Investigating the feasibility of spatially offset Raman spectroscopy for in-vivo monitoring of bone healing in rat calvarial defect models

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Abstract
A wide range of biomaterials and tissue-engineered scaffolds are being investigated to support and stimulate bone healing in animal models. Using phantoms and rat cadavers, we investigated the feasibility of using spatially offset Raman spectroscopy (SORS) to monitor changes in collagen concentration at levels similar to those expected to occur in vivo during bone regeneration (0.0-0.84 g/cm³). A partial least squares (PLS) regression model was developed to quantify collagen concentration in plugs consisting of mixtures or collagen and hydroxyapatite (predictive power of ±0.16 g/cm³). The PLS model was then applied on SORS spectra acquired from rat cadavers after implanting the collagen: hydroxyapatite plugs in drilled skull defects. The PLS model successfully predicting the profile of collagen concentration, but with an increased predictive error of ±0.30 g/cm³. These results demonstrate the potential of SORS to quantify collagen concentrations, in the range relevant to those occurring during new bone formation.

KEYWORDS
bone, connective tissue, regenerative medicine, SORS

1 INTRODUCTION

Bone healing involves complex spatial- and time-dependent molecular processes related to the development of extracellular matrix and deposition of the hydroxyapatite (HA) mineral phase. Understanding these molecular processes is vital for developing optimized scaffolds and grafts that can stimulate bone regeneration in patients. X-ray imaging is the main technique currently used for monitoring bone regeneration in vivo, but provides limited information: it shows the spatial distribution of the mineral components of bone but it is insensitive to the organic molecules forming the extracellular matrix [1]. In addition, X-rays can have health risks to both patients and the clinical practitioners and therefore limits X-rays use for monitoring new bone formation [2]. Current bone healing studies in animal models (e.g., rats, sheep) use micro-computed tomography (CT) to image bone...
resections corresponding to particular time points of the study [3, 4]. Micro-CT provides detailed information regarding the morphology and mineral structure of the bone but lacks molecular specificity. A key challenge in understanding bone regeneration is measuring the subtle molecular processes related to the extracellular matrix, which occur at the earlier stages of the healing process and are the precursors of the later stages of bone mineralization.

In this paper, we investigated the feasibility of spatially offset Raman spectroscopy (SORS) for in-vivo detection and quantification of molecular changes related to bone extracellular matrix deposition as would be observed in a small animal model. Raman spectroscopy is a nondestructive label-free optical spectroscopy technique that has been widely used for molecular analysis of bone tissue [5], soft connective tissue [6] and tissue engineering scaffolds [7]. The diffuse nature of near-infrared light (785-1000 nm wavelength) in connective tissue and porous tissue engineering scaffolds makes diffuse Raman spectroscopy, such as SORS, ideally suited for probing the biomolecular processes deeper into materials, during regeneration of connective tissue in vivo. SORS is a type of diffuse Raman spectroscopy that can measure molecular information deeper into materials. SORS has been used for characterization of bone tissue, ex vivo and in vivo, in animals and humans [8–11]. While most SORS configurations are based on fiber optical probes, digital micro-mirror devices (DMDs) have been proposed recently to offer a flexible approach to optimize the spatial offsets [12], and have been applied to detect hydroxyapatite (HA) in phantom samples mimicking transcutaneous SORS measurements of bone [13, 14]. In the context of bone regeneration in large animal models, SORS studies using 3D-printed polymer scaffolds and HA have shown that HA can be detected even at depths beyond 10 mm at concentrations 10 times lower than in mature bone [14].

While detailed information regarding the mineral phase of bone can be obtained by measuring the intense bands of HA (eg, 960 cm⁻¹ band), the information regarding the extracellular matrix, which is the focus of this study, is more challenging to extract because of the weaker and overlapping bands. Collagen type 1 makes up close to 30% of healthy mature bone and is vitally important in conveying its structural properties [15, 16]. Collagen is also the first structural component of bone to be formed during bone defect healing [17]. Thus, the ability of SORS to measure the molecular processes at the early stages of bone regeneration would expand the usefulness of bone healing studies in in-vivo models.

