Label-free Raman monitoring of extracellular matrix formation in three-dimensional polymeric scaffolds

Aliz Kunstar1,†, Anne M. Leferink1,‡, Paul I. Okagbare3, Michael D. Morris3, Blake J. Roessler4, Cees Otto2, Marcel Karperien1,‡, Clemens A. van Blitterswijk1, Lorenzo Moroni1 and Aart A. van Apeldoorn1,‡

1Department of Tissue Regeneration, and 2Department of Medical Cell Biophysics of MIRA, Institute for Biomedical Technology and Technical Medicine, University of Twente, Drienerlolaan 5, 7522 NB Enschede, The Netherlands
3Department of Chemistry, University of Michigan, 930 N. University Avenue, Ann Arbor, MI 48108, USA
4Division of Rheumatology, Department of Internal Medicine, University of Michigan Medical School, Medical Science Research Building II, 1150 West Medical Center Drive, Room 3560, Ann Arbor, MI 48109-5688, USA

Monitoring extracellular matrix (ECM) components is one of the key methods used to determine tissue quality in three-dimensional scaffolds for regenerative medicine and clinical purposes. Raman spectroscopy can be used for non-invasive sensing of cellular and ECM biochemistry. We have investigated the use of conventional (confocal and semiconfocal) Raman microspectroscopy and fibre-optic Raman spectroscopy for in vitro monitoring of ECM formation in three-dimensional poly(ethylene oxide terephthalate)–poly(butylene terephthalate) (PEOT/PBT) scaffolds. Chondrocyte-seeded PEOT/PBT scaffolds were analysed for ECM formation by Raman microspectroscopy, biochemical analysis, histology and scanning electron microscopy. ECM deposition in these scaffolds was successfully detected by biochemical and histological analysis and by label-free non-destructive Raman microspectroscopy. In the spectra collected by the conventional Raman set-ups, the Raman bands at 937 and at 1062 cm$^{-1}$ which, respectively, correspond to collagen and sulfated glycosaminoglycans could be used as Raman markers for ECM formation in scaffolds. Collagen synthesis was found to be different in single chondrocyte-seeded scaffolds when compared with microaggregate-seeded samples. Normalized band-area ratios for collagen content of single cell-seeded samples gradually decreased during a 21-day culture period, whereas collagen content of the microaggregate-seeded samples significantly increased during this period. Moreover, a fibre-optic Raman set-up allowed for the collection of Raman spectra from multiple pores inside scaffolds in parallel. These fibre-optic measurements could give a representative average of the ECM Raman signal present in tissue-engineered constructs. Results in this study provide proof-of-principle that Raman microspectroscopy is a promising non-invasive tool to monitor ECM production and remodelling in three-dimensional porous cartilage tissue-engineered constructs.

1. Introduction

Articular cartilage is a highly specialized connective tissue with the function to provide a smooth and low-friction buffer between the bones of a joint and to distribute the load over the surface of joints during movement. In several pathological conditions, such as osteoarthritis and rheumatoid arthritis or trauma, cartilage shows limited capacity for regeneration due to poor cellularity and its avascular character [1,2]. The main features of cartilage are closely related to its three-dimensional matrix, which mainly consists of collagen type II, proteoglycans and water [3,4]. Therefore, evaluating the production levels of these essential extracellular matrix (ECM) components is a key to determine tissue quality in tissue-engineered constructs. Conventional quality testing methods such as
immunohistochemistry, histology and microscopy techniques are all destructive and require tissue fixation, labelling, biochemical staining or cell lysis. By contrast, Raman microspectroscopy is a label-free technique, which does not require special sample preparation and can be used for non-invasive characterization of cell and tissue biochemistry [5]. It has been demonstrated that a careful selection of suitable laser wavelengths and intensity eliminates cell damage allowing for the study of cells without inadvertently changing their phenotype or behaviour caused by photo damage [6,7]. This vibrational spectroscopic technique employs an inelastic scattering effect (the Raman effect) to generate a molecular fingerprint of the investigated samples based on detection of specific wavelength shifts caused by chemical bond vibrations.

