21% after 14 days. Carbonate/phosphate is 8% and 5% higher while crystallinity is 7% and 3% lower, respectively. Optical profiling shows that the surface roughness increases 1.2% from controls to in vivo after 7 days, then decreases 13% after 14 days. Overall, the results show maturation of mineral crystals during healing and agree with microscopic assessment.

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OCIS codes: (300.6450) Spectroscopy, Raman; (170.6935) Tissue characterization; (170.6510) Spectroscopy, tissue diagnostics.

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1. Introduction

The bones are complex calcified tissues designed to fulfill important functions like protection, support and locomotion. Bone is a naturally occurring composite material consisting of two main components, the collagen matrix and biological apatite (i.e. bone mineral). The extraordinary toughness and stiffness of the bone is achieved by the unique architecture of the
mineral and matrix. The mineral gives hardness whereas the matrix provides tensile strength and flexibility [1,2].

Millions of people fracture their bones every year. Due to bone injuries, over 6 million patients look for medical care annually in the United States alone. About 5 to 10% of cases require surgical treatment, but most fractures heal without complications [3,4]. Healing occurs through bone formation. During this process, the rapid formation of granulation tissue is replaced by fibrocartilaginous, known as soft callus, which is gradually renewed by a woven bone, known as hard callus [5]. A fracture may fail to heal in some situations [6]. Therefore, it is important to understand the molecular and biochemical mechanisms of normal and impaired healing for the development of effective treatments [7,8]. Traditional methods like radiographic imaging and clinical examination are used to monitor and assess the bone healing process and remain the gold standard. However, these methods are less suited for identifying the composition of the mineral and matrix fashioned during the healing process. Raman spectroscopy is an inelastic light scattering based technique that provides a chemical fingerprint of a sample because the specific molecular bonds vibrate at particular wavenumbers and each molecule has a characteristic spectroscopic fingerprint [9]. In the case of bone, Raman uses the vibrations within the mineral lattice and the protein matrix [10]. Therefore, Raman provides very useful information about the mineral and matrix, complementing the traditional methods.

Raman spectroscopy has been utilized to study the effects of mechanical load on bone, especially tibia/femur fractures. Changes in the phosphate band position as a function of load has been shown [11,12]. Variations in the mineral and collagen spectra have been observed within indents made in bone [13,14] and adjacent to the boundaries of microcracks [15]. In fractured bones, the irregular pattern of mineral loss and reduction in the collagen quality has been shown at fracture boundaries [14]. Bone compositional changes during healing of stress fractures in adult rats has been studied using Raman spectroscopy [5]. After 14 days of healing the bone strength was recovered. Besides studies on limbs bones, calvarial bone defects have gained an extensive reputation amongst the available orthotropic models [16]. Calvaria are the flat bones that comprise the skull. There can be two types of defects based on its size. The critical size defect is the one that does not heal naturally [17,18], whereas in subcritical defects, healing is probable without any assistance. The subcritical calvarial defects that undergo spontaneous healing can be used to monitor the chemical composition. In this study, we prepare the subcritical and shallow calvarial defects to investigate the exact site of fracture injury without any perturbation to the callus formed during healing. This will help to investigate the site of healing for better identification of the variation in bone chemical composition and not require callus removal prior to Raman spectroscopy [19,20].

2. Materials and methods

2.1 Experimental subjects

All aspects of this study were approved by the animal research ethics committees of the City University of Hong Kong, the University of Hong Kong, and the Department of Health of the Hong Kong Special Administrative Region. Male Sprague Dawley (SD) rats (N = 14, 9-11 weeks, ~400 g) were acquired from the Laboratory Animal Unit of the University of Hong Kong. The subjects were housed at the Laboratory Animal Research Unit of the City University of Hong Kong. Subjects were housed in standard cages with temperature of 25 °C, humidity of 60-70%, 12/12 hour light/dark cycle and regular access to chow food and drinking water. Subjects were housed individually to reduce the risk of injuring the surgical site.
2.2 Calvarial fracture model