Here, we used a series of phantoms to mimic the development of the extracellular matrix (ie, increase in collagen concentration over time) and then the mineralization as previously described in a healing bone defect in a rat calvarial model. The phantoms were designed to follow the expected time course changes in collagen and HA concentrations reported in the literature [17, 18]. Using these phantoms, a multivariate model based on SORS spectra was developed to estimate the concentration of collagen. Next, we applied the SORS model to quantify the concentration of collagen when mixtures of collagen and HA, at different concentrations, were implanted in calvarial defects in rat cadavers. We investigated the feasibility of discriminating the collagen concentration changes expected to occur both during the early stages of extracellular remodeling, as well as at the later stages when the Raman bands of collagen overlap with the strong bands corresponding to HA.

2 | MATERIALS AND METHODS

2.1 | Spatially offset Raman spectroscopy instrument

The SORS instrument was equipped with a 785 nm wavelength laser (Xtra II, Toptica). A 75 mm focal length 50.4 mm diameter lens was used to focus the laser beam (power 120 mW) on the sample (spot size ~0.3 mm) and to collect the backscattered Raman photons. After passing through a dichroic filter that reflected the elastically scattered photons, the Raman photons were focused with a lens (focal length 75 mm) onto a software-controlled digital micromirror device (DMD) (size 14.4 mm × 8.8 mm, resolution 1920 × 1080 pixels, DLP6500 Texas Instruments). As the DMD was located in a plane conjugate to the sample, it allowed the selection of multiple spatially offset collection points (diameter each 0.1 mm) distributed in a semi-circle centered on the point conjugate to the laser excitation, equivalent to spatial offset values in the range of 0 to 1.5 mm at 0.5 mm steps [12]. A full schematic of the set up can be found in our earlier work [14]. The Raman photons reflected by the DMD collection points were collimated by a 200 mm focal length lens then focused onto the entrance slit of a spectrometer (Holospec, Andor) equipped with a deep-depletion back-illuminated CCD (iDus 420, Oxford Instruments). The slit was opened to 4 mm width, as the spectral resolution was defined by the size of the DMD collection points [19, 20]. For a selected spatial offset, the SORS spectrum was calculated as the sum of the spectra corresponding to all DMD collection points on the corresponding semi-circle, after aligning and calibrating them along the wavenumber axis [12]. Each SORS spectrum was the average of 10 measured spectra, each
acquired at 50 seconds integration time. A polynomial baseline (third order) was subtracted to remove the background in the Raman spectra.

### 2.2 Calvarial bone regeneration simulation model

Figure 1A describes schematically a simplified pattern of the two main components of bone over time for a healing bone defect in a rat skull using values taken from the literature [17, 21]. This pattern was used to select the concentrations of collagen and HA in the phantom samples, as collagen and HA concentrations change during the healing process (Figure 1B).

After the initial bone damage (day 0), the repair process starts with inflammation around the area. Soft tissue in the form of collagen enters the defect to form the extracellular matrix within the first day or two post-damage. The concentration of collagen continues to increase during the next 4 to 5 days, as HA diffuses into the formed extracellular matrix and mineralization is initiated. After day 8 to 9, the density of collagen decreases as the bone mineralizes until the densities of collagen and HA reach the level found in normal bone. For this study, six time-points were selected between the initial condition to the steady state at the end, with concentrations of collagen between 0.085 and 0.43 g/cm³. The maximum concentration of collagen was 0.84 g/cm³, corresponding to day 7 (stage 4).

### 2.3 Partial least squares model

A prediction model was build using n = 52 phantom samples that spanned the ranges of both collagen and HA concentrations typically encountered during the entire bone regeneration process (as determined in Section 2.2). A PLS model based on the SORS spectra was developed to predict the concentration of collagen in new samples, using the spectral range 800 to 1606 cm⁻¹. The PLS model was evaluated using a leave-one-out bootstrapping method. Four separate PLS models were built, one for each spatial offset (0 mm, 0.5 mm, 1 mm and 1.5 mm). After the training was complete, the models were saved, and no further spectra were added to the training model. The most effective models, found to correspond to spatial offsets 1 and 1.5 mm, were used in conjunction to form an improved model, whereby the collagen concentration was calculated as the mean of the values predicted by the two models.

