

# Three-Dimensional Cell Growth on Structures Fabricated from ORMOCER<sup>®</sup> by Two-Photon Polymerization Technique

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**ABSTRACT:** Two-photon polymerization technique was applied to generate three-dimensional (3D) scaffold-like structures using the photosensitive organic-inorganic hybrid polymer ORMOCER<sup>®</sup>. The structures were studied with respect to potential applications as scaffold for tissue engineering. Cell counting and comet assay, respectively, demonstrated that doubling time and DNA strand breaks of CHO cells, GFSHR-17 granulosa cells, GM-7373 endothelial cells, and SH-SY5Y neuroblastoma cells were not affected by ORMOCER<sup>®</sup>. ORMOCER<sup>®</sup> related alteration of formation of tissue specific cell-to-cell adhesions like gap junctions was ruled out by double whole-cell patch-clamp technique. Additionally, growth of cells on the vertical surfaces of 3D structures composed of ORMOCER<sup>®</sup> is shown.

**KEY WORDS:** tissue engineering, two-photon polymerization, ORMOCER<sup>®</sup>, 3D scaffolds, gap junction, comet assay, DNA strand breaks.

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Figure 6 appears in color online: <http://jba.sagepub.com>

## INTRODUCTION

**A**ge or trauma related tissue degeneration e.g., diabetes, Parkinson disease, bone damage, leads to an increased demand for *in vitro* generated three-dimensional (3D) organized tissues, which can be transplanted into organism (see, for example, review [1,2]). The widely studied approach of tissue engineering is generation of scaffolds, which could guide cell growth in order to produce 3D tissues of interest. Some specific properties requested for these scaffolds are malleability, elasticity, biocompatibility, and, in many cases, biodegradability. Parallel to the development of scaffolds, there is a need for cell culture techniques, allowing cell growth on the vertical/steep surfaces, in order to create true 3D tissue.

In this article, two-photon polymerization (2PP) technique is applied for the fabrication of 3D scaffold-like structures using photosensitive ORMOCER<sup>®</sup> materials. ORMOCER<sup>®</sup>s are produced using sol-gel process from liquid precursors, where inorganic-oxidic units are connected to organic moieties such as urethane- and thioether (meth)acrylate alkoxyxilanes, and contain strong covalent bonds between the inorganic and organic components [3]. The cross-linking of inorganic and organic moieties leads to the formation of a 3D network, which provides ORMOCER<sup>®</sup>s with significant chemical and thermal stability. These hybrid polymeric materials are characterized by a relative high malleability [4] and a low shrinkage coefficient (<2%) [5]. Combined with 2PP technique, ORMOCER<sup>®</sup> can be used to generate any computer designed structures [3,4,6–8]. Moreover, during the sol-gel synthesis, the ratio organic/inorganic network density can be changed, offering a possibility to generate ORMOCER<sup>®</sup> materials with desired mechanical, optical, chemical, and surface properties, and specific elasticity. The generated structures can thereby adapt to different shapes and movements [3,4].

Specific difficulties related to cell growth on vertical surfaces are related to the fact that many cells sink down to the bottom of the culture container, this is especially true for adherent cells [9]. To promote cell growth on vertical surfaces and on the top of a 3D structures, we have developed special techniques, which keep cells in suspension and give them time to adhere onto the steep facets of the 3D structures. Keeping the dish with cells on a shaking table is sufficient to allow cells to grow onto 3D structures composed of ORMOCER<sup>®</sup>. Additionally, it is shown that ORMOCER<sup>®</sup> does not alter cell viability and the capability of cells to form junctions, like gap junctions. Potential applications of 2PP technique on polymers of

the ORMOCER® family to design 3D scaffolds for tissue engineering are discussed.

