

utilized as a FCS probes.

Alexa-albumin is made by adding to label albumin form bovine serum to Alexa-hydrazide. The diffusion behavior of this fluorescent dye was reproduced and embodied with *in vitro* model tracing the movement of albumin. It chanced that Alexa-albumin was commercially available; however, it is necessary to develop FCS probes concerning other molecules to understand physiological diffusion of molecules in the living body. Further studies regarding new FCS molecular probe are essential in the future.

The results of this study reveal that it is possible to measure the diffusion state of fluorescence-dyed albumin applying with FCS measurement regardless of intensity of cartilage degeneration.

As shown in Figure 4., FCS data demonstrated that diffusion coefficients of Alexa-albumin were grater when measuring degenerated cartilage indicating that FCS probes moved more freely in degenerated cartilage than normal tissue. This phenomenon may be occurred in relation to gradually destroyed ECM structures which finally give probes a chance to move wider space than that of normal cartilage. Diffusion coefficients tell us diffusion

characters of certain molecules. The lager molecular weight of molecules showed the lower diffusion coefficients. Simultaneously, a certain molecule of the diffusion coefficients is affected by the solution environments as is proved by tests of HAs showing decrease with increasing their molecular weight. Therefore, lower diffusion coefficients stand for difficulties for molecules to diffuse in the surrounding conditions, which is coincident with present results. Diffusion times of molecule increase with decreasing diffusion coefficients. Contrary, increment of diffusion coefficients are affected by lower molecular weight and diffusion times are shortened.

To the authors' knowledge, there were no previous works demonstrating the feasibility of FCS for direct measurement diffusion behavior of ECM in the cartilage tissue, which may be a potential and useful evaluation tool. If the diffusion of ECM is clarified with this new method by standardization of FCS data of various condition of cartilage, FCS will be applied as an early diagnosis of OA which is accompanied by increased destroyed ECM elements and also as an evaluation tool of bio-engineered artificial cartilage for autologous

chondrocyte implantation.

E. 結論

This work firstly demonstrated the capability of FCS for direct measurement diffusion behavior of ECM in the cartilage tissue.

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

なし

2. 学会発表

李 禎翼、小池 亮一、森戸 崇暁、佐藤 正人、丑田 公規：蛍光相関分光法による関節軟骨中の拡散現象の研究、理研シンポジウム、蛍光相関分光で見る生体系の情報伝達、2008. 2.22（埼玉県和光市）

李 禎翼、丑田 公規 佐藤 正人、小池 亮一、森戸 崇暁、持田 譲治：蛍光相関分光法による関節軟骨中の拡散現象の評価、第23回日本整形外科学会基礎学術集会、2008. 10.23（京都府京都市）

H. 知的財産権の出願・登録状況

なし

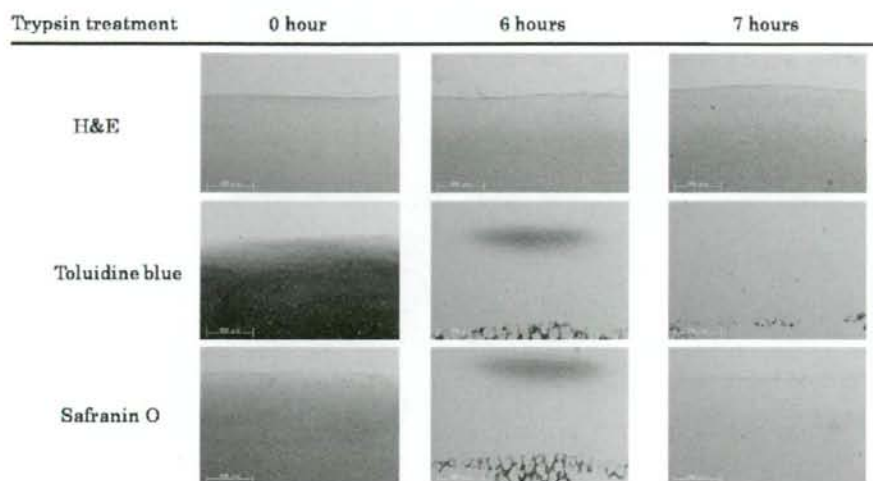


Figure 1. Results of the histological examination, Scale bar = 500 μ m

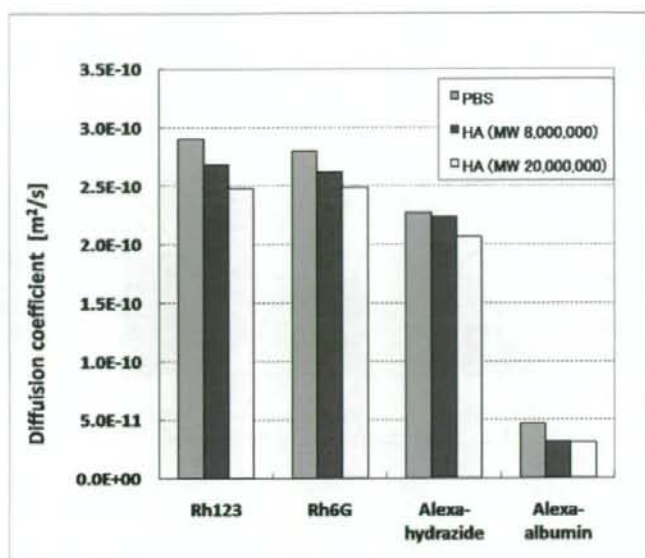


Figure 2. Diffusion coefficients of fluorescent dyes, $E-10=\times 10^{-10}$)

