Innovative TCSPC–SHG microscopy imaging to monitor matrix collagen neo-synthetized in bioscaffolds

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Abstract. We propose an innovative invasiveless technique in the field of nonlinear optical imaging to facilitate monitoring of cell/scaffold combinations for tissue repair. By using a near infrared (NIR) femtosecond excitation, we were able to introduce a new index based on decay time response for fluorescence (F) and Second Harmonic Generation (SHG) obtained with Time Correlated Single Photon Counting (TCSPC) microscopy to monitor structural information on the state of the matrix collagen. Some human Mesenchymal Stem Cells (hMSCs) seeded in 3D scaffolds were tested with different culture times (from D7 to D56) to analyze the effect of Tumor Growth Factor beta 1 (TGF-β1) on type-2 collagen expression in the matrix. After 14 days in the presence of TGF-β1, our results showed an increase in the expression of type-2 collagen synthesized by hMSCs, and a change in collagen conformation, as an indication of its ability to be detected as a harmonophore by TCSPC–SHG without the need for an exogenous probe.

Keywords: Tissue engineering, lifetime, second harmonic microscopy, stem cells, collagen, extracellular matrix

1. Introduction

In bone and cartilage, protein collagen is the major component of extracellular matrix and plays a role in regulating cell proliferation and differentiation. As the mechanical properties and plasticity of protein collagen provide useful information on the physiological stiffness of bio-tissues, the architecture and the state of collagen fibrils in extracellular matrix need to be monitored to validate bio-scaffolds for possible clinical application. The SHG signal is specific to molecules (harmonophores), which do not show centro-symmetry like collagen, the main SHG generator in biological tissues. The triple helical structure of collagen molecules is non-centrosymmetric, highly organized and spatially oriented. As processes involved in SHG are intrinsic properties of the constituent molecules, SHG imaging microscopy provides structural information about the collagen network and fibrils of the cartilage ECM.

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Thus, only a fibrillar arrangement of collagen molecules (like in type I, II or III, for instance), will produce a very strong SHG signal and can be imaged without using exogenous dye and without damaging the collagen proteins [1]. If collagen fibers are oriented in the same direction, the SHG signal will be stronger than if the distribution of the fibers is pseudo-random. In previous results based on SHG analysis of texture [2], we showed that near-infrared SHG tomography allows sufficient tissue penetration to identify the exact degree of collagen network organization and is thus a precise tool for bio-diagnosis or analysis of cartilage degeneration (density and homogeneity of the collagen matrix). In a previous work, we monitored modification of cartilage ECM and dissociation of fibers leading to the loss of the SHG signal. Moreover, SHG not only depends on the structure of molecules, but also on the macroscopic arrangement of ECM and its adaptation to environmental stress [3].

The modification of ECM leads to a variable metabolic response, with a direct impact on cellular morphology, in signal transduction, and perhaps a protective role against environmental constraints, related to mechanical properties. In scaffolds, it would be interesting to correlate the well-known effect of growth factor on cellular metabolic responses, in particular cell proliferation and differentiation leading to collagen synthesis as revealed by SHG imaging. Based on scattered light alone, the SHG signal detected was not related to changes in a particular type of collagen. Multiple signal decay constants may be the reflection of several distinct surroundings of a probe (fluorophore, harmonophore) or of the presence of several conformational states of a molecule. The exact nature of signal decay (natural lifetime) can reveal details about the interactions of the probe with its immediate surroundings (immunolabeling or SHG).

Generally speaking, natural lifetime is the average amount of time a molecule remains in the excited state prior to its return to the ground state, while emitting photons (fluorescent or not). The decay value measured for each pixel and displayed as a “lifetime” contrast by a FLIM setup (Fluorescence Lifetime Imaging Microscopy) is a property of individual molecules and is therefore largely independent of probe concentration and possible photobleaching. FLIM measurements have been developed in a time-related manner (single photon counting) and/or in the time and frequency domain [4]. Measuring fluorescence lifetime distribution by phase fluorimetry enables very rapid determination of the behavior of probes in living cells. As SHG is based on light scattering and not on fluorescence, it does not require the formation of excited state which corresponds to the deactivation of excitation processes, and only a very rapid temporal decay method (Time Correlated Single Photon Counting (TCSPC)) can capture a SHG signal. This is not possible with the Frequency method in visible continuous excitation mode (phase/modulation) used for FLIM.

In this work, by using two-photon microscopy, we applied a Second Harmonic Generation (SHG) technique combined with TCSPC detection to distinguish fluorescence from the SHG signal (TCSPC–SHG). This new TCSPC–SHG multiphoton technique was used to monitor changes in intensity decay induced simultaneously by cell fluorescence (autofluorescence and fluorophore labeling) and by the SHG signal (all types of collagen proteins). The photons that are supplied by the SHG signal (fast time slope for a short decay time) can be contrasted with a fluorescence signal (slow time slope for a long decay time) that is encountered in fluorescence-like auto fluorescence (cell and collagen protein) or after staining in biological tissue [5]. The time slope of the decay is usually the sum of different exponential decay times. To normalize data and to account for cell density and associated autofluorescence, we introduced an index (F–SHG) described as the ratio between the long lifetime (typically extrinsic fluorescence) and fast lifetime (SHG, intrinsic fluorescence), which is obtained by fitting three exponential components.

