Static and dynamic fibroblast seeding and cultivation in porous PEO/PBT scaffolds

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The present study aims at optimizing dermal fibroblast seeding and cultivation in Polyactive scaffolds in order to limit the biopsy size needed for autologous treatment of full-thickness skin defects and chronic wounds. Three methods for seeding and cultivation of fibroblasts in porous scaffolds were compared: dynamic seeding followed by static cultivation (DS), static seeding followed by static cultivation (SS) and dynamic seeding followed by dynamic cultivation (DD). Human dermal fibroblasts isolated from cultured explants were seeded in porous PEO/PBT (Polyactive) scaffolds. Samples were taken from 6 h to 21 days post-seeding for both histological analysis (cell distribution and extracellular matrix (ECM) formation), and quantitative cell number assay. The seeding efficiency 24 h post-seeding was 76% ($\pm$ 3.6%) for dynamically seeded matrices, whereas it was only 30% ($\pm$ 5%) for statically seeded matrices ($p < 0.001$). ECM formation was abundant in DS samples already at day 10, while even after 21 days ECM formation was less pronounced in SS samples. Surprisingly, cells detached from DD samples as aggregates, starting from day 10. Cell numbers as assayed quantitatively correlated with the histological results. At all timepoints cell numbers found for DS samples were significantly higher as compared to SS samples. At day 21, DS samples contained approximately twofold more cells as compared to SS and DD samples and comprised ECM consisting of collagen types I and III. Our results indicate that the combination of dynamic seeding and static cultivation assures efficient utilization of isolated fibroblasts and improved neodermis formation, thereby allowing a reduction in the skin biopsy size needed for the engineering of living skin substitute.

1. Introduction

Current treatment of large full-thickness skin defects and chronic wounds includes grafting with split-skin or full-thickness autografts. However, major drawbacks of such approaches are the creation of a wound at the donor site and/or the relatively scarce amount of donor tissue. Engineered skin substitutes may overcome those limitations by utilizing a small skin biopsy for the isolation of fibroblasts and keratinocytes, followed by cell expansion, subsequent cell seeding onto a scaffold and application of the skin equivalent onto the wound. As a scaffold, a biodegradable synthetic porous copolymer (Polyactive 55/45 (300)) composed of segmented blocks of soft poly (ethylene oxide-terephthalate (PEO)) and hard poly (butylene terephthalate (PBT)) showed suitable mechanical properties and good biocompability both *in vitro* and *in vivo* [1, 2]. For successful autologous skin tissue engineering, not only the selection of a suitable scaffold but also the optimization of cell seeding and cultivation *in vitro* are essential in order to (1) utilize the donor tissue efficiently, and (2) to reduce the time from biopsy harvesting to grafting. Vunjak-Novakovic *et al*. reported that for articular chondrocytes, dynamic conditions were shown to improve both cell seeding onto a scaffold as well as the formation of a cartilageous ECM [3]. However, to the best of our knowledge, it is not known how dynamic conditions might influence dermal fibroblast attachment and proliferation. Therefore we hypothesize that the attachment, spreading and growth of fibroblasts within the porous Polyactive matrices could be further improved by using dynamic seeding and cultivation. Thus, in the present study we compared static and dynamic dermal fibroblast seeding and culture conditions with respect to cell attachment, cell ingrowth and dermal ECM formation in order to optimize dermal fibroblast seeding and cultivation methods.

2. Materials and methods

2.1. Copolymer scaffolds

Porous PEO/PBT copolymer scaffolds (1.55 cm in diameter, 300 μm thick with approximately 70%

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porosity) with a PEO/PBT weight ratio of 55/45 and a PEO molecular weight of 300 Da as produced at IsoTis were used in this study after steam sterilization (121 °C, 30 min).