Subjects were anesthetized by a ketamine/xylazine mixture of 100/10 mg/kg body weight administered via intraperitoneal injection. Their heads were shaved, a 1 cm incision was made, and the skin and subcutaneous tissues were dissected to expose the calvaria. The subject was then mounted on a stereotaxic frame (RWD Life Science Co., Ltd, Shenzhen, P.R. China). A metal burr drill was zeroed at the surface of the parietal bone in one hemisphere. A defect was made with a burr of 1 mm tip diameter with rotation rate of 400 rpm. The defect was made to a depth of ~200 µm, avoiding damage to the dura mater. This in vivo defect was rinsed thoroughly with saline to wash out any bone fragments. After the fracture procedure, the skin was sutured, wiped with 75% ethanol, and antibiotic ointment (polymyxin B) treated to avoid infection (Fig. 1). Post procedure, subjects were given the buprenorphine analgesic, 0.3 mg per 3 ml drinking water, and housed for 7 or 14 days.

![Fig. 1. Subcritical calvarial defect. a) Hair was first removed from the top of the head. b) Skin was then incised. c) A 1 mm calvarial defect was created using a burr (in vivo defect). d) The skin was sutured after the procedure.](image)

2.3 Sample preparation

Subjects were euthanized after 7 or 14 days healing (n = 7 each) using 1 mL/kg body weight of Dorminal 20% (pentobarbital) (Alfasan International B.V., Woerden, Holland) via intraperitoneal injection. One more (control) defect was created using the same procedure on the opposite parietal bone from the in vivo defect. Surgical scissors were then used to decapitate and soft tissues were stripped off to harvest the skull. The harvested skull was wiped thoroughly with 75% ethanol. A bone saw (Extra Fine Razor Saw, X-ACTO, USA) was used to further cut down the skull and obtain the required portion of skull surface (Fig. 2). The samples were soaked in 99.9% acetone for 2 hours to remove remaining fats and blood stains and stored in a −20 °C freezer until Raman spectroscopy.

![Fig. 2. Calvarial defects viewed under magnification (2X). Subject was sacrificed, the skull was harvested, and the calvarium was cut down to approximately 2 x 1 cm for Raman spectroscopy. In vivo and control defect (latter created after sacrificing) on parietal bones are labelled. Red triangles show the spots of laser sites on distant (normal surface) bone around the defects within 2 mm range. In vivo defect is zoomed in to show the laser sites within the defect (red stars). Control defects were also laser scanned in a similar fashion.](image)
2.4 Raman spectroscopy

Raman measurements were attained using a 785 nm, 250 mW excitation laser and a spectrograph with resolution up to 4 cm⁻¹ (RamanStation 400F, Perkin Elmer Inc., MA, USA). The Raman scattering was collected with a high sensitivity open electrode charge coupled device (CCD) with 1024 × 256 pixels. The CCD was stored in a hermetically sealed vacuum and Peltier air cooling was used to operate at a temperature of −50 °C. The spectral range was 400 to 1800 cm⁻¹. The laser spot size of ~100 microns was focused on the sample. Two accumulations and 10 s exposure time were employed. Ten Raman spectra were acquired from each control defect, in vivo defect and at distant bone (normal surface) of the calvaria. The laser sites are shown in Fig. 2. Altogether, 30 Raman spectra/subject were acquired from both 7 and 14 days healing groups. Figure 3 shows the Raman spectrometer setup for this study. The laser was focused on to the defects at ten sites, in a 3-4-3 configuration, as illustrated. Therefore, within each 1 mm calvarial defect, 10 laser exposure sites were selected roughly equidistant from each other to improve the coverage of the defect.

2.5 Optical surface profiling

Optical surface profiling (NT9300, Veeco / Wyko instruments Inc., USA) was used to map the three-dimensional morphology of the defects. Optical profilers are based on interference microscopes that utilize the interference of two beams of light as a ruler for the characterization of surface topographies. During the measurement, light from the illumination goes through an objective lens and is separated into two beams by a beam splitter. The reference beam is reflected from the reference mirror and the test beam is reflected from the sample surface, both back to the objective. The recombination of the two beams form an interference pattern (fringes). These fringes are imaged by the CCD camera and transferred to the computer. The Veeco NT9300 system has an optical resolution minimum of 0.55 µm based on the Sparrow Criteria at 600 nm [21]. A single scan measurement was used and scanning was performed in a vertical scanning interferometry mode in which the objective moves vertically down the full depth range of the sample while collecting frames at the camera frame rate, allowing the measurement of rough surfaces. During the measurement, a 5X objective was used with 0.56X field of view multiplier that gave an effective magnification of 2.8.
2.6 Data analysis