To illustrate the TCSPC–SHG method in multiphoton microscopy, we tested human Mesenchymal Stem Cells (hMSCs) seeded in 3D scaffolds (type-I collagen). It was necessary to distinguish changes in
collagen from the fluorescence signal and from the backward SHG signal [6]. The detected fluorescence was first obtained from the autofluorescence signal with respect to cellular properties or scaffold material (constitutive type-1 collagen) and second from fluorophores coupled to antibody specific type-2 collagen (cell synthesis). The F-SHG index gives the ratio of the signal dedicated to neo-synthesized type-2 collagen expression by hMSCs to the associated SHG signal. The change in the F–SHG index was monitored according to culture time from 7 to 56 days and confirmed the identity of the fiber collagen (type-2) neo-synthesized in the scaffold and enhanced expression in the presence of TGF-β1.

In this paper, we describe complementary TCSPC/SHG optical approaches based on near infrared (NIR) femtosecond excitation for the imaging of the collagen scaffold and discuss the technical limitations and the future outlook of the technique in the biomedical field.

2. Materials and methods

2.1. MSCs and 3D culture in type-1 collagen sponge. Immunofluorescence

Mesenchymal Stem Cells (MSCs) from bone marrow (obtained from the “Service de Chirurgie Orthopédique et Traumatologique” and “Unité de Thérapie Cellulaire et Tissus” of Nancy University Hospital) were plated in a T75 culture flask at 37°C (5% CO₂) in Dulbecco’s modified Eagle Medium supplemented with 10% fetal bovine serum, 1 ng/ml bFGF, glutamine and penicillin-streptomycin. During the last passage (P3), MSCs were cultured with differentiation medium composed of DMEM supplemented with sodium pyruvate, bFGF, penicillin–streptomycin, and PAD (Proline, L-Ascorbic acid-2-Phosphate, and Dexamethasone). MSCs were then seeded in scaffold matrix (sponge) composed of a mixture of type-I and III collagen (bovine dermis purchased from Symatese Biomateriaux, France and placed in 96-well plates).

The resulting constructs were transferred to 48-well plates and cultured for the first three days with chondrogenic medium supplemented with 10% FBS. At D3, collagen sponges were divided into two different culture mediums depending on the supplements added to chondrogenic medium described above: ITS 1% or ITS 1% + TGF-β1 (10 µg/ml). Collagen II from the extracellular matrix expressed by MSC embedded in scaffold was stained by indirect immunostaining. The primary antibody was an Anti-Collagen, type II antibody with suitable secondary antibody, i.e. rabbit polyclonal antibody associated with Alexa 488™. After 15 min washing with PBS, scaffolds were fixed in 4% paraformaldehyde solution for 15 min and placed on glass slides (Labteck) for observation at room temperature.

2.2. TCSPC–FLIM–SHG and index F-SHG

A confocal laser scanning microscope (TCS SP2-AOBS, Leica Microsystems, pinhole fully opened) enabled images in 512 × 512 matrices to be obtained at 400 Hz. The multiphoton imaging system was composed of a femtosecond oscillator (Mira 900F, Coherent), pumped with a solid laser (Verdi 8 W, Coherent) to generate some ultra-short infrared pulses (120 fs, 76 MHz) at λ = 800 nm, and an Electro Optical Modulator enabled control of the power of excitation. The laser beam was focused onto the samples through a water immersion objective with ×40 magnification (NA = 0.8). Fluorescence lifetimes were measured with a Time-Correlated Single Photon Counting method (SPC-730 TCSPC Imaging module, Becker & Hickl, Berlin).

A signal from the scan controller of the microscope enabled decay curves to be traced for each pixel of an image (128 × 128). By sampling the single photon emission with a very large number of multiphoton
excitation flashes at 800 nm, the experiment enabled the probability distribution to be reconstructed [2].

The signal emitted backward (i.e., in the opposite direction to the excitation beam) was collected through a low-pass filter BG 700 to reject the infrared excitation beam (800 nm) and a high-pass filter enabled us to select the spectral range (400–560 nm) to be detected. The instrumental response function (IRF) of the measurement (<170 ps for uric acid) and the transit-time spread of the detector (PMC-100) were small compared to the width of the time channels of the photon histogram (count increment 2). With a reduced Chi-square and an estimated IRF, we used a software (SPC Image, Becker & Hickl) to fit the measurements with three exponential decay models (binning 2, threshold 10) before creating color coded images of the $\tau_2/\tau_1$ ratio (index F–SHG). The distribution histogram and matrix data ($\tau_2/\tau_1$) were exported and stored in ASCII files for calculation.