2.2. Human dermal fibroblast isolation and culture

Fibroblasts were isolated from human *mamma* skin tissue by outgrowth from explant culture. After removing blood vessels and fat tissue, the skin tissue (~ 1.5 g wet weight) was washed three times in phosphate-buffered saline (PBS, 4 °C) supplemented with 1000 U ml^{-1} penicillin/1000 μg ml^{-1} streptomycin, cut into pieces (~ 2 mm^2), spread onto a petri dish (6 cm in diameter), and subsequently 2–3 ml Dulbecco's modified minimum essential medium (D-MEM) (Gibco, BRL) supplemented with 5% fetal bovine serum (FBS) and antibiotics (100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin) was added. More culture medium was added after attachment of explants. Fibroblast outgrowth started at one to two weeks after explant seeding. Cells were harvested by using 0.25% trypsin–ethylenediamine tetra-acetic acid (EDTA) solution (Sigma), and subcultured into T75 flask(s) at a concentration of 10 000–12 500 cells cm^{-2} in 15 ml D-MEM supplemented with 5% FBS and antibiotics.

2.3. Cell seeding and cultivation on 3-D scaffolds

For dynamic seeding, Polyactive matrices were fixed onto needles which were attached to a silicone stopper onto the top-cap of spinner flasks (Bellco Glass, INC). Spinner flasks were filled with 80 ml culture medium and placed into a humidified 37 °C 8% CO_2 incubator. Twelve hours after matrix prewetting in culture medium, cells were dynamically seeded using a non-heated magnetic stirrer (Bellco Glass, INC) at 40 r.p.m. and 1×10^5 cells (passage 2) were seeded per matrix.

For static seeding, Polyactive matrices were kept submerged in multi-well plates (Greiner) during prewetting and seeding by placing glass rings on the matrices. Cells were seeded per matrix at 1×10^5 cells in 1 ml culture medium, and additional 2 ml culture medium was added 3 h later. Twenty-four hours after seeding, samples were transferred to a spinner flask for further cultivation.

In summary, the following three groups were compared: (1) dynamic seeding followed by static cultivation (DS); (2) static seeding followed by static cultivation (SS); and (3) dynamic seeding followed by dynamic cultivation (DD). D-MEM supplemented with 5% PBS was used as a culture medium, and was further supplemented with 0.2 mM ascorbic acid 2-phosphate (Sigma) to facilitate collagen and glycosaminoglycan synthesis [4, 5], starting 24 h after seeding.

2.3. Microscopic evaluation and cell number assay

Samples were taken for stereo light microscopy (LM), scanning electron microscopy (SEM) and quantitative cell number assay, at 6 h ($n = 5$, one for histological analysis and four for quantitative cell number assay), 24 h ($n = 5$), 3 days ($n = 4$, one for histological analysis and three for quantitative cell number assay), 10 days ($n = 4$) and 21 days ($n = 4$) after seeding. Samples (75% of each matrix) for general microscopic evaluation were fixed in 4% glutaraldehyde in 0.14 M cacodylic buffer. Samples were directly stained with methylene blue after rinsing in deionized water, allowing for 3-D analysis of cell distribution and ECM formation under a stereo light microscope (Nikon SMZ-10A). After dehydration, critical point drying and gold-sputtering, samples were analyzed by SEM (Philips SEM 525) to evaluate ECM formation.

To investigate seeding efficiency and cell proliferation in our 3-D culture system, samples were trypsinized at each time point and cell counting was performed using a haemocytometer.

2.4. Immunohistochemistry

An indirect inmmunohistochemical assay (ABC method) was used to analyze for collagen types I and III, which are the main components of dermal extracellular matrix [6, 7]. Samples (25% of each matrix) were fixed in 4% paraformaldehyde phosphate buffer (pH = 7.4) for 12 h at 4 °C. After three washing steps in PBS, samples were immediately used for immunostaining. Briefly, primary rabbit anti-collagen type I (1 : 40) or mouse anti-collagen type III (1 : 40) antibodies (Bionovo) were incubated with the sample overnight, followed by addition of the biotinylated secondary antibody and subsequently the avidin-biotinylated horseradish peroxidase (HRP) complex (ABC reagent) (Bionovo). HRP was visualized by developing the samples for 8 min in 3,3-diamino-benzidine-H_2O_2 (DAB-H_2O_2) substrate (Novacastra). As a negative control 2% normal serum was added instead of the primary antibody. Samples were observed using a stereo light microscope.