The acquired Raman spectra were individually preprocessed with the “Vancouver Raman Algorithm” [22]. A 5th order polynomial fit was used to remove the fluorescence background and perform base line correction on each spectrum. Spectra from in vivo laser sites were rejected when the background fluorescence (mean signal) was more than 500% compared to spectra from control defects. At such a high fluorescence, apart from phosphate at 958 cm$^{-1}$ and CH$_2$ at 1448 cm$^{-1}$, other bands almost completely overlapped, causing distortions that significantly affected later analysis steps such as integrated area calculation. The remaining data was then imported to Origin Pro 8.5 SR1 for further processing. Firstly, smoothing was performed by a local polynomial regression using Savitzky-Golay method with 13 points window and 2nd order polynomial. This filter removed noise without affecting the overall signal shape. Other preprocessing steps included removal of CCD noise spikes, linear baseline treatment, peaks finding, finding integrated areas of selected bands, averaging the respective groups and finally normalization to the phosphate $\nu_1$ band at 958 cm$^{-1}$.

A Raman spectrum shows a strong molecular characteristic related to the internal modes of the PO$_4^{3-}$ tetrahedral [23]. The band in the range 957–962 cm$^{-1}$ correspond to $\nu_1$ stretching of the P-O bond. The 422–454 cm$^{-1}$ band is associated with $\nu_2$ bending of the O-P-O in PO$_4^{3-}$. The 568–617 cm$^{-1}$ band is assigned to $\nu_4$ bending of PO$_4^{3-}$. The 1065-1071 cm$^{-1}$ is related to $\nu_1$ stretching of CO$_3^{2-}$. The above are mineral bands whereas the bands corresponding to the collagen matrix are at 851-855 cm$^{-1}$ (proline), 870-873 cm$^{-1}$ (hydroxyproline), 1243–1269 cm$^{-1}$ (amide III), 1447–1452 cm$^{-1}$ (CH$_2$ wag) and 1595–1720 cm$^{-1}$ (amide I) [1].

Three key parameters used to quantify the bone composition in this study are mineral to matrix ratio, carbonate to phosphate ratio and crystallinity [24]. Mineral to matrix ratio can be obtained by calculating the ratio of integrated areas of any of the phosphate and amide peaks or CH$_2$ bending peak or proline peak [25]. In this study, it was defined using two ways. First, dividing the area of $\nu_1$ phosphate band (at 958 cm$^{-1}$) by the area of Amide III band (at 1245 cm$^{-1}$). Second, dividing the area of the same $\nu_1$ phosphate band by CH$_2$ band (at 1448 cm$^{-1}$). Carbonate to phosphate ratio was defined by the area of the carbonate band (at 1071 cm$^{-1}$) divided by the phosphate band (at 958 cm$^{-1}$). Crystallinity was defined by the inverse of the full width at half maximum (FWHM) of the $\nu_1$ phosphate band (at 958 cm$^{-1}$). All the parameters were calculated per spectrum, and then averaged for each specimen, resulting in a single value per defect and subject.

The data acquired through the optical surface profiler was analyzed using “Vision 32” software (WYKO). The average roughness “R$_a$” was calculated for all the defects within the 1.8 × 1.8 mm profiling area. This parameter is defined as the arithmetic average of all deviations from the mean center line of the roughness profile. This is the most often used surface roughness parameter and has been reported in studies of bone implant materials [26,27]. Root mean square roughness “R$_q$” and the average depth of the defects were also calculated.

Two-way ANOVA was used for statistical analysis at both time points and implemented using GraphPad Prism (version 5.01). Bonferroni post-tests were used to compare the spectroscopic Raman shifts, Raman area intensities and Raman bone parameters for control, in vivo, and surface. p < 0.05 was considered statistically significant.