3. Results

In this study, a double signal detection strategy (fluorescence/SHG) combined with TCSPC–SHG imaging was applied in microscopy to analyze the behavior of cells (proliferation/collagen synthesis) in scaffold made with type-1 and type-3 collagen. To be able to simultaneously monitor type-2 collagen synthesis and cell proliferation, type-2 collagen was specifically labeled with a monoclonal antibody coupled with Alexa488TM fluorophore.

The results revealed a precise range of F–SHG index (from 2 to 8), distributed in four clearly defined areas (A, B, C, D) in relation with cellular behavior and metabolism. The range detection of Alexa488TM fluorophore coupled with Mab against type-2 collagen (Mab col 2) was restricted in area A (from 2 to 2.3). Area B (from 2.3 to 3.3) concerned more specifically global auto fluorescence (AF) from the scaffold (type-1 collagen) and cells. The SHG signal from the scaffold (with or without seeded cells) was strictly confined in area C with a range of from 3.3 to 4.4. Finally, the SHG signal for synthesized collagen by hMSCs was significantly distributed in area D (from 4.4 to 8). For hMSC cultured in the presence of TGF-β1 or not (ITS), type-2 collagen was specifically detected by immunolabeling in area A (Fig. 1). However, the col2-SHG signal appeared only in area D in the presence of TGF-β1, revealing abundant extra-cellular collagen synthesis and organized and structured collagen fibers.

When the hMSCs were cultured in the presence of TGF-β1, the col2-SHG signal increased significantly with an increase in culture time (from 7 to 56 days) (Fig. 2). The col2-SHG signal was detected only after 14 days of culture (in area D) even though type-2 collagen protein was revealed by immunolabeling after 7 days (in area A). This result indicated a change in collagen conformation and its ability to be detected as a harmonophore by TCSPC–SHG imaging. After 56 days of culture, we tested hMSCs seeded in scaffold that was not immunolabeled against type-2 collagen and in control scaffold without cells. In both cases, there were no significant signals in area A, as a valuable probe Alexa488TM fluorescence was confined to this range. Area B was also informative concerning the cellular density in scaffold and concerning proliferation and its effect on global auto-fluorescence. Interestingly, when antibodies against type-2 collagen were added to hMSCs (at D7, D14 and D28), showing an attenuation effect on SHG signal collected in area C, as a potential protecting blanket (bullet-proofing) of collagen fibers.

4. Discussion and conclusion

In this work, multimodality imaging in multiphoton microscopy was used to confirm the identity of the type-2 collagen fibers neo-synthesized by hMSCs cultured from 7 to 56 days in scaffold in the pres-
Fig. 1. Human Mesenchymal Stem Cells (hMSCs) were seeded in 3D scaffold, immunolabeled with Mab directed against type-2 collagen and analyzed with TCSPC–SHG in multiphoton microscopy. After 28 days of culture, in the control scaffold (without cells) and in the presence of TGF-β1 or not (ITS), distribution of F–SHG index was defined in 4 well-defined areas (A, B, C and D) respectively, closely related to detection of type-2 collagen only (Mab-Col2), global autofluorescence (AF), SHG scaffold and SHG synthesized collagen. The SHG signal in area D was similar to that of the control or the ITS culture condition, but increased under the effects of TGF-β1.

ence or not of TGF-β1. As revealed by analysis of the TCSPC–SHG and F–SHG index, type-2 collagen was more expressed by hMSCs in the presence of TGF–β1 (involved in chondrogenic differentiation) than in basic culture medium (ITS). When collagen molecules were organized as a functional and organized protein structure (after 14 days culture), we showed that TCSPC–SHG multiphoton technology can monitor changes in SHG decay versus autofluorescence decay, as an non-invasive approach without the need for exogenous dye. In previous results based on SHG analysis of texture, we showed that near-infrared SHG tomography allows for sufficient tissue penetration to measure the degree of organization of the collagen network and could be used as a precise tool for biodiagnosis or for measuring cartilage degeneration (density and homogeneity collagen matrix). Here, we propose an innovative TCSPC–SHG method of analysis to eliminate problems of photobleaching and phototoxicity that could be useful for non destructive testing of functionalized and seeded biomaterials. This technique could also be used to monitor the state of the collagen network prior to clinical implantation [7], with a new possible application in the field of nonlinear optical imaging applied to multiscale SHG detection (from microscopy to macroscopy).

Acknowledgements

The authors would like to thank Didier Hentsch, and Jean-Luc Vonesh (IGBMC, Strasbourg, France) for their indisensible help with multiphoton imaging. This study was funded in part by grants from
Fig. 2. Contrary to the control scaffold, hMSCs were cultured in the presence of TGF-β1 and seeded in 3D scaffold with a culture time ranging from 7 to 56 days. After immunolabeling (type-2/collagen) except for scaffold at D56, samples were analyzed by TCSPC–SHG microscopy to extract the F–SHG index. In the presence of TGF-β1, SHG collagen expressed by hMSCs increased with an increase in culture time, as shown in area a (upper graph).

CUGN 54, Région Lorraine, RTmfm MRCT CNRS, IBISA, ARC, GDR 2588 CNRS and Agence Nationale de la Recherche (ANR-05-JC5-51629).

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