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Scaffold characterization using NLO multimodal microscopy in metrology for regenerative medicine

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Abstract. Metrology in regenerative medicine aims to develop traceable measurement technologies for characterizing cellular and macromolecule behaviour in regenerative medicine products and processes. One key component in regenerative medicine is using three-dimensional porous scaffolds to guide cells during the regeneration process. The regeneration of specific tissues guided by tissue analogous substrates is dependent on diverse scaffold architectural properties that can be derived quantitatively from scaffolds images. This paper discuss the results obtained with the multimodal NLO microscope recently realized in our laboratory in characterizing 3D tissue engineered (TE) scaffolds colonized from human Mesenchimal stem cells (hMSC), focusing on the study of the three-dimensional metrological parameters.

1. Introduction

Metrology in regenerative medicine addresses a field of research novel at International level, a field where the convergence of nanotechnology and biotechnology will provide a significant contribution to current and future progress. It aims to develop traceable measurement technologies for characterizing cellular and macromolecule behaviour in regenerative medicine products and processes. One key component in regenerative medicine is using three-dimensional porous scaffolds to guide cells during the regeneration process.[1,2] These scaffolds are intended to provide cells with an environment that promotes cell attachment, proliferation, and differentiation. After sufficient tissue regeneration using in vitro culturing methods, the scaffold/tissue structure is implanted into the patient, where the scaffold will degrade away, thereby leaving only regenerated tissue; on a different approach, noncellularised scaffolds are inserted into the patient to elicit in vivo cell recruitment, growth and tissue regeneration. Scaffolds need to meet both the biological goals of tissue formation and the stresses and loading conditions present in the human body. For this reason, any design approach must ensure that the mechanical properties of the resulting scaffold structure are compatible and optimally match the requirements from the environment, that, respectively, are the cell adhesion transmembrane protein, the cytoskeleton structure, the cell population. The need to design scaffold structures, the need for precision control during their fabrication and for determining the metrological indices and the need to characterize their structural behaviour at different scales have lead to numerous experimental and computational challenges. The regeneration of specific tissues guided by tissue analogous substrates is dependent on diverse scaffold architectural properties that can be derived quantitatively from scaffolds

images. Over the last few years a number of imaging techniques have started to be developed which could be applied profitably to tissue engineering. Many of these techniques require labeling of the cells or scaffolds in order to accurately make measurements within the 3D tissue engineered product. However, recently a number of label free technologies have been developed which offer the potential for rapid information rich imaging without compromising or altering the product composition or interfering with the behavior of the cells. In particular, label free imaging technologies such as coherent anti-Stokes Raman scattering (CARS) and other Non-linear Optics (NLO) techniques offer a great deal of potential. A multimodal approach in which different NLO techniques are implemented in a same microscope is particulary powerful [3,4,5].

This paper discuss the results obtained with the multimodal NLO microscope recently realized in our laboratory [6,7,8,9] in characterizing 3D tissue engineered (TE) scaffolds colonized from human Mesenchimal stem cells (hMSC) [6], focusing on the study of the three-dimensional metrological parameters.

2. Materials and methods

2.1. Multimodal microscopy

The multimodal microscope integrate three NLO techniques CARS/SHG/TPEF on a single platform. It is based on a microscope scan unit (Fluoview FV300, Olympus) combined with an upright microscope (BX51WI, Olympus). Near infrared picosecond pulse trains are used to excite the non-linear optical processes in the samples. The optical source system is based on a passively mode-locked Nd:YVO_4 laser (Picotrain, HighQLaser) at 1064 nm and equipped with a SHG unit emitting at 532 nm. The frequency doubled output synchronously pumps an optical parametric oscillator (OPO - Levante Emerald, APE Berlin) with repetition rate 76 MHz and temporal width pulses about 6 ps. The OPO signal and idler waves are simultaneously generated by an LBO crystal in the resonant cavity, tunable in the wavelength range 700 nm - 1020 nm and 1110 nm - 2200 nm respectively. Both waves, exiting the system time and space overlapped and collimated, provides CARS stokes and pump signals. The OPO signal wave excites both SHG and TPEF processes, its photon energy having in the non-linear processes a higher efficiency with respect to the idler photon. The system was implemented with optics and mechanical systems suitable for the simultaneous detection of the different NLO techniques and to provide a z axis combined movement. *Ad hoc* algorithms have been developed for an automatic acquisition of 3D images [6].