3. Results and discussion

Figure 4 shows the normalized Raman spectra (averaged across subjects) acquired from the undrilled surface, control defects and in vivo defects after 7 and 14 days of bone healing. A figure showing average values with standard deviations among the 7 and 14 days subjects corresponding to the surface, control and in vivo defects has been provided in Appendix. The
peaks occurring at 431 cm\(^{-1}\) (\(\nu_2\) phosphate), 588 cm\(^{-1}\) (\(\nu_4\) phosphate), 958 cm\(^{-1}\) (\(\nu_1\) phosphate) and 1071 cm\(^{-1}\) (\(\nu_1\) carbonate) are from bone mineral, which are responsible for vibrations of carbonated calcium phosphate in an apatitic lattice. The peaks at 1245 cm\(^{-1}\) (amide III), 1448 cm\(^{-1}\) (CH\(_2\) bending / deformation) and 1661 cm\(^{-1}\) (amide I) are from the collagen matrix, which are responsible for the protein vibrations [24]. The \(\nu_1\) phosphate band is the strongest marker of the bone mineral and the peaks of amide I and amide III are mainly due to the collagens. Amide I band is mostly linked with the C = O stretching vibration and amide III with C–N vibration [28].

Fracture healing is a complex process where revascularization is vital and adequate blood supply is essential for successful bone repair [29]. Fluorescent blood products in bone cause strong background features, which results in obscured bone Raman spectra [30]. Possible candidates of fluorophores could be residual hemoglobin, proteins, components of the collagenous matrix [31]. The spectra from surface and control defects after both 7 and 14 days healing almost overlap. In contrast, the raw spectra from 7 days and 14 days in vivo defects was considerably higher than the rest of surface or control defects spectra. Figure 4 shows the normalized spectra after background fluorescence removal. The 7 and 14 days in vivo spectra show a vivid difference, especially at collagen peaks. These differences are expected as the in vivo defects have undergone bone regeneration. In contrast, control defects have similar Raman spectra compared with normal bone surface. In general, the 14 days in vivo spectra are more similar to those of control and surface compared with 7 days in vivo spectra, suggesting healing.

Also from Fig. 4, the integrated area as well as the Raman shift (or spectroscopic shift) of the 958 cm\(^{-1}\) phosphate \(\nu_1\) band is similar in all sample types and time points. It was found that Raman shifts are slightly but significantly different (\(p < 0.05\)) between surface and in vivo and between control and in vivo at 1245 cm\(^{-1}\) (Amide III) band. It is noteworthy to mention here and has been discussed by Carden et al. [13] that spectroscopic shifts in amide I
and III bands are observed in response to mechanical loading or deformation and are indicative of ruptured cross links in the organic matrix. Moreover, this damage occurred at the edges of the indents rather than at the center. In our case, there were not any Raman shifts recorded between the surface and control defects. These results suggest that the burring process alone, used to create defects, did not result in any significant spectroscopic shifts. After 14 days healing, there is no statistical difference found (p > 0.05) between surface and in vivo in area peak intensities. This suggests that the defect is healing to resemble normal bone (i.e. surface) after 14 days. Comparing across time points (7 and 14 days), spectra from the corresponding surface and control defects are similar (p > 0.05). The in vivo spectra after 7 and 14 days healing are significantly different (p < 0.05), indicating the impact of the healing process on mineral and matrix composition.