Raman spectral studies have already been performed on structural analysis of collagen [8,9], sulfated glycosaminoglycans (sGAGs) and proteoglycans [10,11]. Furthermore, this technique has been successfully applied to study collagen-containing ECM in a medium-throughput culture system [12] and to monitor chondrocyte behaviour on bioactive scaffolds [13]. In a fibre-optic configuration, Raman spectroscopy has already been used for monitoring tissues in joints in vivo [14] and to investigate the process of bone graft incorporation in bone reconstruction and repair in a transcutanous manner [15]. Other researchers have reported on in vivo measurements from the bladder and prostate [16], oesophagus [17], skin [18], cervix [19,20] and arteries using Raman spectroscopy [21].

In this study, we have investigated the use of label-free Raman microspectroscopy to detect ECM formation and to monitor the production levels of essential ECM components over time in three-dimensional porous poly(ethylene oxide terephthalate)/poly(butylene terephthalate) (PEOT/PBT) scaffolds using conventional and fibre-optic Raman set-ups. Previously, PEOT/PBT copolymers have been extensively studied and proved to be suitable for use as scaffold material in tissue engineering, both in vivo and in vitro [22–25], and reached clinical applications (PolyActive, IsoTis Orthopaedics SA) as dermal substitutes [26] and bone fillers [27,28].

2. Material and methods

2.1. Casting of agarose microwell arrays

Agarose microwell arrays were prepared with a soft-lithography technique. Polydimethylsiloxane negative moulds were used to routinely cast the arrays, with microwells with a diameter of 200 μm, in 3% agarose gel as described earlier [29]. Ultrapure agarose (Invitrogen, Carlsbad, CA, USA) was dissolved with a concentration of 3% w/v in sterile phosphate-buffered saline (PBS) solution (Gibco, Carlsbad, CA, USA) by heating. Seven millilitres of dissolved agarose were pipetted into each well of a six-well plate (in the confocal and fibre-optic Raman study; 48-well plate (in the semiconfocal Raman study; or culture treated 24-well (in the semiconfocal Raman study) or 8-well slide (in the confocal and fibre-optic Raman study). Each scaffold was placed aseptically into a well of a non-tissue culture plate. The cell–fibronectin suspension, adhesion [30], was applied to the chondrocyte suspension before seeding onto the scaffolds. The cell–fibronectin suspension, 3 x 10^6 cells in a final volume of 50 μl for each scaffold, was pipetted onto scaffolds and left to completely adsorb into the porous structure. Samples were left in an incubator at 37°C under a humidified atmosphere of 5% CO₂. Subsequently, at 1 day of culture, the chondrocyte microaggregates spontaneously formed microaggregates (approx. 350 cells per microaggregate) at the bottom of the microwells after 12 h. Subsequently, at 1 day of culture, the chondrocyte microaggregates were flushed out with CM from the agarose microwell arrays, collected and seeded on scaffolds.

2.2. Fabrication of poly(ethylene oxide terephthalate)/poly(butylene terephthalate) scaffolds

300PEOT55PBT45 (PolyActive300/55/45, PolyVation, The Netherlands) is a block copolymer with a weight ratio of 55 to 45 for the two components (PEOT and PBT), respectively, and a molecular weight of the starting poly(ethylene glycol) segments of 300 Da used in the copolymerization process. Three-dimensional regular grids were fabricated by three-dimensional fibre deposition with a bioscaffold (SysENG, Germany) with a fibre to fibre distance of 800 μm, a fibre diameter of approximately 200 μm and a layer thickness of 150 μm. Cylindrical porous scaffolds (6 mm in diameter by 4 mm in height for the semiconfocal Raman study and 4 mm in diameter by 3 mm in height for the confocal and the fibre-optic Raman study) were punched out of the three-dimensional regular grids. These scaffolds were sterilized in 70% ethanol two times for 30 min each, washed in PBS first for 5 min and additionally for other 30 min two times and finally incubated in culture medium overnight prior to cell culture.

2.3. Isolation of bovine chondrocytes and cell culture

Primary bovine chondrocytes were isolated from articular cartilage derived from the femoral-patellar groove of a 10-month-old calf by digestion with 420 Units ml⁻¹ collagenase type II ( Worthington Biochemical, Lakewood, NJ, USA). The freshly isolated passage 0 (P0) chondrocytes were cultured in chondroyte medium (CM) consisting of Dulbecco’s modified Eagle medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% v/v fetal bovine serum (South American origin; Biowhittaker, Lonza, Verviers, Belgium), 100 Units ml⁻¹ penicillin G (Invitrogen, Carlsbad, CA, USA), 100 μg ml⁻¹ streptomycin (Invitrogen), 0.1 mM non-essential amino acids (Sigma, St Louis, MO, USA), 0.4 mM proline (Sigma) and 0.2 mM L-ascorbic-acid-2-phosphate (Sigma) at 37°C under a humidified atmosphere of 5% CO₂.