2.2. Cell culture

Mesenchymal stem cells (hMSCs; Lonza) have been cultured in cell culture flasks with cell culture medium (DMEM from Sigma Aldrich with 10% FBS, 100 U/mL penicillin, 100 g/mL streptomycin) until they reach the 80% of confluence. Cells have been detached from culture flasks by using a trypsin/EDTA solution (Invitrogen) and counted by using a hemocytometer. 10^5 cells have been fluorescently stained with Calcein AM (Invitrogen), a vital staining specific for living cells only: the cell suspension (in cell culture medium) has been centrifuged at 1000 g for 5 minutes in a centrifuge tube to obtain a pellet of cells and 500 µl of PBS containing calcein AM 4µM has been used to re-suspend the pellet. After 10 minutes at 37 °C the cell suspension (in PBS/Calcein AM), stained cells have been washed in PBS to eliminate the excess of Calcien AM: cell suspension has been centrifuged again at 1000 g for 5 minutes. The pellet of stained hMSCs has been re-suspended in 100 µl of cell culture medium and this volume (a drop) has been seeded on the top surface of the scaffold PHBHV. The scaffold, with the drop containing cells, in a Petri dish has been incubated at 37 °C and 5% CO_2 for 1 hour to leave the cells in the drop to invade the scaffold. Then, the scaffold with cells has been attached to a Petri dish to clamp the sample during the imaging. To this purpose it has been used the fibrin gel made by mixing thrombin (12,5 µg/ml) and fibrinogen (5 mg/ml) at 1:2 ratio. This solution is liquid at 4°C but gradually became gel at room temperature. A small drop (10 µl) of fibrin gel is added on a dry Petri dish and very quickly the scaffold with cells is placed on the fibrin gel drop. After

5 minutes at 37 °C the scaffold is clamped to the Petri dish. Then, 10 ml of cell culture medium has been added to the Petri dish and incubated at 37 °C and 5% CO_2 over night to allow cells to adhere to the scaffold and to migrate into the scaffold internal structure.

2.3. Scaffolds

Degradable linear polyurethanes from non toxic building blocks were prepared. Bioactive peptides were incorporated in polyurethane chains, in order to create a synthetic equivalent of the Extracellular Matrix, able to mimic natural tissue. Both cell adhesive peptide (RGD sequences) and enzymatic proteolitically degradable peptides, containing the Ala-Ala sequence, were used as chain extender. Furthermore amino-derived diamines and cyclic diols were used as alternative chain extenders [10,11]

3. Results and discussion

Two complementary experiments have been done with the aim of studying with a non invasive and new methodology one of the most important features in regenerative medicine processes, the interaction of the cells with the scaffold. The metrological approach, studying the uncertainty components in measuring some of the scaffold's characteristic parameters, allows an estimate of the confidence achievable in characterizing those products and processes.

In the first experiment a dry fragment of the polymeric structure was imaged with CARS technique. The Coherent Antistokes Raman spectrum of the polymeric structure was detected and recorded in the wavelength range between 2750 cm^{-1} and 3150 cm^{-1}. Figure 1 shows this spectrum. Two peaks are evident at 2850 cm^{-1} and at 2920 cm^{-1}. This last wavelength was chosen to image the structure.

CARS three-dimensional reconstruction of a dry fragment of the structure architecture was done in a region size of 1414 x1414 x350 μm^3 with a Z-stack of 141 images with resolution 1024 x 1024 pixel and a Z step 2.5 µm. The 3D image was recorded and visualized importing the stack in the free software ImageJ, setting up the voxel size parameters and using 3D viewer plug-in.

Figure 2 shows the 3D reconstruction of the polymeric structure from the CARS signal.