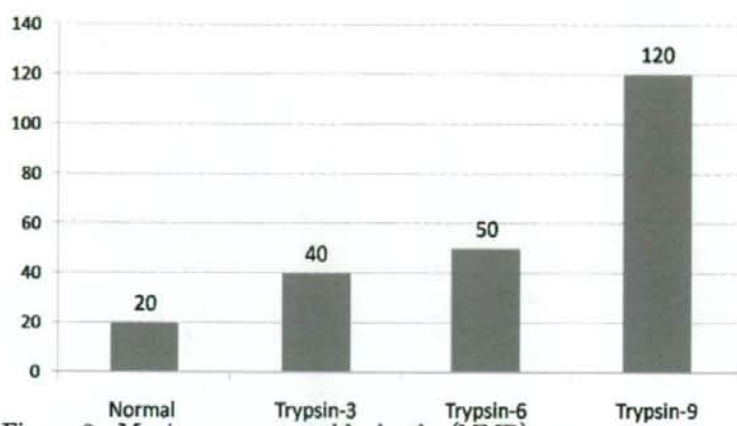


Figure 3. Maximum measurable depths (MMD), μm

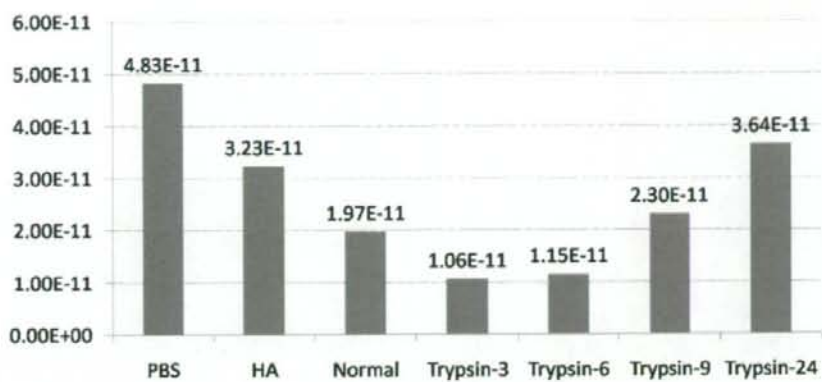


Figure 4. Diffusion coefficients of Alexa-albumin

IV. 研究成果の刊行に関する一覧表

学術論文	雑誌名	タイトル	著者
2008年2月	Proceeding of SPIE 6858, 685804-1-685804-6	Modification of measurement methods for evaluation of tissue engineered cartilage function and biochemical properties using nanosecond pulsed laser	Ishihara M, Sato M, Kutsuna T, Ishihara M, Mochida J, Kikuchi M
2008年4月	別冊整形外科No. 53 変形性関節症の最近の知識	変形性関節症の病態把握と治療効果判定を可能にする定量的機能診断システムの開発ーナノ秒パルスレーザーを用いた力学特性評価と組織性状の同時計測をめざしてー	佐藤正人、石原美弥、杓名寿治、三谷玄弥、菊地眞、持田謙治
2008年7月	第4回集積光デバイス技術時限専門委員会誌, IPD08(25) p.44-49 (2008)	再生医療を光で評価する	石原美弥, 菊地眞
2008年8月	Medical and Biological Engineering and Computing, 46(8), 735-743, 2008	Recent technological advancements related to articular cartilage regeneration	Sato M, Ishihara M, Furukawa SK, Kaneshiro N, Nagai T, Mitani G, Kutsuna T, Ohta N, Kokubo M, Kikuchi T, Sakai H, Ushida T, Kikuchi M, Mochida J
2008年10月	MICROOPTICS NEWS, 26(3) p. 31-36 (2008)	光による再生医療に用いる組織・細胞の評価	石原美弥, 菊地眞

学会発表	学会名	タイトル	著者
2008年3月	第7回日本再生医療学会総会, 名古屋, 2008.03.13-14	軟骨再生医療評価のための光計測技術開発	石原美弥, 佐藤正人, 持田讓治, 菊地眞
2008年4月	2007年度東海大学医学部研究助成金(重点研究)報告会, 伊勢原	ナノ秒パルスレーザーによるScaffold Free組織工学的軟骨の非侵襲的性状評価	佐藤正人
2008年5月	第47回日本生体医工学会大会, 神戸 2008.05.08-10,	再生医学における光技術	石原美弥, 佐藤正人, 持田讓治, 菊地眞
2008年7月	第4回集積光デバイス技術研究会, 東京, 2008.07.30	再生医療を光で評価する	石原美弥, 菊地眞
2008年9月	第9回 医療・バイオデバイス応用レーザー専門委員会, 福岡, 2008.9.26	光技術による再生医療の細胞・組織の評価	石原美弥
2008年10月	第23回日本整形外科基礎学術集会, 京都, 2008.10.23-24	光による軟骨再生医療の評価	石原美弥, 佐藤正人, 持田讓治, 菊地眞
2008年10月	第109回微小光学研究会, 東京, 2008.10.9	光による再生医療に用いる組織・細胞の評価	石原美弥, 菊地眞
2008年10月	BioJapan2008, 横浜, 2008.10	再生医療の早期実用化を目指した再生評価技術開発ー軟骨の再生医療プロセスの計測・評価技術開発	佐藤正人, 石原美弥
2008年10月	第23回日本整形外科基礎学術集会, 京都, 2008.10.23-24	ナノ秒パルスレーザーによる組織工学的軟骨の非侵襲的性状評価	杓名寿治, 佐藤正人, 石原美弥, 長井敏洋, 古川克子, 菊地眞, 牛田多加志, 持田讓治
2008年10月	第23回日本整形外科基礎学術集会, 京都, 2008.10.23-24	蛍光相関分光法による関節軟骨中の拡散現象の評価	李禎翼, 丑田公規, 佐藤正人, 小池亮一, 森戸崇暁, 持田讓治
2009年1月	レーザー学会学術講演会第29回年次大会, 徳島, 2009.01.10-12	再生医療に用いられる組織・細胞のレーザーによる評価	石原美弥, 菊地眞
2009年3月	第8回日本再生医療学会総会, 東京	ナノ秒パルスレーザーによるScaffold Free組織工学的軟骨の非侵襲的性状評価	杓名寿治, 佐藤正人, 石原美弥, 長井敏洋, 古川克子, 菊地眞, 牛田多加志, 持田讓治
2009年3月	第8回日本再生医療学会総会, 東京	光による関節軟骨の機能評価とASTM(米国材料試験協会)標準化	佐藤正人, 石原美弥, 三谷玄弥, 杓名寿治, 菊地眞, 持田讓治

V. 研究成果の刊行物・別刷

Recent technological advancements related to articular cartilage regeneration

Masato Sato · Miya Ishihara · Katsuko Furukawa · Nagatoshi Kaneshiro · Toshihiro Nagai · Genya Mitani · Toshiharu Kutsuna · Naoshi Ohta · Mami Kokubo · Tetsutaro Kikuchi · Hideaki Sakai · Takashi Ushida · Makoto Kikuchi · Joji Mochida

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Abstract Some treatments for full thickness defects of the articular cartilage, such as the transplantation of cultured chondrocytes have already been performed. However, in order to overcome osteoarthritis, we must further study the partial thickness defects of articular cartilage. It is much more difficult to repair a partial thickness defect because few repair cells can address such injured sites. We herein show that bioengineered and layered chondrocyte sheets using temperature-responsive culture dishes may be a potentially useful treatment for the repair

of partial thickness defects. We also show that a chondrocyte-plate using a rotational culture system without the use of a scaffold may also be useful as a core cartilage of an articular cartilage defect. We evaluated the properties of these sheets and plates using histological findings, scanning electrical microscopy, and photoacoustic measurement methods, which we developed to evaluate the biomechanical properties of tissue-engineered cartilage. In conclusion, the layered chondrocyte sheets and chondrocyte-plates were able to maintain the cartilage phenotype, thus suggesting that they could be a new and potentially effective therapeutic product when attached to the sites of cartilage defects.

