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Parallel high-resolution confocal Raman SEM analysis of inorganic and organic bone matrix constituents

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In many multi-disciplinary fields of science, such as tissue engineering, where material and biological sciences are combined, there is a need for a tool that combines ultrastructural and chemical data analysis in a non-destructive manner at high resolution. We show that a combination of confocal Raman spectroscopy (CRS) and scanning electron microscopy (SEM) can be used for such analysis. Studies of atomic composition can be done by X-ray microanalysis in SEM, but this is only possible for atomic numbers greater than five and does not reveal molecular identity. Raman spectroscopy, however, can provide information on molecular composition and identity by detection of wavelength shifts caused by molecular vibrations. In this study, CRS–SEM revealed that early *in vitro*-formed bone extracellular matrix (ECM) produced by rat osteoprogenitor cells resembles mature bone chemically. We gained insight into the structure and chemical composition of the ECM, which was composed of mainly mineralized collagen type I fibres and areas of dense carbonated calcium phosphate related to the collagen fibre density, as revealed by Raman imaging of SEM samples. We found that CRS–SEM allows the study of specimens in a non-destructive manner and provides high-resolution structural and chemical information about inorganic and organic constituents by parallel measurements on the same sample.

Keywords: scanning electron microscopy; confocal Raman spectroscopy; osteoblasts; extracellular matrix; Raman imaging

1. INTRODUCTION

Raman spectroscopy provides information on molecular vibrations. Raman spectroscopy is a method based on an inelastic scattering effect. In short, a monochromatic light source (laser) irradiation can excite molecules to a higher vibration state, which then relax to a different vibration level from their original state. As a result, a photon is emitted with less energy and, therefore, a longer wavelength than the initial photons from the laser. This event is called Stokes Raman scattering. The energy difference between the incident and scattered radiations appears as a frequency shift from the incident light. These frequency shifts are specific for a given chemical bond and, therefore, allow molecular analysis. Not only can scanning electron microscopy (SEM) be used for structural analysis, but also it can provide chemical information as well, by using X-ray microanalysis (XRMA). XRMA is, however, limited because no molecular information can be obtained from these data and moreover, detection of elements is limited to the higher atomic numbers (greater than five). In contrast, Raman spectroscopy can give information on molecular identity, composition, orientation and crystal form by detection of wavelength shifts caused by molecular vibrations. Therefore, combining SEM with confocal Raman spectroscopy (CRS) not only allows structural and elemental analysis of a sample, but also can provide information about the presence of inorganic and organic molecules in a non-invasive manner. In general, highly sensitive systems, containing high-power lasers and liquid-cooled CCD cameras are needed to obtain high signal-to-noise ratio data when studying biological samples with Raman spectroscopy. A clear example of such a system was shown in a study done on polytene chromosomes showing different DNA-protein ratios