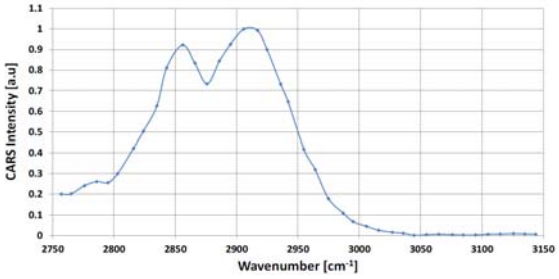

Figure 1 - Coherent Antistokes Raman spectrum of the polymeric structure in the wavelength range 2750 cm^{-1} and 3150 cm^{-1}

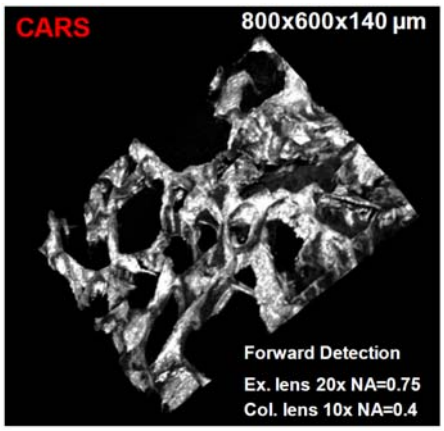

Figure 2 – CARS three-dimensional reconstruction of a dry fragment of the structure architecture

Recent literature and the standard guides [2,12] define specific geometrical parameters characterizing the micro-structure of scaffolds, fundamental to the cell attachment and in-depth penetration (and tissue vascularization), in conjunction with biological factors as cell seeding and nutrients supply. In particular, being an adequate transport of nutrient through the scaffold fundamental for culturing cells, the capability of scaffolds to be permeated by nutrients and metabolites, i.e., the ease with which fluids/species move through the scaffold, has a marked impact in

Tissue Engineered processes. As a consequence a metrological measurement of parameters as porosity, pore size and distribution, surface area, and tortuosity is the key argument for Tissue Engineered product knowledge and quality. This immediate translate in safety of regenerative medicine processes.

Different techniques are typically used to determine and to quantify these parameters; a drawback of the used techniques is their invasiveness with respect to the sample [2,13]. CARS imaging could bypass this limit through non-invasive measurements of samples, allowing the samples after the measurements to be used for cell culture and possibly for implants.

With this in mind a preliminary metrological study of the CARS imaging of the scaffold has been done, key parameters have been measured and an estimate uncertainty have been defined.

The 3D CARS images have been processed using an ad hoc developed computational algorithm [6] to extract a number of morphological parameters. The parameters are: the Pore Circularity (a value between 0 and 1 indicating a thin long shape when approaches 0 and a perfect circle when approaches 1), the Pore Roundness (a value between 0 and 1 indicating an elongated shape when approaches 0 and a perfect circle when approaches 1), the Pore Feret'sDiametre (the maximum distance between two points along the pore boundary), the Average Porosity (the ratio between the total pores area and the total scaffold area) and the Void Space Ratio (the ratio between the total pores volume and the total scaffold volume), following the standard guide [12] terminology and definitions.

With the aim of measuring uncertainty components due to measurement artifacts lied to (i) CARS technique itself, (ii) optical attenuation and distortion phenomena depending on the sample characteristics (iii) uncertainties in software threshold definition, different measurement have been performed of the same sample, changing optical parameters and software threshold analysis. Uncertainties have been estimated as reproducibility over these parameters.

Table 1 shows the results obtained by the measurement of morphological parameters of the polymeric scaffold in analysis, with associate estimated uncertainties.

Parameter	**Measured value from CARS Images**
Average Porosity	56.5%
Void Space Ratio	89.6%
Pore Circularity	0.16 ± 0.1
Pore Roundness	0.55 ± 0.2
Pore Feret's Diameter	(179 ± 180) μm

Table 1- Measured values of the shape descriptor parameters obtained by image processing of the CARS 3D reconstruction of the polymeric structure

A second study has been done on the ability of the specific polymeric structure to act, for human Mesenchimal stem cells (hMsc), as a scaffold; that means to be a suitable artificial matrix where cells could survive, proliferate, and colonize the structure in 3D. This study, novel for its capacity to image living samples without perturbing its behaviour, has the potential to quantify two main parameters: proliferation of cells and localization with respect to the 3D scaffold. Nevertheless this quantification is out of the scope of this paper.