M. Sato (✉) · M. Ishihara · N. Kaneshiro · T. Nagai · G. Mitani · T. Kutsuna · N. Ohta · M. Kokubo · J. Mochida
Department of Orthopaedic Surgery, Surgical Science,
Tokai University School of Medicine, 143 Shimokasuya,
Ishihara, Kanagawa 259-1193, Japan
e-mail: sato-m@is.icc.u-tokai.ac.jp

M. Ishihara · M. Kikuchi
Department of Medical Engineering, National Defense Medical
College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan

K. Furukawa
Department of Bioengineering, School of Engineering,
The University of Tokyo, 7-3-1 Hongo, Bunkyo,
Tokyo 113-8656, Japan

K. Furukawa
Department of Mechanical Engineering, School of Engineering,
The University of Tokyo, 7-3-1 Hongo, Bunkyo,
Tokyo 113-8656, Japan

T. Ushida
Laboratory of Regenerative Medical Engineering,
The University of Tokyo, 7-3-1 Hongo, Bunkyo,
Tokyo 113-8656, Japan

T. Kikuchi · H. Sakai
CellSeed Inc., 33-8 Wakamatsucho, Shinjuku,
Tokyo 162-0056, Japan

Keywords Tissue engineering · Articular cartilage · Chondrocyte · Cell sheet · Scaffold free · Photoacoustic measurement

1 Introduction

Adult articular cartilage shows a poor self-repairing ability after either degeneration or injury occurs and is, therefore, unlikely to be restored to normal condition once it has been damaged. The currently available treatments for cartilage defects include the application of a periosteal patch to cover the defect [1] and mosaicplasty, in which an osteochondral pillar is grafted from a non-weight-bearing site [2]. However, the use of periosteal patches has limitations due to problems with ossification and the limited area that can be treated. Although microfracture techniques, in which drilling is employed to induce bone marrow cells to differentiate into chondrocytes, are now widely used, the cartilage obtained by this technique is fibrocartilage with different characteristics from those observed in hyaline

cartilage. Since promising results for the transplantation of cultured autologous cartilage cells have been reported [3], various articular cartilage regeneration techniques have thus been clinically applied, including the use of scaffolds such as atelocollagen [4] and cell transplantation therapy with bone marrow-derived mesenchymal stem cells (MSC) [5]. However, the current cartilage regeneration techniques are intended for the treatment of full thickness defects, and there have so far been no reports regarding the clinical application of a technique for treating partial thickness defects in patients with early osteoarthritis as far as we could determine in a comprehensive literature search.

In this study, we introduce two different approaches for the treatment of cartilage defects without using a scaffold. One is using chondrocyte sheets for the treatment of partial thickness defects of the articular cartilage; while, the other is using a chondrocyte-plate for the core of an articular cartilage defect. Finally, we mention the findings of a study describing a non-invasive measurement method to evaluate the biomechanical properties of such tissue-engineered cartilage using photoacoustic measurements.

2 Materials and methods

2.1 Isolation of chondrocytes

2.1.1 Human articular chondrocytes

The cells used for the *in vitro* experiment included human articular chondrocytes derived from patients who had undergone anterior cruciate ligament reconstruction and gave their informed consent at the Tokai University Oiso Hospital from December 2004 to August 2005. The chondrocytes were obtained while forming the interfoveolar ligament, and then they were isolated by enzymatic treatment. Twenty-five knees from 25 patients aged 14–49 years (average 23 years old, 19 males and 6 females) were used as the source of these cells. Next, the chondrocytes were subjected to enzymatic treatment, and then were seeded and cultured according to the method of Sato et al. [6].

2.1.2 Articular chondrocytes from Japanese white rabbits

Twelve Japanese white rabbits aged 3–4 weeks and weighing about 800–1,000 g were used as the source of articular cartilage cells. Cartilage samples were collected from the femoral compartment of the knee joint, and then were subjected to the same enzymatic treatment process as that used for human articular cartilage cells. Thereafter, the isolated cells were seeded and cultured in temperature-responsive culture dishes.

2.2 Preparation of the tissue-engineered cartilage with ACHMS scaffold

The cells (0.2 ml) were then seeded at a high density (2×10^6 cells/scaffold) on the top (open side) of an atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold, Fig. 2a–c) [7–15] (diameter 6 mm, thickness 2 mm) in 96-well plates (Sumitomo Bakelite Corp., Tokyo, Japan) by centrifugation at 500 rpm (45 g) for 5 min and then were cultured in F12/DMEM supplemented with a 10% fetal bovine serum (Iwaki) and 50 g/ml of ascorbic acid (Sigma Corp., St Louis, MO) at 37°C in an atmosphere of 5% CO₂ in air and 100% relative humidity. After the indicated period of incubation, the cultures were studied (1) by a scanning electron microscope, (2) for the DNA content as an indicator of cell proliferation, as well as (3) for the glycosaminoglycan (GAG) accumulation as the marker of matrix formation [17]. Prior to the transplantation of the chondrocyte-containing ACHMS scaffold into an articular cartilage defect *in vivo*, the chondrocytes were cultured for 21 days in the scaffold. All cells were plated at a density of 2×10^6 cells/dish (Falcon culture dish; diameter 10 cm; Nippon Becton Dickinson Co., Ltd., Tokyo, Japan).