The mineral to matrix ratio is a compositional Raman parameter linked to bone mechanical strength, which can be degraded by the increase or decrease of mineral content [32]. Mineral/matrix is used to estimate the overall bone mineralization. Figure 5 presents the mean with standard deviations of the Raman parameters and Table 1 and 2 lists the corresponding p-values for comparisons between the three sample types and two time points. After 7 days healing, both mineral/matrix ratios (υ1 PO₄⁻³ / Amide III and υ1 PO₄⁻³ / CH₂ wag) of in vivo defects are decreased relative to control defects and normal surface, indicating significantly lower bone mineralization (p < 0.05). Using the υ1 PO₄⁻³ / Amide III definition, the percentage decrease of mineral/matrix from the surface to in vivo defect is 22% and from control to in vivo is 34%. Using the υ1 PO₄⁻³ / CH₂ wag definition, the decrease from surface to in vivo is 24% and from control to in vivo is 34%. After 14 days healing, mineral/matrix is still significantly lower (p < 0.001) in in vivo defects compared with control defects using both definitions. But compared to normal surface, it became non-significant (p > 0.05, via υ1 PO₄⁻³ / Amide III definition) or slightly significant (p < 0.05, via υ1 PO₄⁻³ / CH₂ wag definition). The decrease in mineral/matrix from surface to in vivo is just 6% (using υ1 PO₄⁻³ / Amide III) and 21% (using υ1 PO₄⁻³ / CH₂ wag). The decrease from control to in vivo defects is 21% (using υ1 PO₄⁻³ / Amide III) and 31% (using υ1 PO₄⁻³ / CH₂ wag). P-values are summarized in Tables 1 and 2. The difference in mineralization of 14 days samples by the two definitions is likely due to the different orientations of specimen under the objective of the Raman microscope. It has been shown by Nyman et al. that υ1 PO₄⁻³ / Amide III is the Raman property likely to detect a compositional difference when there is a limited sample size and sufficiently large number of Raman spectra per specimen [25], similar to our study. Both definitions show similar trend. Also, extending from 7 to 14 days healing yields 36% improvement in mineral/matrix using υ1 PO₄⁻³ / Amide III and 18% improvement using υ1 PO₄⁻³ / CH₂ wag. These results are indicative of new mineral deposition and is consistent with the theory that significantly increased remodeling begins to overt fracture and as a result, much of the bone tissue is relatively young [33]. The average mineral/matrix ratio decreases as a result of increased remodeling because younger bone tissue contains relatively less mineral than older existing bone. These results are consistent with McCreadie et. al finding of decreased bone mineralization in the fractured region [14]. Moreover, there is a consistent increase found in mineralization from surface to control defects at both times (19%, via υ1 PO₄⁻³ / Amide III definition, 14% via υ1 PO₄⁻³ / CH₂ wag definition). The possible explanation for this finding is that calvarial bones consist of layers of compact bone, separated by diploë, which is a cancellous bone made of trabeculae. Each bone sample is composed of complex structural units created at different time points therefore have different level of mineralization. Recently studied computational approach of localized tissue mineralization by Berli et al. found that a less mineralized bone is situated closer to the surface while a more mature and mineralized bone is buried deeper in the inner cores of the trabeculae [34]. It should also be noted that lipids have characteristic bands around 1442-1444 cm⁻¹ [35]. In the in vivo spectra, there might be overlap of lipid and protein bands close to the
1448 cm$^{-1}$ band in this study. However, the Raman differences between in vivo and control/surface are driven primarily by other bands like $\nu_1$ phosphate and Amide III.

![Raman parameters after 7 and 14 days healing in the three sample types. Data are represented as means ± standard deviation. Mineral to matrix ratio a) $\nu_1$ PO$_4^{3-}$/Amide III and b) $\nu_1$ PO$_4^{3-}$/CH$_2$ wag, c) carbonate to phosphate ratio and d) crystallinity.](image)

**Table 1.** Percentage differences along with statistical comparison of Raman parameters used to assess bone composition in this study. Comparisons are made between sample types at the two time points. S indicates surface, C indicates control, IV indicates in vivo, (*) indicates percentage increase, and (↓) indicates percentage decrease. Two-way ANOVA with Bonferroni posthoc tests were used to calculate significance. NS stands for not significant (p ≥ 0.05), * indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001.

| Raman parameter  | Quantification | Percentage differences with p-values |
|------------------|----------------|-------------------------------------|
|                  |                | 7 days | 14 days | 7 days | 14 days | 7 days | 14 days |
|                  | S / C | S / IV | C / IV | S / C | S / IV | C / IV |
| Mineral / Matrix | $\nu_1$ PO$_4^{3-}$/Amide III | 19% (↑) | 22% (↓) | 34% (↓) | 19% (↑) | 6% (↓) | 21% (↓) |
|                  | *     | **     | ***    | *     | NS     | ***    |
|                  | $\nu_1$ PO$_4^{3-}$/CH$_2$ wag | 14% (↑) | 24% (↓) | 34% (↓) | 13% (↑) | 21% (↓) | 31% (↓) |
|                  | NS    | **     | ***    | NS    | *      | ***    |
| Carbonate / Phosphate | CO$_3^{2-}$/ $\nu_1$ PO$_4^{3-}$ | 3% (↓) | 5% (↑) | 8% (↑) | 3% (↓) | 1.5% (↑) | 5% (↑) |
|                  | NS    | *      | *      | NS    | NS     | NS     |
| Crystallinity    | Inverse of FWHM of $\nu_1$ PO$_4^{3-}$ | 1% (↑) | 6% (↓) | 7% (↓) | 1% (↑) | 2% (↓) | 3% (↓) |
|                  | NS    | ***    | ***    | NS    | NS     | NS     |
Table 2. Comparisons are made between two-time points corresponding to sample types. Legends are explained in Table 1’s caption.