2.5. Statistical analysis

Cell numbers on scaffolds were expressed as the average $\pm$ SD ($n = 3$ or 4). Two-tailed Student's *t*-test was used to analyze the significance of differences between groups.

3. Results

From SEM and stereo LM (3-D analysis), cell spreading within Polyactive scaffolds could already be observed 24 h after seeding both in dynamically and statically seeded samples. However, significantly more cells were present in the scaffolds of the dynamically seeded groups when compared to static seeding conditions (Fig. 1). The seeding efficiency 24 h post-seeding as determined by direct quantification of the cell number in the scaffolds was 76% ($\pm$ 3.6%) for the dynamically seeded matrices, whereas it was only 30% ($\pm$ 5%) for the statically seeded scaffolds ($p < 0.001$) (Table I). After 3 days, a pronounced cell ingrowth and cell proliferation could be seen in DS samples while fewer cells were detected in SS samples. Although no statistically significant difference could be found between DS and DD samples related to

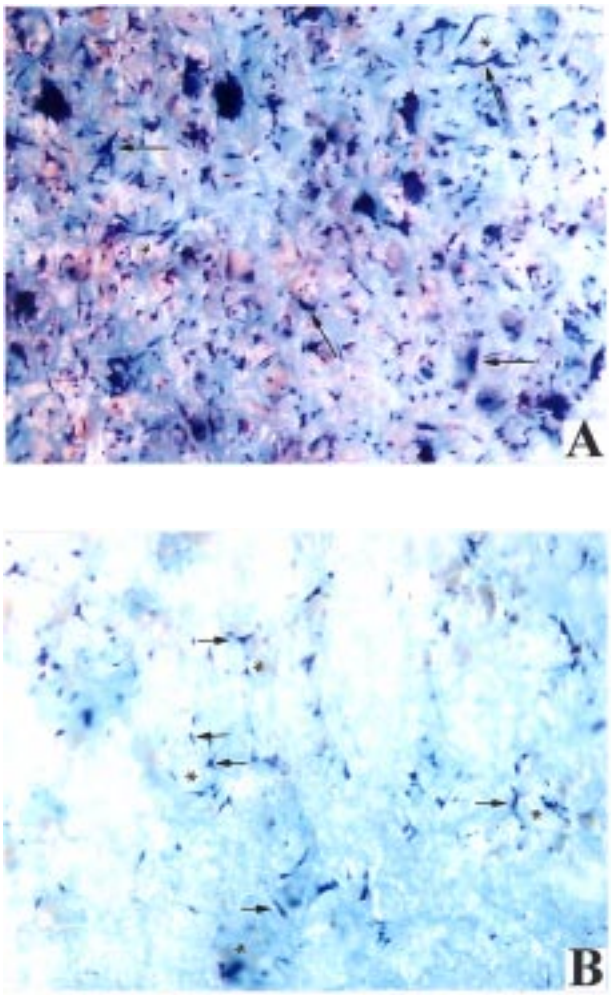

Figure 1 Dermal fibroblasts attached onto Polyactive scaffolds 24 h after seeding (3-D image of methylene blue-stained samples). (A) dynamic seeding; (B) static seeding; (*) pores within Polyactive scaffolds; arrow: cells (field length: 2.45 mm).