2.3 Preparation of the chondrocyte sheet (without scaffold)

Each culture dish was removed from the incubator when the cells reached confluence and then was left to stand at about 25°C for 30 min. After the culture medium was removed, the cell sheet was collected using a polyvinylidene difluoride (PVDF) membrane according to the method reported by Kaneshiro [16]. In brief, the PVDF membrane was placed on the cell sheet and then the sheet was rolled up with the membrane from one corner. The cultured human chondrocytes could be successfully collected as cell sheets by this method. Next, each cell sheet was placed on top of another confluent cell sheet in order to prepare multilayered sheets. Three-layered sheets of the cartilage cells from Japanese white rabbits were also prepared. In addition, a 0.4- μ m filter was also used in order to compress these sheets onto the culture dish and thereafter incubation was performed for 1 week in order to prepare the multilayered sheets for transplantation.

2.4 Preparation of chondrocyte-plate (without scaffold)

In order to create a chondrocyte-plate that is hard enough and with a sufficiently regular appearance, a primary static culture is required before the rotational culture is performed.

2.4.1 Primary static culture

We harvested all chondrocytes after passages 1–3 in culture. Thereafter, we adjusted the cell suspension to a density of 1.0×10^7 cells/ml in DMEM/F12 supplemented with 20% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone, and 50 µg/ml ascorbic acid. Four cylindrical molds (diameter 10 mm, height 10 mm) were then placed on each culture insert having a pore size of 0.4 µm (Corning Coastrar Japan, Tokyo, Japan: Cat.3419). At first, 13 ml of culture medium was added under the culture insert. Subsequently, 13 ml of medium was added on the culture insert around the molds. (The surrounding culture medium was identified whether or not the medium invaded from the lateral side of the bottom of the molds.) Next, 600 µl of the cell suspension (0.6×10^7 cells) was inoculated in the mold and the cell suspension subsided 15–20-min later. Thereafter, approximately another 40 ml of culture medium was added to the culture insert dish until the molds were completely filled with medium (total medium was 65 ml). Adding the medium after cellular sedimentation by gravity was found to prevent cellular leakage from the top of the mold. The culture insert was then put into a dish measuring 150 mm in diameter (Becton Dickinson Labware), without a culture insert dish cover. Then 8 h later, the cell suspension in the mold showed cell aggregation, namely producing a chondrocyte-plate. Thereafter, the mold was removed. The chondrocyte-plate was primary static cultured for 7 days until it formed a regular cylindrical shape under the dynamic culture conditions. The dishes were then placed in a humidified 37°C/5% CO₂ incubator.

2.4.2 Rotational culture system

In the rotational culture studies, all of the constructs were cultured under primary static culture conditions and then were cultured under dynamic conditions using a rotational culture system. The chondrocyte-plate was moved to a non-adherent six-well dish (Corning Cat 3411) using a medicine spoon. Next, an orbital shaker; rotational culture system was used [17]. The construct was cultured at a speed of 70 rpm for 3 weeks. The culture medium was completely replaced every 2–3 days (the medium renewal rate was 2.5 ml/plate per day). The dishes were thereafter placed in a humidified 37°C/5% CO₂ incubator.

2.5 Histological study

The samples were fixed in 4% paraformaldehyde, embedded in paraffin, and then sectioned (4 µm thick). Histological staining was performed with Safranin-O for the glycosaminoglycans. The sections were immunostained

with mouse antihuman type II collagen monoclonal antibody (Daiichi Fine Chemical Corp., Toyama, Japan) by incubating with diaminobenzidine and 5 µl hydrogen peroxide in 100 ml PBS for 5 min at room temperature.

2.6 Photoacoustic measurement method

A nanosecond pulse light was used for the excitation of the stress waves. The light beam was focused with a lens and then coupled to a silica fiber of 600 µm in core diameter. The stress waves induced by the light pulses were detected by a piezoelectric transducer consisting of a poly(vinylidene fluoride-trifluoroethylene) [P(VdF/TrEE)] film of 55 µm in thickness. The output signals of the photoacoustic transducer were then amplified (bandwidth, 1 GHz, TDS 3054B, Tektronics, Beaverton, OR). The relaxation time theoretically has a relationship with the viscous-to-elastic modulus rate (Fig. 1). The relaxation time (τ) was calculated using the Levenberg–Marquardt algorithm, a non-linear least squares method, as described below. When the attenuation of the stress wave intensity is affected only by the reflection at the boundaries and the relaxation during transmission through the viscoelastic materials, then the time course of the stress wave intensity is thus expected based on the following equation

$$I_{\delta} = I_0 \times R \times \exp(-\delta t/\tau). \quad (1)$$

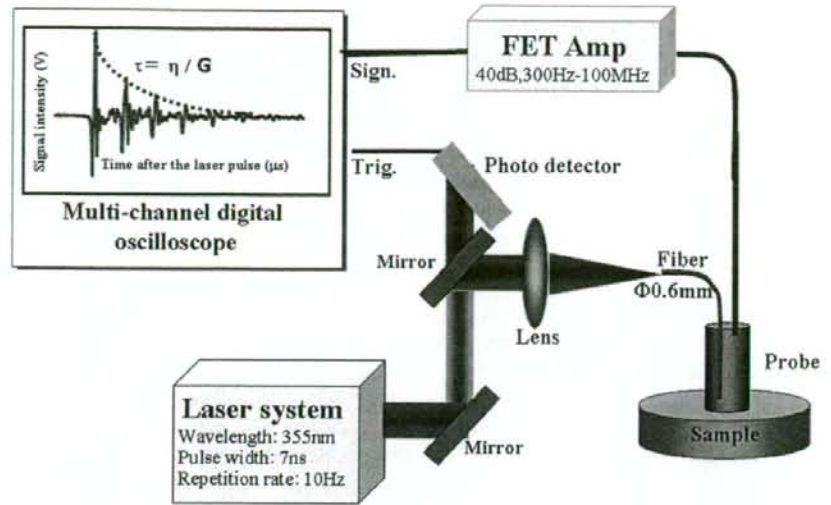
where τ is relaxation time, I_0 initial intensity of the stress wave, R product of the reflectance at the front surface and posterior boundary, δt time required to transmit the stress wave back and forth between the sample, and I_{δ} first peak intensity of each wave packet of the measured acoustic pulse sequence at $t = \delta t$. We investigated the characteristics of the measured signal in order to determine whether or not the diffraction affected the calculation of the relaxation time from the measured stress wave intensity. It was shown that there was no need to consider the diffraction effect when measuring the articular cartilage with only a few millimeters in thickness in comparison to the diffraction length (>20 mm). The tissue-engineered cartilage with the ACHMS scaffold was measured using a photoacoustic measurement system [13, 18].