### 2.4 Phantom construction

Figure 2A describes schematically the phantom design for the rat model used for developing the PLS model. The

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**FIGURE 1**  A, A schematic description of the four main phases of a bone critical defect healing (based on [16]. B, The time dependence model for collagen (red line) and hydroxyapatite (HA) (black line) concentrations during bone healing [based on [21]. Stages 1–6 are labeled as the points of main interest in this study and are referenced in Table 1.
skull bone was mimicked by a Teflon sheet 1.5 mm thick with a 6.3 mm diameter defect drilled through it. Teflon was selected as it has similar scattering properties to bone [22] while the main Raman band at 734 cm\(^{-1}\) does not overlap with the main bands present in the Raman spectra of the biological materials investigated here. Chicken thigh skin (sourced from local shop) was used as a substitute for the rat skin as the thickness was roughly comparable (~1 mm thick) and more easily sourced. Porcine fat (adipose tissue; also sourced from a local shop) was used to mimic the brain by acting as a bulk scattering material. Fat has a more consistent distribution of material than brain, which led to a more stable phantom for repeated measurements.

Collagen and HA plugs were created by packing the correct amount of collagen fleece and HA into a 6.3 mm diameter mold, with a small amount of water to act as a binding agent, and allowed to dry for 48 hours to make sure there was no residual water. For each measurement a single collagen/HA plug was placed into the Teflon defect. The defect was covered with the skin layer. For SORS measurements, the samples were placed on the
SORS instrument with the skin side facing the laser, which laser focused on the skin at the position corresponding to the defect center.

2.5 Rat cadavers and skull defect

Male Sprague-Dawley rats were purchased from Charles River Ltd, 550 g at time of death, 20 weeks old and sacrificed using a schedule 1 method. All animal studies were conducted in compliance with ethical approval and in accordance with institutional guidelines.

First, the skin was shaved and peeled back from the skull to create a skin flap that could cover the defect. The skull was then drilled with a high speed Dremel rotary tool to minimize cracking or crushing the skull. The defect was drilled just through the skull layer with minimal damage to the brain underneath. The experiment was designed to maximize the similarity between the phantom and the actual skull so the hole was drilled at the center of the skull with a diameter of 6 mm. Collagen and HA plugs were created in the same way as the phantom experiment (Section 2.4) by packing the correct amount of collagen and HA into a 6 mm molds. For each measurement, a single collagen/HA plug was placed into the defect, then the defect was covered with the skin flap.

3 RESULTS AND DISCUSSION

3.1 Investigation on phantom samples: training and validation of the PLS model for prediction of collagen concentration

Figure 2A describes schematically the phantoms used in this study to model the rat calvarial model. SORS spectra obtained from these phantom samples were used to train a PLS model that was used for prediction of collagen concentration for collagen:HA plugs inserted in rat cadavers. Figure 2B compares the SORS spectra (black lines) measured at 1.5 mm offsets with spectra measured at 0 mm offset, for samples containing increasing concentration of collagen and HA, from the minimum 0.085 g/cm³ (Stage 1) to 0.86 g/cm³ (Stages 3-4). For collagen:HA concentrations corresponding to Stage 1 (0.085 g/cm³ collagen, 0 g/cm³ HA), the spectrum measured with 0 mm spatial offset is dominated by the bands at 1445 cm⁻¹ (assigned to C–H deformation vibrations in lipids and proteins), 1200-1400 cm⁻¹ (assigned mainly to Amide III in proteins), 1000-1150 cm⁻¹ (assigned to C=O stretching), 850 and 938 cm⁻¹ bands assigned to proline and hydroxyproline in collagen [23]. A small band at 1002 cm⁻¹, assigned to phenylalanine, can also be observed [23]. These Raman bands are associated to collagen, lipids and other proteins in the skin layer, which is the main contributor to the Raman spectrum.