cell attachment at 3 days (Table I), cell morphology as observed in DD samples deviated from a well elongated fibroblast phenotype as found in DS samples (data not shown). After 10 days, DS samples showed ECM covering the majority of the Polyactive matrix (as determined by methylene blue staining, Fig. 2A), while SS samples only showed accumulation of cells and minimal ECM formation (Fig. 2B). At day 21, ECM eventually covered the scaffold as a dense layer in DS samples (Fig. 3A) as compared to less extensive ECM formation in SS samples (Fig. 3B). Surprisingly, cells detached from DD samples as aggregates which were previously located within the pores starting from day 10, (Fig. 2C), as concluded from detaching cell aggregates during sample harvesting at day 21. At all time points cell numbers as assayed quantitatively for DS samples were significantly higher when compared to SS samples. At day 21, cell numbers for DS samples were approximately two-fold higher as compared to SS and DD matrices (Table I, Fig. 4).

Immunostaining showed that ECM formation was extensive and consisted of both collagen types I and III. Staining for collagen type I was more pronounced when compared to collagen type III, and was distributed homogeneously within the matrix (Fig. 5).

4. Discussion

The focus of the present study was to utilize hydrodynamic forces in order to improve and better understand neodermis formation *in vitro,* as well as to limit the biopsy size needed for autologous treatment of full-thickness skin defects and chronic wounds. We showed that dynamic conditions applied during seeding and cultivation of dermal fibroblasts on 3-D scaffolds affected seeding efficiency as well as dermal ECM formation, which was crucial for the stimulation of continuous neodermis formation [8].

In particular, fibroblast seeding efficiency could be significantly increased (46% more cells attached) by using dynamic conditions as compared to static conditions (Table I and Fig. 4). Therefore, dynamic seeding conditions were shown to be beneficial not only for articular chondrocytes as reported by Vunjak-Novakovic *et al*. [3], but also for dermal fibroblasts. In both studies, the stirring speed was well below the range of 150–300 r.p.m. at which cell damage was observed [9]; therefore, dynamic conditions at a stirring speed of 40–50 r.p.m. are not expected to compromise cell survival during seeding. On the other hand, and surprisingly, we found that the effect of dynamic conditions on dermal ECM formation, as characterized by the presence of collagen types I and III, is significantly different as compared to neocartilage development *in vitro*. Whereas continuous dynamic conditions yielded cartilaginous constructs which contained more collagen per wet weight and which were more homogeneously

TABLE I Cell numbers ($\times 10^5$) as determined on 3-D scaffolding from 6 h to 21 days post-seeding

	6 h ($n = 4$)	24 h ($n = 4$)	3 days ($n = 3$)	10 days ($n = 3$)	21 days ($n = 3$)
1. Dynamic seeding + Static culture	0.37 ± 0.038	0.76 ± 0.036	1.60 ± 0.21	3.13 ± 0.23	4.20 ± 0.17
2. Static seeding + Static culture	0.16 ± 0.050	0.30 ± 0.050	0.38 ± 0.08	1.05 ± 0.08	2.60 ± 0.21
3. Dynamic seeding + Dynamic culture	N/A	N/A	1.74 ± 0.13	1.98 ± 0.05	1.89 ± 0.29
p* 1 vs 2	< 0.001	< 0.001	< 0.001	< 0.001	< 0.005
p* 1 vs 3	N/A	N/A	NSD	< 0.005	< 0.001

1×10^5 cells were seeded per matrix.
N/A = not applicable.
NSD = not significantly different.
* = according to a two-tailed Student's *t*-test.