## MATERIALS AND METHODS

### Application of Two-Photon Polymerization Technique for the Fabrication of 3D Structures

Near-infrared Ti:sapphire femtosecond laser pulses were applied for the fabrication of 3D structures. The laser wavelength, pulse duration, and repetition rate, were respectively 780 nm, 80 fs, and 94 MHz. The detailed description of the experimental setup can be found in our previous publications [6]. In our experiments, femtosecond laser pulses were tightly focused into a volume of the hybrid organic–inorganic polymer ORMOCOMP®, a member of the ORMOCER® family (Microresist Technology GmbH, Germany), containing 1.8% of photoinitiator Irgacure 369 (Ciba Specialty Chemicals, Switzerland). This hybrid material was developed for optical applications and is synthesized such that almost no residual O–H groups are present within the final polymer [10]. ORMOCOMP® contains methacrylate groups and can be cross-linked via free-radical polymerization reaction. The laser pulses initiate 2PP process in the focal area, and so locally transfer the liquid into solid state. A 3D polymerized structure is created by moving the laser focus in 3D within the volume of ORMOCOMP®. After irradiation, the nonsolidified material was removed by a 1 : 1 solution of 4-methyl-2-pentanone and isopropanol to reveal the generated structure. Manufactured structures were seeded with various cell types in order to determine the possibility of growing a tissue on 3D structures.

### Biocompatibility of ORMOCOMP®

To test biocompatibility of ORMOCOMP®, flat film of ORMOCOMP were generated by spin-coating the material on cover slips ( $24 \times 24 \text{ mm}^2$ ). The film was solidified by UV radiation using a 16 W UV lamp (250–380 nm) for at least 4 h. After washing with a 1 : 1 solution of 4-methyl-2-pentanone : isopropanol and drying under sterile conditions, the ORMOCOMP covered cover slips were placed in Petri dishes (35 mm  $\phi$ ), filled with 2 mL of a DMEM/F12 medium (Sigma, Taufkirchen, Germany), supplemented with penicillin/streptomycin (100 U/mL and 10 mg/mL, respectively) and 5–10% fetal calf serum. CHO cells, GFSHR-17 granulosa cells, GM7373 endothelial cells, and SH-SY5Y neuroblastoma cells were added at respectively (cells/mL)

$5.9 \times 10^6$ ,  $5.8 \times 10^6$ ,  $3.7 \times 10^6$ , and  $4.6 \times 10^6$ . The cells were cultivated in a cell culture incubator, in which a 95%/5% air/CO<sub>2</sub> atmosphere and a 80% humidity were maintained. Every 24 h, the cells were trypsinized and counted, in order to determine their specific growth parameters. Cells were also grown on control cover slips to serve as a reference. As illustration of the growth behavior, the results of GM7373 endothelial cells are shown in bulk diagram for at least five experiments of cells grown on ORMOCOMP® and under control conditions. The error bars indicate the standard error of the mean (SEM).

To analyze the formation of cell-to-cell junctions, the double whole-cell patch-clamp technique [11] was applied. The conductance of gap junction ( $G_j$ ) which was measured within 5 min after establishment of the double whole-cell configuration is given for 10 cell pairs of cells grown on three cover slips with ORMOCOMP®, and nine cell pairs grown on three cover slips under control conditions. The error bars indicate SEM.

### Comet Assay

To test whether ORMOCOMP® has DNA damage effects, DNA strand breaks were analyzed by use of the comet assay for CHO cells, GFSHR-17 granulosa cells, GM7373 endothelial cells, and SH-S5Y neuroblastoma cells. Detailed description of the comet assay procedure can be found in previous publications [12,13]. Cells grown on control cover slips and on cover slips covered with ORMOCOMP® were trypsinized, collected, and centrifuged for 10 min at 800g. The pellets were resuspended in PBS to  $2 \times 10^6$  cells/mL. Later 20  $\mu$ L of the cell suspension was mixed with 100  $\mu$ L of low melting agarose (0.6%). A 100  $\mu$ L of this mixture was given onto agarose-coated glass slides and covered with a cover slip. The slides conserved for 10 min at 4°C for solidification. The cover slip was removed and further 100  $\mu$ L of agarose was added. After solidification at 4°C, the slides were incubated for 90 min in a lysis buffer, containing 2.5 M NaCl; 100 mM Na<sub>2</sub>EDTA; 10 mM Tris; 1% lauryl sarcosin; 1% Triton X-100; 10% DMSO; pH 10. Subsequently, the cover slips were placed in a horizontal gel electrophoresis chamber, filled with electrophoresis buffer for alkaline comet assay (1 mM Na<sub>2</sub>EDTA; 300 mM NaOH; pH > 13). After 40 min adaptation to the buffer, electrophoresis was performed (25 V; 300 mA; 4°C; 20 min). For neutralization, the slides were washed three times with Tris-buffer (400 mM Tris; pH 7.5) and dried at room temperature. Comets were visualized by ethidium bromide staining (20  $\mu$ g/mL) and examined at 200-fold magnification with a fluorescence microscope