After being cultured on tissue culture plastic (T-flask; Nunc; Thermo Fischer Scientific, Roskilde, Denmark), in control samples chondrocytes (P1) were seeded on scaffolds in CM. To compare samples seeded with single chondrocytes and with chondrocyte aggregates (confocal Raman study), P1 chondrocytes were used to seed directly onto scaffolds, while in parallel cell aggregates were made using agarose microwell arrays in CM (2865 microwells and 1 x 10^6 cells per agarose microwell array) for subsequent scaffold seeding. Chondrocytes seeded in these microwells spontaneously formed microaggregates (approx. 350 cells per microaggregate) at the bottom of the microwells after 12 h. Subsequently, at 1 day of culture, the chondrocyte microaggregates were flushed out with CM from the agarose microwell arrays, collected and seeded on scaffolds.

2.4. Cell seeding of poly(ethylene oxide terephthalate)/poly(butylene terephthalate) scaffolds

Each scaffold was placed aseptically into a well of a non-tissue culture treated 24-well (in the semiconfocal Raman study) or 48-well plate (in the confocal and fibre-optic Raman study; both Nunc, Thermo Fisher Scientific, Roskilde, Denmark).

In the semiconfocal Raman study, human fibronectin (300 μg ml⁻¹, BD Biosciences), which is known to support cell adhesion [30], was applied to the chondrocyte suspension before seeding onto the scaffolds. The cell–fibronectin suspension, 3 x 10^6 cells in a final volume of 50 μl for each scaffold, was pipetted onto scaffolds and left to completely adsorb into the porous structure. Samples were left in an incubator at 37°C under a humidified atmosphere of 5% CO₂ for 45 min. Subsequently, 2 ml of culture medium was carefully added drop wise. After 7 and 21 days of culture, the samples were studied using Raman microspectroscopy. Chondrocytes of C-28/12 human immortalized chondrocyte cell line were also seeded on PEOT/PBT scaffolds. C-28/12 cells were used as a negative control for matrix formation, since they were previously described showing mainly proliferation.
and low gene expression involved in ECM synthesis and turnover [31]. C-28/2 cell-seeded samples were cultured in CM under a humidified atmosphere of 5% CO₂ and 85% humidity, 1001 cm⁻¹, 1624, 2921 and 3054 cm⁻¹. Scanning, Bedford, MA, USA). Toluene, a Raman calibration standard, which has accurately known peak frequencies (521, 785, 1004, 1624, 2921 and 3054 cm⁻¹), was used for wavenumber calibration of the spectra. Prior to Raman measurements, samples were washed with PBS, fixed in 10% formalin for 10 min, washed extensively with PBS afterwards and placed onto UV grade calcium fluoride slides (Crystlan Ltd, UK).

2.7. Raman data analysis

Spectra obtained from the semiconfocal Raman study were corrected for curvature, dark current and variations in the CCD quantum efficiency. A mean spectrum was calculated and baseline-corrected from each transect after correction for the fused silica background.

Preprocessing of spectra obtained from the confocal Raman study (single cells versus microaggregates study) was performed as described previously [34–36]. The spectra were preprocessed by: (i) removing cosmic ray events, (ii) subtracting the camera offset noise (dark current), and (iii) calibrating the wavenumber axis. The well-known band positions were used to relate wavenumbers to pixels. The frequency-dependent optical detection efficiency of the set-up was corrected using a tungsten halogen light source (Avilight-HAL; Avantes BV, Eerbeek, The Netherlands) with a known emission spectrum. The detector-induced etaloning effect was compensated by this procedure. After data correction, the spectra of the Raman scans on respective measurement days were averaged to generate mean spectra for each sample.

In both the semiconfocal and confocal Raman study, semiquantitative univariate data analysis was performed by selecting specific vibrational bands of collagen or/and sGAGs in the averaged spectra from each time point and integrating each band after local baseline subtraction. Subsequently, normalized band-area ratios were obtained from the collagen and proteoglycan bands separately over the integrated band of the phenylalanine ring breathing mode at 1001 cm⁻¹. The calculations were made using normalized band-area ratios, not absolute values, so the system is a semiquantitative rather than a quantitative monitoring of the collagen and sGAG formation [12]. All data manipulations were performed using routines written in Matlab 7.4 (The Math Works Inc., Natick, MA, USA).