3 Results

3.1 Tissue-engineered cartilage with the ACHMS scaffold

The high density of the chondrocytes (2×10^6 cells/scaffold) could be seeded into the ACHMS scaffold by gentle centrifugation without spilling the cells due to the scaffold membrane sealing. Scanning electron microscopy after

Fig. 1 Experimental setup of the photoacoustic measurement. System for measuring the relaxation time by the photoacoustic method. The lens is a silica lens with a 70-mm focal length, and the photodetector, which is used to trigger the photoacoustic signal, is a biplanar phototube. The diameter of the transducer is 4 mm. The monitor of the digital oscilloscope shows a typical observed waveform of photoacoustic signal. FET Amp field effect transistor amplifier



21 days of culturing (Fig. 2e, f) revealed the scaffold to be filled with grown chondrocytes with their produced and accumulated matrix molecules. The chondrocytes in the scaffold were also found to retain their spherical shape, namely, the typical appearance of the chondrocyte cells, during the culturing period. Figure 2 also shows microphotographs of the chondrocyte cultures inside the scaffold after 21 days. The chondrocytes were able to proliferate in the scaffold, thus resulting in a strong expression of type II collagen (Fig. 2d).

After 1 week of culture, the tissue-engineered cartilage was considered to be sufficiently stable to be handled with surgical pincers. The DNA content of the chondrocytes in the scaffold culturing increased at a lower rate in comparison to the monolayer culturing during the initial 21 days (Fig. 3a). The increase in the total GAG content from the cells and matrix components of the scaffold culture occurred at a higher rate than in the monolayer culture, and this difference was observed throughout the entire culture period for up to 21 days (Fig. 3b). Therefore, chondrocytes are able to gradually grow for a longer period of time (at least 21 days), and although they have a lower growth rate than in a monolayer culture, the formation of the extracellular matrix in the scaffold culture is higher.

3.2 Chondrocyte sheet (without a scaffold)

The chondrocyte sheets could be easily prepared by placing one sheet on another and then culturing them together. It was thus possible to continue the culture of such three-layered sheets. The three-layer human articular chondrocyte sheets that were cultured for 1 week were extendable

and thus were not damaged by any mild external force. Figure 4 shows that the layered rabbit articular chondrocyte sheets cultured for 3 weeks maintained their original shape without shrinking when the PVDF was removed. As a result, it was thus possible to create round grafts which demonstrated a chondrocytic phenotype.

3.3 Chondrocyte-plate (without scaffold)

After a primary static culture for 7 days, the chondrocyte-plate was able to be collected in a spoon while maintaining the form of a circle, then it was conducted to the rotational culture system. It was thus preserved without losing its regular cylindrical shape even if we pinched it with tweezers after a rotational culture for 3 days. After 2 weeks of rotational culture, the chondrocyte-plate was considered to be sufficiently stable to be handled using surgical pincers. The chondrocyte-plates measuring about 1.1 mm in thickness after 2 weeks and about 1.4 mm after 3 weeks of cultivation had a regular cylindrical shape and the macroscopic appearance of cartilage. The chondrocyte-plate was thus able to be made without losing its regular cylindrical shape regardless of the cellular passage number (Fig. 5).

3.4 Evaluation of photoacoustic measurement method

The tissue-engineered cartilage with the ACHMS scaffold cultured for a longer period had smaller relaxation times. The relaxation times obtained by photoacoustic measurement closely correlated with the measured intrinsic relaxation parameters, with a correlation coefficient of over 0.9 (Fig. 6).

Fig. 2 An atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold). **a** Top of the scaffold. **b** Bottom of the scaffold with the membrane seal. **c** Microscopic appearance of the bottom of the scaffold. **d** Type II collagen immunostaining of the tissue-engineered cartilage after 21 days. **e** A scanning electron microphotograph of the scaffold cultured chondrocytes after 21 days (original magnification $\times 150$). **f** A scanning electron microphotograph of the scaffold cultured chondrocytes after 21 days (original magnification $\times 1,000$)

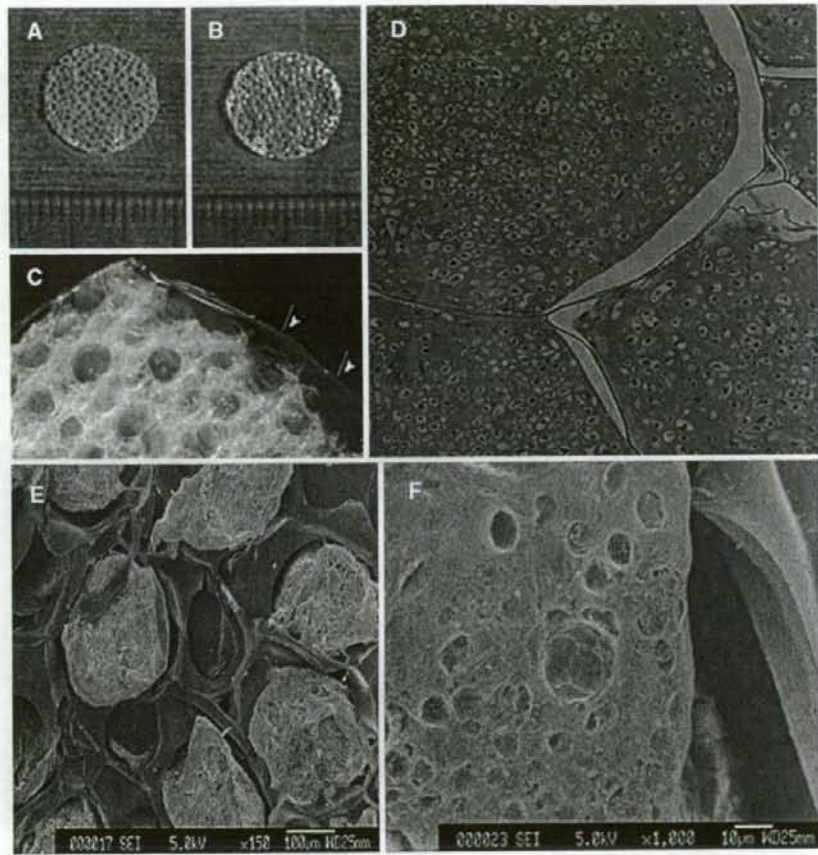
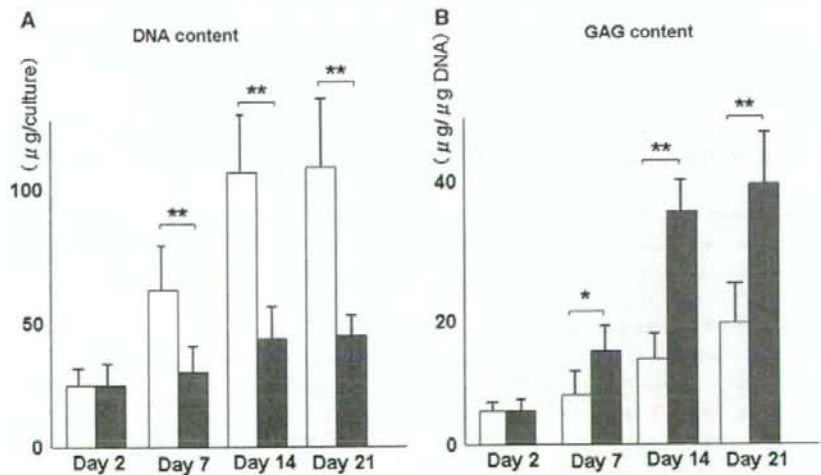