A 3D polymeric structure 3 mm x 3 mm x 0,350 mm have been cultured as previously described. The construct was observed under the multimodal microscope for three days in a time-lapse experiment [6, 14] with the aim of studying the interaction between scaffold and hMsc cells.

Figure 3 shows the cells colonizing the structure at different times during three days in culture. Fluorescent calcein, metabolized by the cells was detected by the multimodal microscope in Two Photon Emission Fluorescence (TPEF) EPI detection modality, showing the 3D cell cytoplasma

morphology. Simultaneously the 3D structure of the scaffold was detected by the multimodal microscope in Coherent Antistokes Raman Scattering (CARS) Forward modality.

In each measurement a stack of 66 images of 512x512 pixels averaged 2 times with a Kalman filter, with a Z step of 1 μm were collected in around six minutes. All the Z stacks were converted in a 3D view using 3D viewer imageJ plug-in.

The analysis of the 3D images shows the position of the single cells with respect to the static position of the scaffold. It is possible to observe the number of cells per area increasing during the time in culture, showing a strong proliferation. After the first 24 hours cells start to colonize the scaffold pores from the pore's external surface to its centre. Some of the cells disappeared, due to different death processes (apoptosis). These results demonstrate that this specific construct is a good example of regenerative medicine product, useful for metrological studies of regenerative medicine processes.

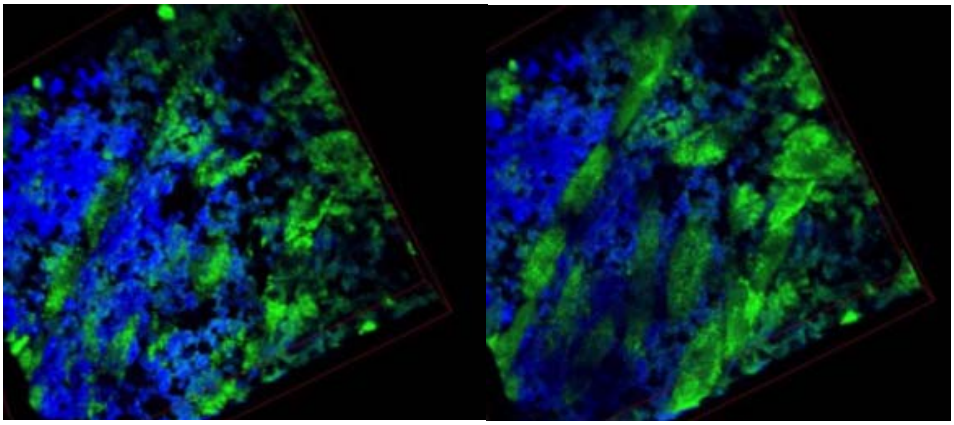

Figure 1 – NLO multimodal microscope images of living hMsc cells (green) colonizing the 3D scaffold (blue) at different times (24h, 30h) in a three day experiment.

With respect to the measurement method used for this analysis, it seems extraordinary promising for its intrinsic image resolution, for its ability to a deep 3D imaging, for the independent but simultaneous acquisition of scaffold and cells without any perturbation of their native processes.

4. Conclusion

The results obtained with the multimodal NLO microscope recently realized in our laboratory in characterizing 3D tissue engineered (TE) scaffolds colonized from human Mesenchimal stem cells (hMSC), have been focused on the study of the three-dimensional scaffold parameters and of the real time cell behaviour when cultured on a specific polymeric structure. Such studies demonstrate NLO microscopy as a powerful, not invasive and reproducible instrument for research in regenerative medicine.

These results show the possibility to develop traceable measurement technologies for characterizing cellular behavior in regenerative medicine products and processes. Further studies have to be done in order to better define eventual artifacts in measuring scaffold and cells morphology with the different imaging techniques.

Time-lapse experiments allowed by multimodal NLO microscopes are a real novelty in studying one of the most important features in regenerative medicine processes, the interaction of the cells with the scaffold.

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