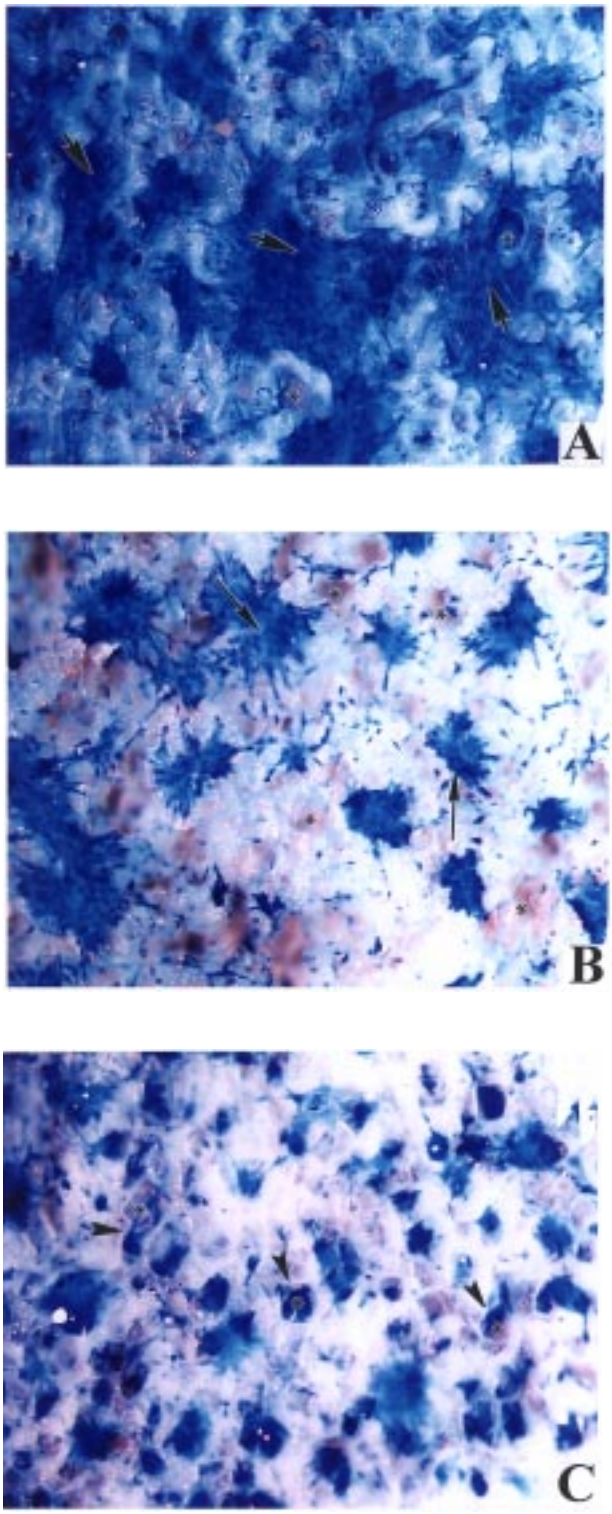

Figure 2 Dermal fibroblasts and ECM on Polyactive scaffolds 10 days after seeding (3-D image of methylene blue-stained samples). (A) dynamic seeding + static culture; (B) static seeding + static culture; (C) dynamic seeding + dynamic culture; (*) pores within Polyactive scaffolds; arrow in (A) abundant ECM formation; long arrow in (B) cell accumulation + appearance of ECM formation; arrowhead in (C) cell aggregates located within the pores (field length: 2.45 mm).