Figure 2B shows a reference SORS spectrum of collagen in blue line (bovine source). Collagen has a number of bands that can be used to identify collagen from other biomolecules: 938 cm⁻¹ band assigned to C–C stretch/α-helix band [23], and the 1002 cm⁻¹ ring breathing peak. The 1220 to 1280 cm⁻¹ band is also significantly different in shape to the broad 1200 to 1400 cm⁻¹ band seen from the skin. These three spectral features are highlighted on the spectra with the gray highlighted sections. Figure 2B shows that increasing the concentration of collagen in the defect (spectra B and C) does not change significantly the spectra measured using a 0 mm spatial offset. This indicates that the spectra are not affected by changes in collagen concentration in the defect region. When HA is added to the defect region (spectrum d), the 960 cm⁻¹ phosphate peak becomes the most intense Raman band in the spectrum, while no other significant spectral changes are observed. This shows that when selecting a 0 mm offset, the defect volume does not contribute significantly to the majority of the Raman signal, except for HA 960 cm⁻¹ band, which has a much higher Raman scattering cross-section compared to the other components of the phantom sample. Spectral changes reflecting the increase in collagen concentration in the defect are better observed in the SORS spectra measured using a 1.5 mm offset, at wavenumbers corresponding to collagen Raman bands, in particular 938 cm⁻¹ C–C stretch/α-helix band. For the sample corresponding to Stage 4, HA is easily detected by the 960 cm⁻¹ band, at similar intensity as for the spectrum measured at 0 mm offset.

Figure 3 presents the PLS models trained to predict the collagen concentration in the phantom defect based on the SORS spectra. The PLS model was built from the spectral range 800 to 1606 cm⁻¹ in order to avoid the Teflon band at 734 cm⁻¹ (absent when applying the model on rat cadavers). The data points in Figure 3 represent the predicted value of collagen concentration by the leave-one-out bootstrapping. The concentration of collagen ranged from 0 to 1.1 g/cm³ and the concentration of HA ranged from 0.0 to 2.2 g/cm³. These collagen and HA blends were selected to cover the full range relevant to bone healing as presented in Figure 1.

The results in Figure 3 show that the PLS model corresponding to the 0 mm spatial offset has the lowest correlation between the predicted concentration and actual concentration of collagen with a (RMSE) of 0.24 g/cm³. Increasing the spatial offset provides stronger signal from the collagen concentration and the performance of the prediction models increase from RMSE = 0.21 g/cm³.
Increasing offset has the effect of increasing the amount of signal that is detected from deep within the sample relative to the total signal generated. This means the variation in signal from a change in collagen concentration will have a greater effect on the relevant spectral bands. This increase in signal to background ratio would improve the correlation between concentration and spectra for the PLS model to be trained on. These results show that using spatial offsets of 1 to 1.5 mm, it is feasible to detect changes in collagen concentration occurring within the first 2 to 4 days of bone regeneration [17]. Nevertheless, Figure 3 also shows that the lowest model performance occurs for concentrations of collagen between ~0.7 and 0.9 g/cm³, which corresponds to the later stage of bone regeneration. This region has an RMSE of 0.31 g/cm³ in the 1.5 mm offset compared to the 0.17 g/cm³ for the rest of the range. At these stages, in addition to collagen, the samples contain collagen and HA at higher concentration. The intense band at 960 cm⁻¹ assigned to HA partially overlaps with the main Raman bands of collagen in the 900 to 1000 cm⁻¹ range, affecting the prediction accuracy. In the building of the PLS model, a range of skin thicknesses were used, with a range of approximately 0.5 to 1.0 mm. This combined with the heterogeneous nature of skin led to an unpredictable variance in the spectral contribution from skin due to the varying concentrations of collagen and fat from the skin itself. In order to build a model that takes into account skin thickness a much larger data set would be needed. As well as the samples varying from the minimum to maximum concentration of HA and collagen, they would also have skin thicknesses ranging from thinnest to the thickest. Using a single offset, differentiating between high concentrations deep within the material and low concentrations close to the surface can be difficult so a model that accounts for skin thickness would likely utilize multiple offsets.