| Raman parameter | Quantification | Percentage differences with p-values |
|-----------------|----------------|--------------------------------------|
|                 |                | 7d S / 14d S | 7d C / 14d C | 7d IV / 14d IV |
| Mineral / Matrix| $v_1 \text{PO}_4^{3-}$ / Amide III | 13% (↑) | 14% (↑) | 36% (↑) |
|                 | $v_1 \text{PO}_4^{3-}$ / CH$_2$ wag | NS | NS | NS |
| Carbonate / Phosphate| $\text{CO}_3^{2-}$ / $v_1 \text{PO}_4^{3-}$ | 0.5% (↑) | 0.04% (↑) | 3% (↓) |
| Crystallinity | Inverse of FWHM of $v_1 \text{PO}_4^{3-}$ | 0.3% (↓) | 0.6% (↓) | 4% (↑) |

The carbonate to phosphate ratio is another important Raman parameter that determines the changing composition during fracture healing. Carbonate/phosphate shows the substitution of carbonate group to phosphate group in bone mineral crystals [30]. After 7 days healing, the percentage increase of carbonate/phosphate is significant (p < 0.05), 5% from surface to in vivo and 8% from control to in vivo. After 14 days healing, the percentage increase becomes nonsignificant (p > 0.05), 1.5% from surface to in vivo and 5% from control to in vivo. Comparing the 7 and 14 days in vivo defects, 3% decrease in carbonate substitution was found after 14 days. Increase in carbonate/phosphate ratios reflects the concentration and availability of carbonate ions in the mineral environment [36]. Based on the results, fracture healing causes new mineral deposition and remodeling. The carbonate ions present in the mineral environment are incorporated into the apatitic lattice, resulting in the increase of carbonate substitution to phosphate ions. This substitution tends to decrease after 14 days.

The mineral crystallinity reflects the mineral crystal size [30]. After 7 days, the percentage decrease of crystallinity is highly significant (p < 0.001), 6% from surface to in vivo and 7% from control to in vivo. After 14 days, the percentage decrease becomes nonsignificant (p > 0.05), 2% from surface to in vivo and 3% from control to in vivo. The increase in crystallinity from 7 to 14 days healing is 4%. The reduced crystallinity suggests that younger and hence, smaller crystals are formed at the injured site. As the bone heals, the crystallinity increase is related to the maturation of crystals. It is interesting to note that during 7 and 14 days healing, the increase in carbonate/phosphate (Fig. 5(c)) occurs alongside the decrease in crystallinity (Fig. 5(d)). Previous studies of synthetic carbonated apatites have shown that higher carbonate content resulted in decreased crystallinity and vice versa [37]. Affirming the trend, our results also suggest that as the bone heals, there is less carbonate substitution and improved crystallinity.