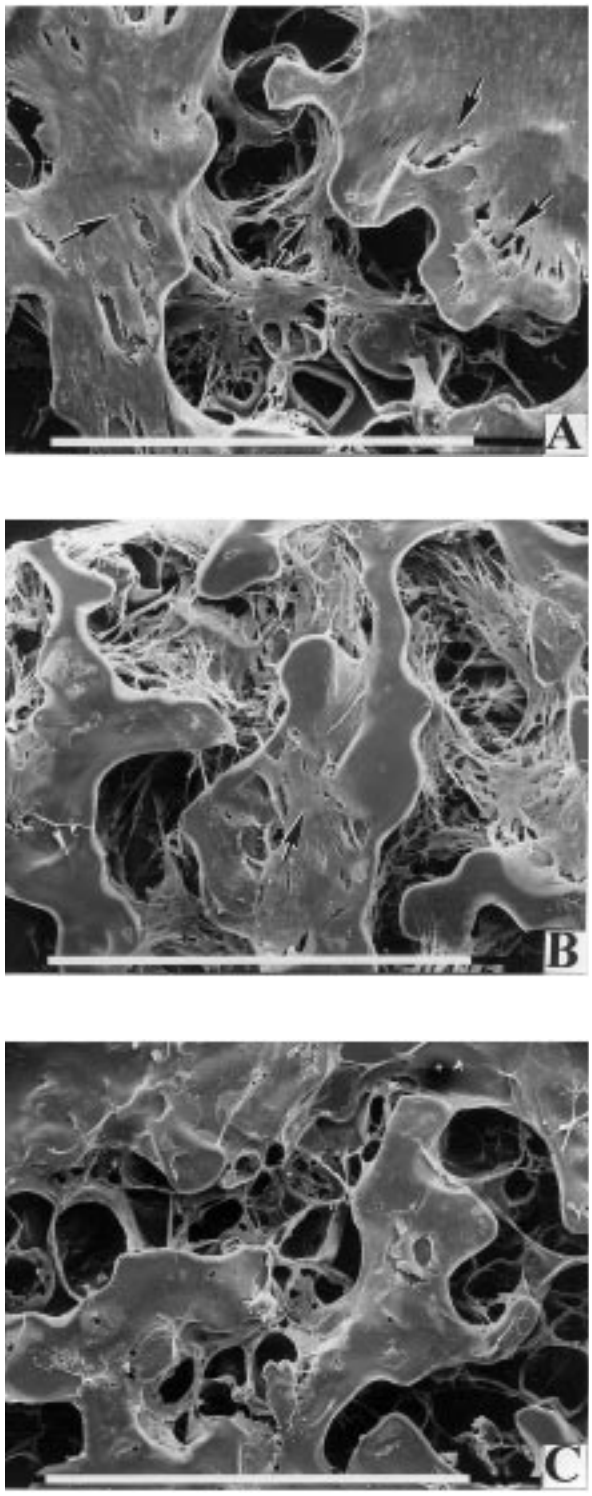

Figure 3 Dermal fibroblasts and ECM on Polyactive scaffolds 21 days after seeding (SEM). (A) dynamic seeding + static culture; (B) static seeding + static culture; (C) dynamic seeding + dynamic culture; arrow: ECM. (Bar = 1 mm).

stained for glycosaminoglycan [10], dermal fibroblast cell numbers decreased starting 10 days post-seeding and dermal ECM formation was less pronounced as compared to static conditions (Figs 3 and 4). In contrast, fibroblast ingrowth and ECM formation within Polyactive matrices were most pronounced for static cultivation conditions (DS). These results correlate well with the extent and frequency of mechanical forces experienced by the cell types mentioned above *in vivo*: articular chondrocytes being embedded in a weight-bearing cartilage matrix are exposed to dynamic forces of several MPa [11], whereas human dermal fibroblasts are considered to experience mechanical stress less frequently and with a lower amplitude. Those considerations as well as our results not only exemplify the importance of mechanical forces for tissue engi-

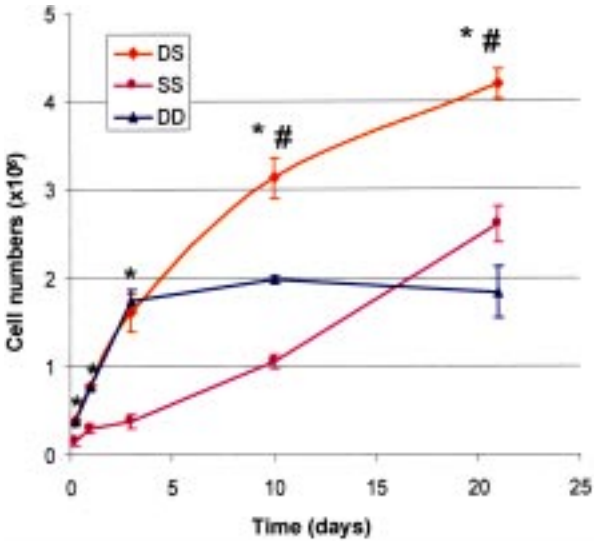

Figure 4 Cell proliferation as a function of cultivation time in a 3-D culture system. (*) Significant difference between DS and SS; (#) significant difference between DS and DD. (two-tailed Student's *t*-test)