In both PLS models corresponding to 1.0 and 1.5 mm spatial offsets, there is a stronger prediction accuracy at lower concentrations of collagen. It is possible that this reduced performance at larger concentrations could be linked to a variety of factors. Over the course of a long measurement, the collagen fibers absorb moisture from sources such as the skin. Greater concentrations of collagen could increase how much the skin dries out, affecting signal scattering. At high concentrations of collagen and HA, the packing of the material into the defect was a limiting factor, as the collagen needed to be packed more...
densely. The change in optical properties is due to the change in the concentration of scattering material within the defect. This could lead to a concentration dependence of prediction performance.

The PLS models were then tested on new phantom samples. For these samples, the concentrations of collagen and hydroxyapatite were selected to match specific points of interest along the time course of bone regeneration. These samples were not included in the training dataset. The selected points were demonstrated in Table 1 referencing stages along the healing model demonstrated in Figure 1. These stages were selected to investigate the ability to determine the early deposition of collagen in the sample as well as ongoing soft tissue remodeling.

The results in Figure 4 show a strong correlation between the actual concentration of collagen in the test samples and the values predicted by the PLS model. The predicted values of the concentration follow the overall profile of the true concentration values and the majority are within the expected uncertainties of the PLS model, with the larger concentrations exhibiting exhibiting a larger variance. The model over predicts at low collagen concentrations and under predicts high concentrations. The observed variance between the predicted concentrations for different samples is likely to be caused by variations in the thickness of the skin layers, as each sample used a different layer of skin. It is worth noting that for in-vivo measurements on the animal, the SORS spectra will be acquired from the same location therefore variations in skin thickness are less likely to occur within the same animal.

### Table 1

Densities of collagen and HA selected to represent different stages during bone healing

| Stage | Day | Collagen density (g/cm³) | HA density (g/cm³) |
|-------|-----|--------------------------|-------------------|
| 1     | 0   | 0.085                    | 0.00              |
| 2     | 2   | 0.24                     | 0.00              |
| 3     | 3   | 0.64                     | 0.00              |
| 4     | 7   | 0.86                     | 0.32              |
| 5     | 14  | 0.43                     | 1.07              |
| 6     | 21  | 0.43                     | 1.84              |

**FIGURE 4** Prediction of collagen concentration for a time-course model of bone healing using phantom samples. Black triangles represent the predicted concentrations by the PLS modes (5 different samples), with the black dotted line as a guiding line for the average values. Error bars denote the uncertainties of the prediction model obtained from the boot-strapping evaluation. Red line is a guiding line for the actual collagen concentrations in the sample.

### 3.2 Quantification of collagen concentration in bone defects in rat cadavers

In order to investigate the feasibility of measuring collagen concentrations directly on animals, prior to live animals, experiments were carried out using rat cadavers. Bone defects were induced in the skulls of the rats and plugs of collagen:HA were inserted, then covered by the skin flaps (Figure 5A). The PLS model from Section 3.1 was applied on the SORS spectra measured transcutaneously by focusing the laser spot at the center of the defect location, mimicking in-vivo measurements. For each animal, six collagen:HA plugs were used, using concentrations covering all six stages of bone regeneration. In total, three animals were used to evaluate inter-animal variance. Figure 5B presents SORS spectra measured from one of the animals covering the first four stages of simulated bone healing taken with a 1.5 mm offset. In all SORS spectra, Raman bands associated to collagen can be identified mainly in the 1200 to 1350 cm⁻¹ (amide III bands with specific higher intensity at 1240 to 1270 cm⁻¹ compared to 1300 to 1350 cm⁻¹) and 850 and 950 cm⁻¹. As the spectra were normalized using the 1445 cm⁻¹ band, the SORS spectra measured for the
samples with lower concentration of collagen (e.g., Stages 1, 2) have lower signal to noise ratio compared to the samples corresponding to Stages 3 to 5. The Raman band assigned to HA phosphate vibrations at 960 cm$^{-1}$ can be identified in the SORS spectra starting with Stage 4.