2.8. Fibre-optic Raman spectroscopy and data analysis

Fiber-optic Raman measurement was carried out using a custom designed fibre-optic Raman probe. Details of the Raman probe design have been previously described [15,37]. Briefly, the fibre-optic Raman probe consisted of 50 collection fibres that were
Figure 1. Baseline-corrected mean Raman transects from samples of PEOT/PBT scaffolds, seeded with bovine chondrocytes, acquired after 7 and 21 days of culture period and collected by the confocal Raman set-up in the region (a) from 600 to 1800 cm\(^{-1}\) and (b) from 900 to 1200 cm\(^{-1}\). Increased intensities of the Raman bands at 937 (collagen) and 1062 cm\(^{-1}\) (proteoglycans) were clearly visible from day 7 to day 21 of the culture period. (c) Semiquantitative univariate data analysis showed normalized integrated band-area ratios of the collagen and proteoglycan bands after 7 and 21 days of culture period. The normalized band-area ratios were obtained from the ratio of the collagen or proteoglycan band over the band of phenylalanine (1001 cm\(^{-1}\)). All bands were integrated after baseline subtraction. The normalized band-area ratios for both collagen and proteoglycan content were significantly increased from day 7 to day 21 of culture period (collagen: \(p = 0.0003\); sGAG: \(p = 0.0065\)). (Online version in colour.)

arranged into 10 branches. The fibres employed in the probe design were silica fibres with core diameter of 100 \(\mu\)m. Each branch housed five fibres and terminated in a stainless steel ferrule with an outer diameter of 1.25 mm. The Raman probe was used with a custom designed aluminium probe holder to facilitate contact of each fibre with the scaffold. The Raman collection probes were bundled at the distal end into a linear array for coupling to the Raman spectrograph (RamanRxn1, Kaiser Optical Systems Inc.). Laser illumination was achieved using a 300 \(\mu\)m core optical fibre that terminated in a stainless steel ferrule (outer diameter of 400 \(\mu\)m) and coupled to an 830 nm laser source. Raman data were acquired with the fibre-optic Raman probe and processed with Matlab—including silica subtraction—to generate 10 Raman spectra for all collection probes. Data processing included dark subtraction, removal of spikes due to cosmic rays and correction for grating induced curvature. Silica contribution from the optical fibres was subtracted by derivative subtraction of silica background spectra that were acquired by reflecting the excitation laser from a frosted aluminium surface into the optical fibres. Further processing included baseline subtraction using a fifth-order polynomial [38]. The 10 Raman spectra were averaged to generate mean spectra for each sample (each single cell-seeded scaffold).

2.9. Glycosaminoglycan detection assay

Following 7 and 14 days of culture, samples from the confocal Raman study (single cells versus microaggregates study) were washed with PBS and frozen at 80°C. After thawing, the constructs were digested for 16 h at 56°C with 1 mg ml\(^{-1}\) proteinase K (Sigma-Aldrich) in Tris/EDTA buffer (pH 7.6). This solution contained 185 \(\mu\)g ml\(^{-1}\) iodoacetamide and 10 \(\mu\)g ml\(^{-1}\) Pepstatin A (both Sigma-Aldrich). Quantification of total DNA was done using the CyQuant DNA assay (MolecularProbes) and a spectrophotometer (excitation 480 nm, emission 520 nm, Victor3, Perkin Elmer). The amount of GAG was determined spectrophotometrically after reaction with dimethylmethylen blue dye (Sigma-Aldrich) in a 10 mM hydrochloric acid (HCl) solution containing 3.04 g l\(^{-1}\) of chloramine T/oxidation buffer mix (excitation 480 nm, emission 520 nm). A microplate reader (Multiskan GO, Thermo Fisher) was used to determine the absorbance at 520 nm. The amount of GAG was calculated using a standard of chondroitin sulfate (Sigma-Aldrich).