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in their interbands (Puppels *et al.* 1990). However, the above-mentioned systems are in general too large to fit into a vacuum chamber of a SEM. We have built a compact confocal Raman microscope that was incorporated into the vacuum chamber of an environmental SEM, thus allowing us to do non-invasive chemical and ultrastructure research on typical biological samples. In tissue engineering, (bio)materials are commonly combined with biological components, such as the addition of specific cells or proteins for drug release, etc. In most cases, inorganic and organic components are present and preferably need to be evaluated in the most non-invasive way possible, in order to maintain the structure of the sample. Several different authors investigated the mineralization of a collagen matrix made by osteoprogenitor cells *in vitro* on different substrata by electron microscopy. In these cases, a network of collagen fibres was observed that had undergone calcification (Oghusi *et al.* 1992; Yoshikawa *et al.* 1997; Yamamoto *et al.* 2002). Separate FTIR and XRD analysis showed that the observed mineralization consisted of carbonated apatite-like calcium phosphate (CO_3-AP) comparable to bone (Oghusi *et al.* 1992; Ou-Yang *et al.* 2001). In previous studies on bone, by using Raman spectroscopy in all cases, the samples used were bulk-like and sometimes treated specifically with hydrazine (Walters *et al.* 1990; Rehman *et al.* 1995) or H_2O_2 (Penel *et al.* 1998; Freeman & Silva 2002) to decrease fluorescence of the samples. Although these approaches produce compositional data, they do not allow for both chemical and ultrastructural analysis at the same time. Recently, a combination of transmission light microscopy and Raman spectroscopy has been used to study microdamage in bovine bone and to image damage at low magnifications (Timlin *et al.* 2000), showing that different types of carbonated apatite were present around micro-cracks. This proved that high-resolution Raman analysis on a micrometer scale can reveal crucial information about sample composition, which can then be related to 2D histology. In 3D cell cultures, it was found by CRS that human osteoblasts produced bone cell spheroids, under the influence of TGF-β1, containing microspicules, composed of CO_3-AP similar, to mature bone (Kale *et al.* 2000). These examples illustrate that there is an increasing demand for combining ultrastructural with chemical analysis at high resolution, to gain a better understanding of the formation of extracellular matrix (ECM) in active cellular processes. In this study, we investigated *in vitro*-formed bone ECM, produced by osteoprogenitor cells obtained from rats, on titanium alloy plates.

2. METHODS

Biomimetic CO_3-AP coatings were provided by IsoTis SA (The Netherlands). The coatings were prepared according to a precipitation method by Barrere *et al.* (1999, 2001) on sandblasted Ti6Al4V (Smitford Staal BV, The Netherlands) square plates with a surface area of 100 mm^2, and 1 mm thickness. The plates were ultrasonically cleaned for 15 min in acetone, 70% ethanol and demi-water, in sequence. The coatings were analysed by XRMA, SEM (FEI, model XL-30 ESEM-FEG) and combined confocal Raman scanning electron microscopy (CRSEM) as described in the following.

2.1. Cell culture

Rat bone marrow cells were isolated from the femora of young (150 g) male Wistar rats (Maniatopoulos *et al.* 1988) and cultured in T75 flasks. Cell culture medium used during culture and seeding was composed of α-MEM medium (Life technologies, The Netherlands) containing 0.2×10^{-3} M L-ascorbic-acid-2 phosphate (ASAP, Life technologies), 0.01 M β-glycerophosphate (βGP, Sigma, The Netherlands), 1×10^{-8} M dexamethasone (DEX, Sigma), 15% foetal bovine serum (FBS, Life technologies) and 1% penicillin–streptomycin. At the third passage, the cells were seeded onto Ti6Al4V plates at a density of 1×10^4 cells cm^{-2} and then cultured for two weeks to allow sufficient ECM formation. As a control, the cells were also seeded on tissue culture polystyrene to monitor the growth and morphology of the cells. After 14 days of culture the cells were fixated with 1.5% glutaraldehyde in 0.14 M cacoadylate buffer (pH 7.4). After fixation, the cells were dehydrated by immersion in an increasing ethanol series (70–100%) and then critical point dried (Balzers CPD 030). In order to reveal the ECM structure underneath the cultured cells, the top cell layer was removed by gently applying either pressurized air to blow of the cell layer, or Scotch tape to reveal the underlying ECM and cells. The samples were analysed by CRSEM and XRMA. Pure collagen type I (Sigma) from bovine tendon was used as a positive control for collagen present in the ECM measured by Raman spectroscopy.