Fig. 3 **a** DNA content in the cultured chondrocytes using ACHMS scaffold (gray bars) and monolayer culture (white bars). **b** GAG content in the cultured chondrocytes using ACHMS scaffold (gray bars) and monolayer culture (white bars). Measurements of the DNA and GAG contents have been carried out on the indicated days. Data represent the mean \pm SD of six determinations. * $P < 0.05$, ** $P < 0.01$



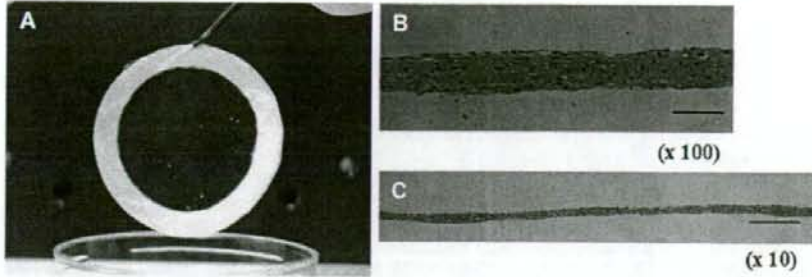


Fig. 4 A layered chondrocyte sheet. **a** Harvest of the cell sheets. The cultured chondrocytes using a temperature-responsive surface could be released from the dish (ϕ 35 mm) only by reducing the temperature without a proteolytic enzyme. The confluent cultured chondrocytes were harvested as a single contiguous cell sheet

retaining cell-cell junction as well as the deposited ECM on the basal side. **b** The chondrocyte sheets could be layered and thereafter adhere to the other cell sheets. The culture of the five-layered chondrocyte sheets could be continued for 1 week. **c** Low magnification of **b**. *Bar* 400 μ m

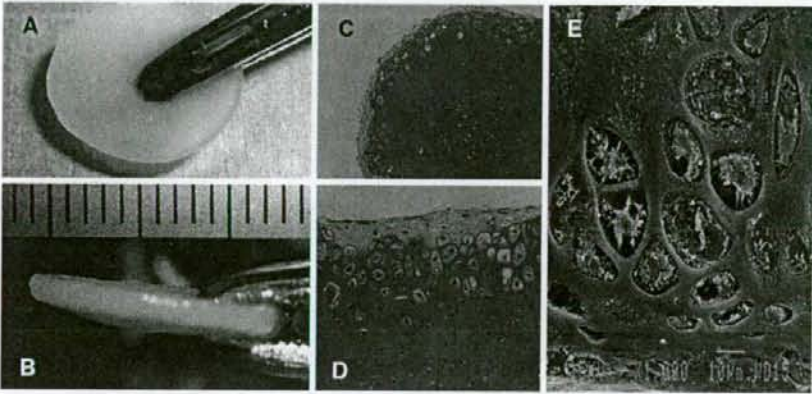


Fig. 5 A chondrocyte-plate. **a, b** The macroscopic appearance of the chondrocyte-plate which formed after 7 days of primary static culture, and subsequently, a chondrocyte-plate formed after 2 weeks of rotational culture. The bottom of the scaffold with the membrane seal. **c** Safranin-O stained paraffin sections of a chondrocyte-plate. **e** A high magnification of **c**. The peripheral region, which was not

stained by safranin-O forms elongated fibroblastic-like cells. **e** The scanning electron microscopic appearance of the chondrocytes-plate which formed after 8 h of inoculation in the mold, and sequentially, the appearance of the chondrocyte-plate which formed after 3 weeks of rotational culture. *Bar* 10 μ m

4 Discussion

A high density of chondrocytes from the articular cartilage of Japanese white rabbits was three-dimensionally cultured in an ACHMS scaffold for up to 21 days. Although the DNA content in the scaffold increased at a lower rate than in monolayer culturing, the SEM data showed the scaffold to be filled with cultured chondrocytes which produced an extracellular matrix after 21 days. In addition, GAG accumulation in the scaffold culture occurred at a higher level than the monolayer culture. Masuoka et al. [11] revealed that the implantation of allogenic chondrocytes cultured in ACHMS scaffolds could be effective for repairing articular cartilage defect using a rabbit model. The use of a proper scaffold contributes to tissue construction and the scaffold is a critical item for tissue engineering. However, it does not

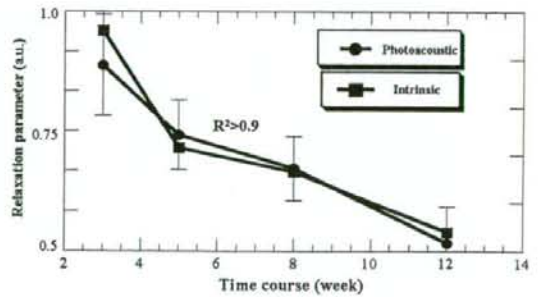


Fig. 6 Comparison between the relaxation times as measured by the photoacoustic method and the values of the intrinsic viscoelastic properties measured by a rheometer. The tissue engineered cartilage cultured for a longer period had smaller relaxation times. *Error bars* standard deviations

always work well with the repair process *in vivo*. For instance, some biodegradable polymers, such as PLLA, may generate inflammation in the late phase, and some non-biodegradable collagens may also have a harmful effect on tissue remodeling. We speculate that tissue-engineered cartilage without a scaffold may, therefore, be suitable to obtain a satisfactory repair of injured cartilage, thus providing a fast recovery from injuries. We herein introduce the findings of our recent studies concerning cartilage regeneration.