During the healing of calvarial defects a cartilaginous callus (or soft callus) is formed that later undergoes mineralization, resorption and is then replaced by a hard callus and ultimately remodeled to new bone [38]. In animal models (rat, mouse, rabbit), the peak of soft callus formation happens 7 to 9 days post trauma [39]. During this time, intramembranous ossification response generates a hard callus. The peak of the hard callus development is usually reached by day 14 as defined by histomorphometry of mineralized tissue [39]. The calcified cartilage is replaced with woven bone as the hard callus formation progresses and it becomes mechanically rigid and more solid [40].
Figure 6 shows digital microscope images of surface, control and in vivo defects on parietal bones after 7 and 14 days under 300X magnification. Callus formation can be seen after 7 days in in vivo defects when compared with control defects and normal calvarial surface. The red arrows in the figure indicate soft callus whereas the green and blue arrows show hard callus and matured or healed bone, respectively. In Fig. 6(c), the soft callus formation can be seen at discrete sites throughout the defect with gaps between. In Fig. 6(d), the defect after 14 days is in the process of bridging gaps with new callus formation. Figure 7(a) shows the image acquired from a 14 days in vivo defect using the Raman spectrometer’s microscope. As discussed in section 2.4, ten Raman spectra were acquired from multiple locations for each defect. Here, three locations (Fig. 7(a)) and their corresponding Raman spectra (Fig. 7(b) are shown. Location 2 is a newly formed soft callus, location 3 is a matured or almost healed bone while location 1 is a hard callus. The resulting Raman spectra show clear differences. Lower signal to noise ratio of Raman spectra from location 2 is indicative of large fluorescence that arise because of the complexity of newly formed soft callus, naturally accompanied by large blood vessels and proteins. Compared to Fig. 4, the soft callus spectrum is similar to the 7 days in vivo spectra whereas the healed bone spectrum is similar to the control and surface spectra. The hard callus spectrum lies between the soft callus and matured bone spectra. Therefore, the results in Figs. 4 and 5 and Table 1 and 2 are closely related to the extent of soft callus, hard callus and healed bone in the sample at 7 and 14 days.
Fig. 7. a) Raman spectroscopy measurement sites in an in vivo defect after 14 days of healing. The red arrow indicates the laser focused on a site of soft callus formation. The green arrow shows a hard callus site and the blue arrow shows a healed bone site. b) The corresponding normalized spectra acquired from the sites in a).

To further assess callus formation and its maturation after 7 and 14 days of healing, optical surface profiling was performed. Figure 8 shows the depth profiles of control and in vivo defects after 7 and 14 days of healing. The surface roughness parameters $R_a$ and $R_q$ are shown in the top left of the figure panels. Comparing with control, the in vivo defect after 7 days (Fig. 8(b)) showed a slight increase in surface roughness whereas the in vivo defect after 14 days (Fig. 8(c)) showed a decrease in surface roughness as indicated by $R_a$ and $R_q$. The depth profile shows that the control defects are relatively flat. The 7 days in vivo defect shows relatively rough surface due to soft callus formation and with depth of $170 \pm 5 \mu m$ (± indicates the standard deviation among the samples). For reference, control defects are $190 \pm 5 \mu m$ deep. The depth of 14 days defects was $130 \pm 5 \mu m$. These results show the pattern of surface healing over a two weeks span. Callus formation at discrete sites leads to increased roughness after 7 days. By 14 days, bony bridges and hard callus form, reducing the roughness. This profiling supports that the differences between 7 and 14 days in vivo Raman parameters are closely related to the callus formation during healing.

Fig. 8. 3D morphology of control and in vivo defects after 7 and 14 days of healing. a) Control, b) 7 days in vivo and c) 14 days in vivo. Measurements were performed using a surface optical profiler (NT9300, Veeco / Wyko) as described in the methods section. Vision 32 was used to calculate the arithmetic average “$R_a$” and root mean square “$R_q$” of surface roughness.

4. Conclusion

Bone compositional changes are vital to the fracture healing process. The aim of this study was to assess the compositional changes during healing using optical methods. We studied a subcritical calvarial defect model which allows monitoring and identifying the changes in bone composition during healing at the exact site of injury. At both 7 and 14 days healing time points, the Raman spectra and parameters largely overlapped in normal calvarial surface.
and control defects while the main difference was between in vivo defects and normal bone surface or control defects. This supports that the model can study healing without complications from heterogeneity in bone or mechanical perturbations of the healing surface. After 7 days of healing, lower mineral to matrix ratio, higher carbonate to phosphate ratio and decreased crystallinity was found in in vivo defects compared with surface or control. After 14 days, as the bone further heals, mineral/matrix increased, carbonate/phosphate reduced and crystallinity increased towards normal. These Raman results were supported by microscopy images and optical surface profiling that show the formation of soft callus after 7 days healing and replacement of soft by hard callus after 14 days.

Appendix

![Average Raman spectra with standard deviations among the subjects](image)

Fig. 9. Average Raman spectra with standard deviations among the subjects (a) 7d surface, (b) 7d control, (c) 7d in vivo, (d) 14d surface, (e) 14d control, and (f) 14d in vivo defects. Error bars indicate standard deviation. Spectra from 7 days in vivo defects show relatively large standard deviations.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.