neering but also suggest that by mimicking the forces experienced by the cells *in vivo* (i.e. by applying shear stress) one might well enhance cell proliferation or/and ECM synthesis, thereby improving the quality of the engineered tissue. In conclusion the present study suggests that dynamic conditions may (a) improve cell seeding by making cell–scaffold contacts more likely, but may (b) be not beneficial for further cultivation because of the shear stress experienced by dermal fibroblasts.

Our results suggest that for human dermal fibroblasts, dynamic seeding followed by static cultivation (DS) is an appropriate method leading to efficient as well as homogeneous fibroblast attachment, ingrowth and pronounced ECM formation. Future studies will focus on the evaluation of the skin equivalent in animal models as well as on using more elaborate culture techniques such as applying intermitted shear forces.

5. Conclusion

The optimized seeding method presented herein will allow reduction of the size of the skin biopsy needed for the engineering of autologous living skin substitutes aiming at the treatment of large full-thickness skin defects and/or chronic wounds.

Acknowledgments

The authors would like to thank Dr Schuurman from the Plastic & Reconstructive Surgery Department of the Academic Hospital of Utrecht for supplying us with skin tissue, and Dr Lamme for critical reading of the manuscript.

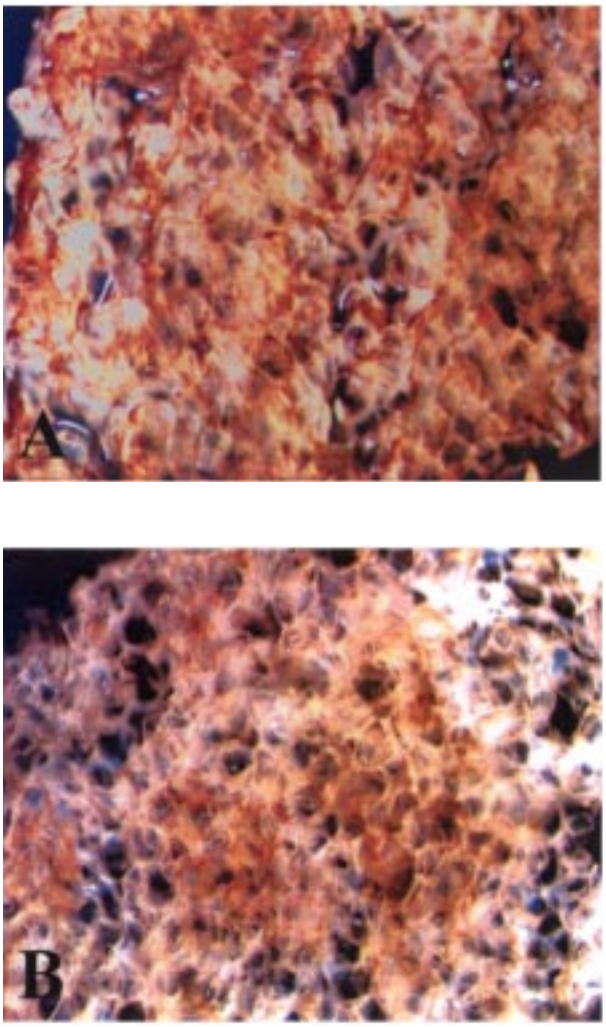

Figure 5 Immunohistochemistry of DS samples 21 days after seeding (3-D image). Cell–polymer constructs were stained with antibodies against collagen types I and III. (A) Anti-collagen type I; (B) anti-collagen type III (field length: 2.45 mm).

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Received 10 May
and accepted 17 May 1999