Temperature-responsive culture dishes have already been applied to conduct research in various fields of regenerative medicine, including the regeneration of the myocardium [19, 20], vascular epithelium [21], cornea [22], hepatocytes [23], and renal cells [24]. This method has also been clinically applied for the myocardium and cornea [22]. The surface of a temperature-responsive culture dish is coated with a polymer (poly-*N*-isopropylacrylamide) which thus becomes hydrophilic or hydrophobic in a reversible manner, depending on the temperature [25]. The polymer has a low critical solution temperature of 32°C, below which it becomes soluble in water. Based on this characteristic, the temperature-responsive culture dish has a weakly hydrophobic surface, similar to that of commercially available dishes, and it can, therefore, be used to culture cells in a conventional manner when the temperature is 37°C or higher. However, the surface of the dish becomes hydrophilic when the temperature falls below the critical solution temperature of 32°C. Therefore, the confluent sheets of cultured cells can be spontaneously released from the hydrophilic dish surface by reducing the temperature to below 32°C [26]. Using this method, the cultured cells can thus be harvested as a sheet without damaging the cell–cell junctions and the ECM because it eliminates the need for conventional enzymatic harvesting with trypsin. Such cell sheets have been reported to have various advantages, including the preservation of the normal phenotype and the expression of the adhesion proteins on the base [27]. Furthermore, these cell sheets can also be placed on each other to prepare layered “tissue” because the ECM is preserved on the base and such 3D manufactured tissue has already been successfully used for transplantations [20]. We already confirmed that the layered chondrocyte sheets were able to maintain a normal phenotype of the chondrocytes, and they could, therefore, be attached to the sites of the injured cartilage, thereby acting as a barrier to prevent the loss of proteoglycan from these sites, while also protecting them from catabolic factors in the knee joints of the rabbits [16].

We also succeeded in forming 3D tissue-engineered cartilage using a rotational culture system without the use of a scaffold, and thus showing a sufficient biomechanical strength. To date, only a few scaffold-free approaches have been available for 3D cultivations [28–30]. We have chosen a rotational culture system in a dense dissemination

using a mold, thus inducing cell aggregation and eventually forming a 3D chondrocyte-plate, and thereafter suitable tissue-engineered cartilage was constructed using an orbital shaker, thus making it easy and simple to create a rotational flow in the medium, and thereby inducing an appropriate shearing stress to the chondrocyte-plate. Using the above-mentioned chondrocyte sheets and the chondrocyte-plate, we are now planning to conduct further studies in translational research in order to make this method clinically available in the near future.

Furthermore, we also recognized the importance of the non-invasive measurement method for the tissue-engineered cartilage, and developed photoacoustic measurement method to evaluate the biomechanical properties of the tissue-engineered cartilage. This photoacoustic measurement method can be used not only for evaluating engineered tissue both *in vitro* and *in vivo*, but also for the diagnosis of the disease and for surgical treatment planning [13, 18]. After transplantation of tissue-engineered cartilage and chondrocyte sheet, the subchondral bone of the joints would also have to be evaluated. In such cases, a micro CT-imaging could be a powerful tool for the structural properties of the hard tissues [31]. Although these technologies were originally independent, and handled separately, we would like to combine them in order to regenerate articular cartilage after obtaining appropriate institutional approval.

5 Conclusions

1. The tissue-engineered cartilage with an ACHMS scaffold, the layered chondrocyte sheets, and the chondrocyte-plate were able to maintain the normal phenotype of the chondrocytes, thus suggesting their potential effectiveness for the treatment of the defects of articular cartilage.
2. The newly developed photoacoustic measurement method is also considered to be useful for the evaluation of tissue-engineered cartilage *in vitro*.

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References

1. O'Driscoll SW, Salter RB (1986) The repair of major osteochondral defects in joint surfaces by neochondrogenesis with autogenous osteoperiosteal grafts stimulated by continuous passive motion. An experimental investigation in the rabbit. *Clin Orthop Relat Res* 208:131–140