2.10. Hydroxyproline assay

Hydroxyproline assay was carried out on the same proteinase K-digested samples as for the GAG assay and DNA quantification. From the digested sample, 15 \(\mu\)l was transferred to a Teflon-capped glass bottle, 15 \(\mu\)l of concentrated HCl (12 M) was added and samples were hydrolysed at 120°C for 3 h. The complete supernatant was transferred to a 96-well plate and left to evaporate at 60°C. Subsequently, 100 \(\mu\)l of chloramine T/oxidation buffer mix was added to each well and incubated at room temperature for 5 min. Finally, 100 \(\mu\)l of 4-dimethylaminobenzaldehyde reagent was added to each including the hydroxyproline standard and 4-methyl-2,6-dinitrophenol (Sigma-Aldrich).

Hydroxyproline was determined using the method of Engvall and Premium [40]. The reaction was carried out for 10 min at 22°C. A standard curve was generated using 15 \(\mu\)l of the 4-dimethylaminobenzaldehyde reagent and 4-methyl-2,6-dinitrophenol (Sigma-Aldrich) in a 10 mM hydrochloric acid (HCl) solution containing 3.04 g l\(^{-1}\) of chloramine T/oxidation buffer mix (excitation 480 nm, emission 520 nm). The amount of hydroxyproline was determined using a spectrophotometer (excitation 520 nm, emission 550 nm, Victor3, Perkin Elmer) and a standard of hydroxyproline (Sigma-Aldrich).

---

(a) 7 days
- 1277 1448
- 1614
- 1716
- 21 days
- 1614 1716

(b) intensity (arb. units) vs. Raman shift (cm\(^{-1}\))
- 937
- 1062

(c) normalized band-area ratios (arb. units)
- collagen (937 cm\(^{-1}\))
- proteoglycans (1062 cm\(^{-1}\))

Table 2. Relative quantification of samples for each collection probe. The normalized band-area ratios were obtained from the ratio of the collagen or proteoglycan band over the band of phenylalanine (1001 cm\(^{-1}\)). The normalized band-area ratios were increased from day 7 to day 21 of culture period. (a) All bands were integrated after baseline subtraction. The normalized band-area ratios for both collagen and proteoglycan content were significantly increased from day 7 to day 21 of culture period (collagen: \(p = 0.0003\); sGAG: \(p = 0.0065\)). (Online version in colour.)
incubated for 90 min at 60°C. A microplate reader (Multiskan GO, Thermo Fisher) was used to determine the absorbance at 560 nm.

2.11. Histology
In the confocal Raman study (single cells versus microaggregates study), after Raman measurements, samples were embedded in glycol methacrylate for histological analysis, sectioned at 10 μm intervals and stained with Safranin-O (Sigma) for visualization of sGAGs, and haematoxylin and eosin (H&E; Sigma) for visualization of the nuclei and cytoplasm.

2.12. Scanning electron microscopy
Cell morphology and attachment were characterized by SEM analysis with a Philips XL 30 ESEM-FEG. Samples (from the confocal Raman study) were fixed for 30 min in 10% formalin. Subsequently, the samples were dehydrated in sequential ethanol series and critical point dried from liquid carbon dioxide using a Balzers CPD 030 critical point dryer. The constructs were gold sputter coated (Cressington) prior to SEM analysis.

2.13. Statistical analysis
The results are presented as mean ± standard deviation (s.d.). Experimental data were analysed for statistical significance using a Student t-test. Statistical significance was set to p-value < 0.05 (*).

3. Results and discussion
The aim of this study is to investigate the use of conventional (semiconfocal and confocal) and fibre-optic Raman microspectroscopy for monitoring ECM formation during cell culture in three-dimensional fibre-deposited porous PEOT/PBT scaffolds. Moreover, chondrocytes have been seeded as single cells and as microaggregates onto the PEOT/PBT scaffolds, to investigate whether there are differences in ECM formation between single cell- and cell aggregate-seeded scaffolds. The rationale of using microaggregates instead of single chondrocytes was to initiate increased ECM formation. It is known that three-dimensional aggregation is an essential step in chondrogenesis and sustains matrix production of chondrocytes [39–41].