2.2. Combined confocal Raman scanning electron microscope

A collimated and circularly symmetrical beam from a diode laser with a frequency of 685 nm is reflected by a dichroïc beam splitter (BS) into the vacuum chamber of the SEM through a coupling window (figure 1). The beam is then focused by a 60× objective (numerical aperture 0.65) on a sample of interest. The excited Raman scattering is collected by the same objective, and the Stokes components of Raman frequencies pass through the BS, a notch filter and a pinhole (∅ 0.25 μm), which allows for confocality of the system. The scattering is collected by spectrograph–monochromator, in which the incoming light is decomposed by a concave holographic diffraction grating and focused on a thermo-electrically cooled CCD (1056×256 pixels, Princeton Instruments Spec10). The theoretical spatial resolution of the system is ∼700 nm with an effective laser power of 6 mW on the sample. The CCD is connected with a computer for data collection and analysis using WinSpec (Roper Scientific Inc., USA) and Microcal Origin (Microcal Software Inc., USA) data analysis software. The set-up was calibrated by using 2, 5 and 10 μm ∅ polystyrene beads to establish the coordinates needed for lateral movement from the electron

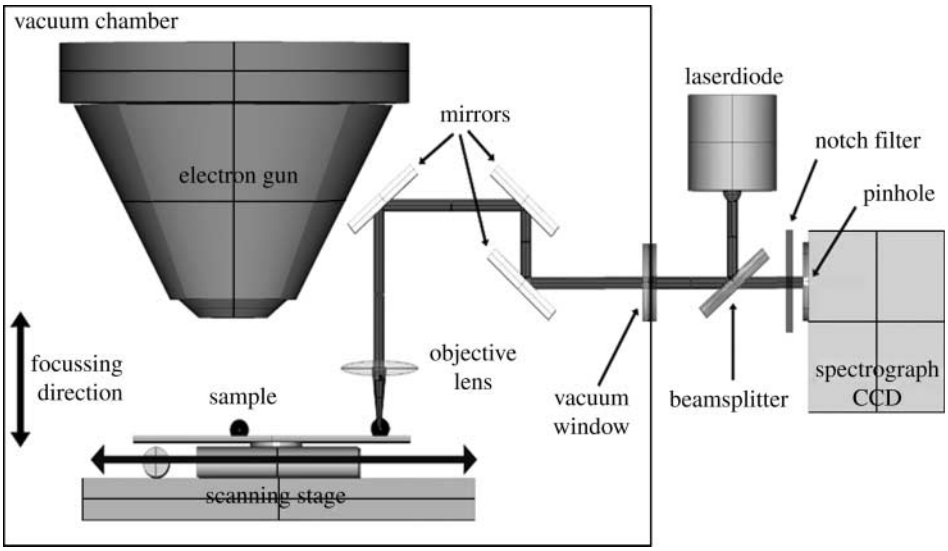

Figure 1. Schematic of the CRSEM. Samples are analysed by selecting a location by EM and then shifting the sample into the confocal laser spot at exact coordinates. The laser source consists of a laser diode with a wavelength of 685 nm and is diverted through a side port into the vacuum chamber by a BS. The laser is then emitted onto the sample by a set of gold-coated mirrors through a 60× objective. The induced Raman scatter is then collected by the same objective and diverted in the opposite direction, through a pinhole allowing for confocality of the system. Inside the spectrograph, the Raman scattering is subsequently focused on a holographic diffraction grating, and the decomposed wavelengths are then detected by a thermo-electrically cooled CCD. The scanning stage (nanometre movement) is equipped with a small light source to use transmission light microscopic observation for easy calibration of stage movement.

gun to the laser spot. Raman measurements were taken of the ECM observed by SEM in such a way that the focal plane always just touched the titanium alloy substrate surface; in this way all measurements were done the same. After collection of whole Raman spectra, images were collected on chosen frequencies by using a nanometre scanning stage (Kleindiek, Germany). The stage was controlled by LabView software (National Instruments, USA), and images were then generated from WinSpec data files by programming in LabView.

3. RESULTS

3.1. Confocal Raman scanning electron microscopy

We used polystyrene beads of sizes 10–2 μm to calibrate sample stage positioning. In this manner, we established the exact movement of our samples from our electron micrograph to the laser spot of the confocal Raman microscope. We were able to obtain clear polystyrene spectra (figure 2*a*) from these samples and, furthermore, were able to select a specific band (1004 cm^{-1}) for