2. Matsusue Y, Yamamuro T, Hama H (1993) Arthroscopic multiple osteochondral transplantation to the chondral defect in the knee associated with anterior cruciate ligament disruption. *Arthroscopy* 9:318–321. doi:10.1016/S0749-8063(05)80428-1
3. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med* 331:889–895. doi:10.1056/NEJM199410063311401
4. Ochi M, Uchio Y, Kawasaki K, Wakitani S, Iwasa J (2002) Transplantation of cartilage-like tissue made by tissue engineering in the treatment of cartilage defects of the knee. *J Bone Joint Surg Br* 84:571–578. doi:10.1302/0301-620X.84B4.11947
5. Wakitani S, Imoto K, Yamamoto T, Saito M, Murata N, Yoneda M (2002) Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. *Osteoarthritis Cartil* 10:199–206. doi:10.1053/joca.2001.0504
6. Sato M, Asazuma T, Ishihara M, Kikuchi M, Masuoka K, Ichimura S et al (2003) An atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) for the culture of annulus fibrosus cells from an intervertebral disc. *J Biomed Mater Res A* 64:248–256. doi:10.1002/jbm.a.10287
7. Sato M, Asazuma T, Ishihara M, Ishihara M, Kikuchi T, Kikuchi M et al (2003) An experimental study of the regeneration of the intervertebral disc with an allograft of cultured annulus fibrosus cells using a tissueengineering method. *Spine* 28:548–553. doi:10.1097/00007632-200303150-00007
8. Sato M, Asazuma T, Ishihara M, Kikuchi T, Masuoka K, Ichimura S et al (2003) An atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) for the culture of annulus fibrosus cells from an intervertebral disc. *J Biomed Mater Res A* 64:249–256
9. Sato M, Ishihara M, Ishihara M, Kaneshiro N, Mitani G, Nagai T et al (2007) Effects of growth factors on heparin-carrying polystyrene-coated atelocollagen scaffold for articular cartilage tissue engineering. *J Biomed Mater Res B Appl Biomater* 83:181–188. doi:10.1002/jbm.b.30782
10. Sato M, Asazuma T, Ishihara M, Kikuchi T, Masuoka K, Ishihara M et al (2003) Tissue engineering of the intervertebral disc with an allograft of cultured annulus fibrosus cells using atelocollagen honeycomb-shaped scaffold having a membrane sealing (ACHMS-scaffold). *Med Biol Eng Comput* 41:365–371. Incorporating Cellular Engineering. doi:10.1007/BF02348444
11. Masuoka K, Asazuma T, Ishihara M, Sato M, Hattori H, Ishihara M et al (2005) Tissue engineering of articular cartilage using an allograft of cultured chondrocytes in a membrane-sealed atelocollagen honeycomb-shaped scaffold (ACHMS-scaffold). *J Biomed Mater Res* 75B:177–184. doi:10.1002/jbm.b.30284
12. Hattori H, Sato M, Masuoka K, Ishihara M, Kikuchi T, Matsui T et al (2004) Osteogenic potential of human adipose tissue-derived stromal cells as an alternative stem cell source. *Cells Tissues Organs* 178:2–12. doi:10.1159/000081088
13. Ishihara M, Sato M, Sato S, Kikuchi T, Mochida J, Kikuchi M (2005) Usefulness of photoacoustic measurements for evaluation of biomechanical properties of tissue-engineered cartilage. *Tissue Eng* 11:1234–1243. doi:10.1089/ten.2005.11.1234
14. Hattori H, Masuoka K, Sato M, Ishihara M, Asazuma T, Takase B et al (2006) Bone formation using human adipose tissue-derived stromal cells and a biodegradable scaffold. *J Biomed Mater Res B Appl Biomater* 76:230–239. doi:10.1002/jbm.b.30357
15. Masuoka K, Asazuma T, Hattori H, Yoshihara Y, Sato M, Matsumura K et al (2006) Tissue engineering of articular cartilage with autologous cultured adipose tissue-derived stromal cells using atelocollagen honeycomb-shaped scaffold with a membrane sealing in rabbits. *J Biomed Mater Res B Appl Biomater* 79:25–34. doi:10.1002/jbm.b.30507
16. Kaneshiro N, Sato M, Ishihara M, Mitani G, Sakai H, Mochida J (2006) Bioengineered chondrocyte sheets may be potentially useful for the treatment of partial thickness defects of articular cartilage. *Biochem Biophys Res Commun* 349:723–731. doi:10.1016/j.bbrc.2006.08.096
17. Furukawa K, Suenaga H, Toita K, Numata A, Tanaka J, Ushida T et al (2003) Rapid and large-scale formation of chondrocyte aggregates by rotational culture. *Cell Transplant* 12:475–479
18. Ishihara M, Sato M, Kaneshiro N, Mitani G, Sato S, Mochida J et al (2006) Development of a diagnostic system for osteoarthritis using a photoacoustic measurement method. *Lasers Surg Med* 38:249–255. doi:10.1002/lsm.20285
19. Shimizu T, Yamato M, Kikuchi A, Okano T (2001) Two-dimensional manipulation of cardiac myocyte sheets utilizing temperature-responsive culture dishes augments the pulsatile amplitude. *Tissue Eng* 7:141–151. doi:10.1089/107632701300062732
20. Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K et al (2002) Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. *Circ Res* 90:40–48. doi:10.1161/hh0302.105722
21. Hirose M, Kwon OH, Yamato M, Kikuchi A, Okano T (2000) Creation of designed shape cell sheets that are noninvasively harvested and moved onto another surface. *Biomacromolecules* 1:377–381. doi:10.1021/bm0002961
22. Nishida K, Yamato M, Hayashida Y, Watanabe K, Yamamoto K, Adachi E et al (2004) Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N Engl J Med* 351:1187–1196. doi:10.1056/NEJMoa040455
23. Harimoto M, Yamato M, Hirose M, Takahashi C, Isoi Y, Kikuchi A et al (2002) Novel approach for achieving double-layered cell sheets co-culture: overlaying endothelial cell sheets onto monolayer hepatocytes utilizing temperature-responsive culture dishes. *J Biomed Mater Res* 62:464–470. doi:10.1002/jbm.10228
24. Kushida A, Yamato M, Konno C, Kikuchi A, Sakurai Y, Okano T (2000) Temperature-responsive culture dishes allow nonenzymatic harvest of differentiated Madin–Darby canine kidney (MDCK) cell sheets. *J Biomed Mater Res* 51:216–223 (doi:10.1002/(SICI)1097-4636(200008)51:2<216::AID-JBM10>3.0.CO;2-K)
25. Okano T, Yamada N, Okuhara M, Sakai H, Sakurai Y (1995) Mechanism of cell detachment from temperature-modulated, hydrophilic–hydrophobic polymer surfaces. *Biomaterials* 16:297–303. doi:10.1016/0142-9612(95)93257-E
26. Kikuchi A, Okuhara M, Karikusa F, Sakurai Y, Okano T (1998) Two-dimensional manipulation of confluent cultured vascular endothelial cells using temperature-responsive poly (*N*-isopropylacrylamide)-grafted surfaces. *J Biomater Sci Polym Ed* 9:1331–1348. doi:10.1163/156856298X00424
27. Okano T, Yamada N, Sakai H, Sakurai Y (1993) A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(*N*-isopropylacrylamide). *J Biomed Mater Res* 27:1243–1251. doi:10.1002/jbm.820271005
28. Mainil-Varlet P, Rieser F, Grogan S, Mueller W, Saeger C, Jakob RP (2006) Scaffold-free articular cartilage repair using a tissue-engineered cartilage-like implant: an animal study. *Osteoarthritis Cartil* 9:S6. doi:10.1053/joca.2001.0438
29. Marlovits S, Tichy B, Truppe M, Gruber D, Vecsei V (2003) Chondrogenesis of aged human articular cartilage in a scaffold-free bioreactor. *Tissue Eng* 9:1215–1226. doi:10.1089/10763270360728125

30. Grogan SP, Rieser F, Winkelmann V, Berardi S, Mainil-Varlet P (2003) A static, closed and scaffold-free bioreactor system that permits chondrogenesis in vivo. *Osteoarthr Cartil* 11:403–411. doi:10.1016/S1063-4584(03)00053-0
31. Van Cleynenbreugel T, Schrooten J, Van Oosterwyck H, Vander Sloten J (2006) Micro-CT-based screening of biomechanical and structural properties of bone tissue engineering scaffolds. *Med Biol Eng Comput* 44:517–525. doi:10.1007/s11517-006-0071-z



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