In the semiconfocal Raman study, Raman transects from PEOT/PBT scaffolds seeded with bovine chondrocytes were acquired after 7 and 21 days of culture period using a conventional Raman set-up. Mean Raman transects of these samples showed increased intensities of the bands at 937 and 1062 cm⁻¹ from day 7 to day 21 of the culture period (figure 1a,b, truncated). These bands correspond to collagen (proline, hydroxyproline and C–C vibrations of collagen backbone) [42] and chondroitin sulfate and proteoglycans (including aggrecan) [10,43,44], respectively, which are essential components of the ECM in cartilage. It was previously described that the band of collagen found at 937 cm⁻¹ can be used as a Raman marker for collagen-containing ECM.
form over time in chondrocyte pellet cultures [12]. Furthermore, chondrocyte behaviour on bioactive scaffolds has already been monitored by using Raman spectra from the ECM deposited onto such scaffolds and by assessing Raman bands indicative for collagen and amide I in the spectral region between 1200 and 1800 cm$^{-1}$ [13]. In order to quantify changes in intensities of bands at 937 and 1062 cm$^{-1}$, we used semiquantitative univariate data analysis. The results of the univariate data analysis are shown in figure 1c with the normalized band-area ratios for the band signifying collagen and proteoglycans. Subsequently, band-area ratios were obtained from collagen and proteoglycan bands separately over the integrated band of the phenylalanine ring breathing mode at 1001 cm$^{-1}$ [45]. Phenylalanine was found to be the best-resolved band and is not sensitive to local chemical environments [32]. The normalized band-area ratio for both collagen and proteoglycan content increased from day 7 to day 21 of culture period. Mean Raman transects were also obtained from PEOT/PBT scaffolds without cells (figure 2a,b, truncated) as reference spectra. Comparing these spectra with the mean Raman spectra of cell-seeded scaffolds in figure 1a,b, Raman contributions of PEOT/PBT polymer can be distinguished at 630 and 854 cm$^{-1}$ and between 1100 and 1800 cm$^{-1}$ in Raman spectra from cell-seeded scaffolds. The area of measurement on ECM overlaps in some cases with part of the polymer scaffold during Raman imaging, leading to Raman contributions from the polymer in the obtained spectra. Yet, PEOT/PBT-specific bands can be easily recognized and do not interfere with ECM-specific bands at 937 and 1062 cm$^{-1}$.

Raman transects from PEOT/PBT scaffolds seeded with the C-28/I2 human immortalized chondrocyte cell line, used as negative control for ECM formation, were collected after 21 days of culture period similar to bovine chondrocyte-seeded samples. The average of the Raman transects of these samples did not show bands at 937 cm$^{-1}$ (collagen) and at 1062 cm$^{-1}$ (proteoglycans), even after 21 days of culture (figure 3). Furthermore, bright field microscopy of these samples showed that no ECM was present (figure 4a) which confirmed the analysis done based on Raman spectra. By contrast, the bright field micrographs of bovine chondrocyte-seeded samples (figure 4b) showed collagen-like fibre formation at day 7 of the culture period indicative of ECM production. Moreover, the amount of ECM further increased in time completely covering the scaffolds at day 21 (figure 4b).

In the confocal Raman study presented here, the seeding of single chondrocytes was compared with seeding of chondrocyte microaggregates on three-dimensional fibredeposited PEOT/PBT scaffolds. In a previous study, similar three-dimensional polymer scaffolds were seeded with bovine articular chondrocytes in spinner flasks, so that stirred cell culture promoted the formation of cell aggregates as were used in this study [46]. In that previous study [46], cell aggregation enhanced the kinetics of cell attachment without compromising the uniformity of cell distribution on the scaffolds. In our confocal Raman study, chondrogenic differentiation medium was used to stimulate proteoglycan synthesis from chondrocytes in three-dimensional cell culture in the same way as was done by Mauck et al. [47]. Mean Raman spectra from single chondrocyte- and chondrocyte microaggregate-seeded scaffolds (figure 5) showed bands corresponding to phenylalanine (C–C aromatic ring) at 1001 cm$^{-1}$ [45], lipids/proteins (CH$_2$ bending mode) at 1448 cm$^{-1}$ [48] and amide I at 1657 cm$^{-1}$ [49]. Additionally, Raman bands at 937 cm$^{-1}$ (collagen) and at 1062 cm$^{-1}$ (proteoglycans) were also detected indicating ECM deposition. Although normalized band-area ratios for proteoglycan content did not change over time, collagen synthesis was found to be different in single chondrocyte-seeded scaffolds when compared with microaggregate-seeded samples (figure 5b,c). The normalized band-area ratios for total collagen content of single cell-seeded samples gradually
showed a significant increase during this period (collagen content of single cell-seeded samples gradually decreased during the 21-day culture period, the collagen content of the microaggregate-seeded samples was found to be different in single chondrocyte-seeded scaffolds when compared with microaggregate-seeded samples. While normalized band-area ratios for All bands were integrated after baseline subtraction. Although normalized band-area ratios for proteoglycan content did not change over time, collagen synthesis showed Raman bands at 920 and 972 cm$^{-1}$ traction. However, Raman spectra of cell-seeded scaffolds showed Raman contributions from the optical fibres (i.e. residual silica Raman spectra) even after silica background subtraction. Additionally, we investigated ECM deposition in the single cell- and microaggregate-seeded samples by more conventional methods. GAG formation was successfully detected by biochemical analysis (GAG assay), both in the single cell- and microaggregate-seeded samples at day 7 and 14 of culture period; however, significant differences were not observed (figure 7a). The hydroxyproline analysis indicated that cells produce significantly more collagen-like matrix per cell when seeded on the scaffolds as single cells compared with microaggregates (figure 7b). Furthermore, higher collagen production per cell was found after 7 days compared with 14 days for single cells. By contrast, the absolute bands in the spectral region between 900 and 1100 cm$^{-1}$. Therefore, bands arising in this region in spectra of bare scaffolds, as can be seen in figure 6c, originate from Raman scattering generated from the silica within the optical fibres, which were not completely removed by derivative silica subtraction algorithm performed with codes written in Matlab. A suitable algorithm in Matlab for more accurate silica background subtraction and/or further data processing using a multivariate statistical technique such as band target entropy minimization greatly reduces background bands and maximizes the Raman bands specific for ECM.