Next, the PLS model developed in Section 3 was applied on the SORS spectra measured from the rat cadavers (Figure 6). The results show that, for all three animals used here, the predicted collagen values follow the correct pattern of increasing collagen from Stage 1 to Stage 4, followed by a decrease at Stages 5 and 6 of the prediction. This consistency can also be attributed to the fact that, for each animal, the thickness of the skin covering the collagen:HA plugs remained the same when changing the concentrations of collagen and HA (skin thickness was considered the main source of variance for the phantom samples). Nevertheless, the results in Figure 6 show that the predicted values of collagen concentration were higher than the real values, by a factor of 2 to 2.5. We speculate that several factors contributed to

FIGURE 5  A, Photographs of a rat head used in this study, showing the defect drilled in the skull. B, SORS spectra obtained using collagen:HA plugs with concentrations corresponding to Stages 1 to 4 of bone regeneration (1.5 mm spatial offset). Spectra have the baseline subtracted

FIGURE 6  Time course model of simulated bone regeneration in a rat skull. Each symbol denotes a different rat skull, and the red line is a guideline for the actual concentration of collagen placed in the sample
this over prediction, both caused by differences between the samples used for training the PLS model and the actual rat samples. Rat skin was found to be significantly thinner than the chicken skin used for training the model, there was also left-over hair on the rat skin and bone tissue surrounding the defect volume instead of Teflon for phantom sample. Furthermore, the cadaver had brain tissue as the scattering medium whereas the phantoms had porcine fat. While multiplying the predicted values of the concentration by a factor of 0.5 could provide an empirical correction, the results show that a preferable improvement in prediction accuracy could be obtained in future studies by training the PLS models with samples based on rat cadavers rather than phantoms.

4 | CONCLUSIONS

A wide range of biomaterials and tissue-engineered scaffolds are being investigated to support and stimulate bone healing in humans. Prior to use in humans, animal models are widely used to evaluate biocompatibility and optimize the properties of the materials in order to reduce healing time and improve the quality of the newly formed bone. In this study, we investigated the feasibility of using spatially offset Raman spectroscopy to monitor changes in collagen concentration at levels similar to those expected to occur in animal models.

We showed that a SORS-based PLS model can be used to create a quantitative prediction model of collagen concentration within a phantom sample mimicking bone regeneration in a rat calvarial defect model. The concentrations of collagen were selected to have relevant values to in-vivo bone healing (0-0.86 g/cm³) as determined from literature. The predictive power was 0.16 g/cm³, and the main source of uncertainty was linked to variations in thickness of the skin layers used. It may be possible to link this method with another method, such as optical coherent tomography, in order to measure the thickness of the skin covering the defect [24]. This dimension could then be added to the prediction model to account for variability in skin thickness.

The PLS model was then used to predict the collagen concentration in collagen:HA plugs inserted in defects induced in the skull of rat cadavers. The PLS model successfully retrieved the overall pattern of collagen concentration across the concentrations simulating all stages of bone healing. Nevertheless, the actual concentration values were predicted to be approximately twofold higher than the actual values. These overprediction errors were associated to differences in structure and composition between the phantom samples used for training the PLS model and the actual rat cadaver samples. While the use of phantom samples for training the PLS models provides simplicity and avoids using animals, we assume that the predictive power could be improved if training is carried out on samples as similar as possible to those on which the actual measurements are to be performed.

These feasibility results indicate that SORS could be used in a non-invasive way to measure molecular changes related to bone regeneration in vivo. Such studies could generate unique longitudinal data that will provide a better understanding of the spatial and temporal molecular processes underpinning the repair of bone, that would enable the optimization the physical and chemical properties of scaffolds. The ability to follow the bone healing process on the same animal, with high chemical specificity, will provide higher quality data with ethical and economic benefits from reducing the number of animals used during the research. Future studies could also investigate the feasibility of monitoring in-vivo bone regeneration in humans.

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