Figure 5. Mean Raman spectra and corresponding normalized band-area ratios of PEOT/PBT scaffolds seeded with bovine single chondrocytes or chondrocyte microaggregates. (a) Mean Raman spectra acquired after 7, 14 and 21 days of culture period and collected by the confocal Raman set-up in the region from 600 to 1800 cm$^{-1}$. The spectra are vertically displaced for clarity: (a) 7 days, (b) 14 days and (c) 21 days cultured scaffolds seeded with single chondrocytes, and (d) 7 days, (e) 14 days and (f) 21 days cultured scaffolds seeded with chondrocyte microaggregates. Semiquantitative univariate data analysis showed normalized integrated band-area ratios of collagen and proteoglycans bands for the Raman spectra of (b) single cell- and (c) microaggregate-seeded scaffolds at day 7, 14 and 21 of culture period. The normalized band-area ratio acquired from the ratio of the collagen or proteoglycan band to the band of phenylalanine (1001 cm$^{-1}$). All bands were integrated after baseline subtraction. Although normalized band-area ratios for proteoglycan content did not change over time, collagen synthesis was found to be different in single chondrocyte-seeded scaffolds when compared with microaggregate-seeded samples. While normalized band-area ratios for collagen content of single cell-seeded samples gradually decreased during the 21-day culture period, the collagen content of the microaggregate-seeded samples showed a significant increase during this period ($p = 0.0353$).
amount of total collagen was comparable for both time points in both conditions. The cell number, determined by DNA assay, showed an increase in single cell-seeded samples, whereas the DNA content for microaggregates remained stable during culture. This could explain the decrease in time for total collagen produced by single cell-seeded samples.

Furthermore, sGAG formation was also successfully shown by Safranin-O staining, and the nuclei and cytoplasm were visualized by H&E staining both in the single cell- and microaggregate-seeded samples at day 7 and 14 of culture period (figure 7c). The results of the Safranin-O indicated milder level of sGAG production in the microaggregate-seeded samples compared with the single cell-seeded ones, which can be explained by the fact that, especially after 14 days, the number of cells in the histological section is lower for microaggregate-seeded samples. This subtle difference in sGAG production was also observed by the GAG assay, although it was not statistically significant.

Furthermore, both the single cell- (figure 8a) and microaggregate-seeded sample (figure 8b) matrix-like structure formation could be observed by electron microscopy. Chondrocyte microaggregates adhering to the scaffold surface could be seen in the SEM micrographs of the microaggregate-seeded samples at day 7 of culture period. After 21 days of culture, the majority of microaggregates had a flattened appearance and collagen-like fibrous structures were observed in the vicinity of cells suggesting active ECM formation ultimately covering the surface of the scaffold used.

4. Conclusions
In this study, the use of conventional (semiconfocal and confocal) Raman microspectroscopy and fibre-optic Raman spectroscopy has been demonstrated for studying the formation of cartilage ECM in three-dimensional fibre-deposited PEOT/PBT scaffolds. The three different Raman microscope set-ups used in this study were all found to be feasible tools to monitor tissue formation inside tissue-engineered porous three-dimensional scaffolds. We studied ECM production of primary bovine chondrocytes seeded into three-dimensional fibre-deposited PEOT/PBT scaffolds. The Raman bands at 937 and 1062 cm\(^{-1}\) (attributes of ECM) were shown to be indicative for ECM production in the Raman spectra as analysed by fibre-optic Raman and confocal Raman
measurements inside the pores of the three-dimensional tissue-engineered construct. Normalized band-area ratios for proteoglycan content did not show clear differences over time when comparing single chondrocytes with microaggregates seeded into samples. However, we observed that collagen synthesis was different in single chondrocyte-seeded scaffolds when compared with microaggregate-seeded samples. Normalized band-area ratios for collagen content of single cell-seeded samples gradually decreased during the 21-day culture period, whereas the collagen content of the microaggregate-seeded samples significantly increased during this period. Results obtained in this study suggest that seeding microaggregates, instead of single chondrocytes, seems to improve ECM formation and therefore potentially can be used to improve cartilage tissue-engineered constructs. Future studies will focus on studying matrix (and pericellular matrix) organization in scaffolds more in detail.

A limitation of the fibre-optic system was the inherent background signal generated by silica within the optical fibre. Careful data analysis using Matlab revealed that bands at 920 and 972 cm\(^{-1}\) attributed to ECM components were recoverable. The use of a new generation of fluorocarbon fibre-optic Raman probes, which generate a reference Raman band for quantitative Raman spectroscopy, will eliminate silica background signals and possibly lead to well-resolved Raman spectra of ECM [37,53]. If so and as opposed to the conventional Raman set-ups, which are only able to measure a relatively small volume of the scaffolds, this would allow collection of Raman spectra from multiple pores in tissue-engineered scaffolds with one measurement in parallel. This generates a representation of the overall Raman signal from ECM within these scaffolds.

In order to further improve such analysis, further investigation is needed to validate the potential of fibre-optic evaluation of cartilaginous matrix formation in tissue-engineered constructs. Additionally, more profound knowledge on the discrimination of specific collagen types is a key element to be considered in future studies regarding Raman measurements of cartilage ECM.

Funding statement. The authors gratefully acknowledge the funding from the Dutch Program for Tissue Engineering (DPTE) through grant no. TGT. 6737, from The Netherlands Institute for Regenerative Medicine (NIRM) through grant no. FES0908 and from the National Institutes of Health (NIH) through grant nos. R01AR055222 and R01AR056646. C-28/I2 human immortalized chondrocyte cell line was established using cells derived from tissue that was provided by the National Disease Research Interchange.

**Figure 7.** Matrix production. Results of (a) GAG assay and (b) hydroxyproline assay, a measure for total collagen, both normalized to DNA content of single chondrocyte- or microaggregate-seeded PEOT/PBT scaffolds at day 7 and day 14 of culture period. GAG production was successfully detected in all samples, although major significant differences were not observed. Collagen production decreased significantly for single cells when comparing day 7 with day 14 (\(p = 0.042\)). Microaggregates show significantly less collagen production than single cells (\(p = 0.016\)) after 7 days of culture. (c) Result of Safranin-O and H&E staining visualizing sGAG production and nuclei and cytoplasm, respectively, in single chondrocyte- and microaggregate-seeded scaffold at day 7 and day 14 of culture period. Scale bars, 100 µm. (Online version in colour.)
References


Figure 8. SEM micrographs of (a) single chondrocyte- and (b) microaggregate-seeded scaffolds at day 7 and day 21 of culture period. Collagen-like fibres, indicating ECM formation, were observed in all samples. Chondrocyte microaggregates can be seen adhered to the scaffolds and showing the onset of cell spreading at day 7 of culture period. After 21 days of culture period, the majority of the microaggregates gained a flattened appearance and collagen-like fibrils were observed indicating active ECM formation by the cells covering the surface of the scaffold. Magnifications and scale bars: 100×: 200 μm; 250×: 100 μm; 1000×: 20 μm; 2500×: 10 μm; 4000×: 5 μm; 10 000×: 2 μm.