(Zeiss, Oberkochen Germany), using xenon lamp and ethidium bromide filter set (excitation at  $\lambda = 520$  nm). The images were recorded with a CCD Camera. For a quantitative analysis of the DNA breaks, the tail moment was evaluated using comet scoring software (<http://www.autocomet.com>). The results are given as a relative tail moment  $\pm$  SEM. At least 1000 cells/treatment were evaluated.

### Cell Culture on ORMOCER® 3D Structures

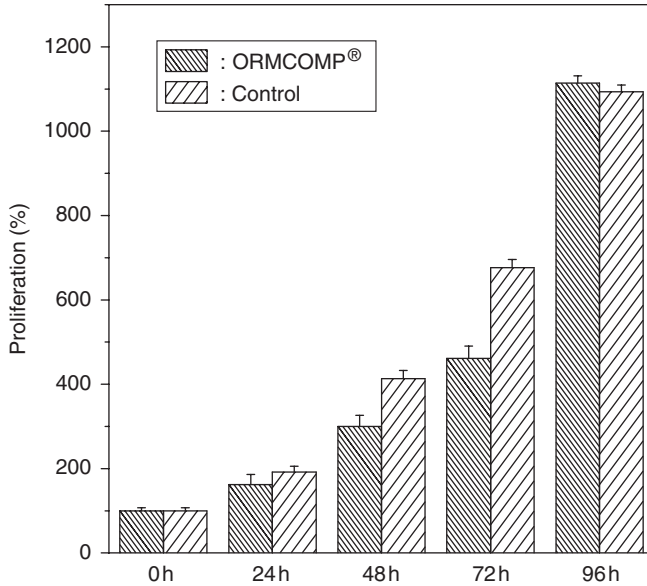
Three-dimensional structures designed on computer using the ordinary rapid prototyping software for CAD/CAM were generated by 2PP on cover slips, which were inserted into a Plexiglas holder. The whole construction was placed inside plastic Petri dishes (60 mm  $\phi$ ), containing 10 mL of cell culture medium (DMEM/F12) as described earlier. The holders with the structures mounted on them were placed so that the structures were oriented upside down. To avoid the sedimentation of the cells on the bottom of the Petri dish and to promote the cell adhesion onto the 3D structures, the Petri dishes with cells were placed on a shaking machine in the cell culture incubator. The culture medium was changed every 3–4 days. To observe the cells grown on the lateral surfaces of the 3D structures, a Nikon stereo microscope (Nikon, Düsseldorf, Germany) was used.

## RESULTS

### Biocompatibility of ORMOCOMP®

In order to test the biocompatibility of ORMOCOMP®, GFSHR-17 granulosa cells, GM-7373 endothelial cells, and SH-SY5Y neuroblastoma cells were seeded on a glass cover slip covered with ORMOCOMP®. The cells were counted after different period of cultivation, and their proliferation rates were statistically compared to those of the cells cultivated on the cover glass without ORMOCOMP®. Using the Student's *t*-test ( $p < 0.05$ ), it could be concluded that ORMOCOMP® did not significantly affect the cell growth. Cells cultivated on ORMOCOMP® grew as fast as the cells cultivated at control conditions (Figure 1). Comparison of the doubling time of the different cells cultivated on ORMOCOMP® and under control conditions, showed that the growth parameter of the cells were not affected by the presence of ORMOCOMP® (Table 1).

To test whether ORMOCOMP® has a DNA damage effects on the cells, DNA strand breaks was analyzed using comet assay method.

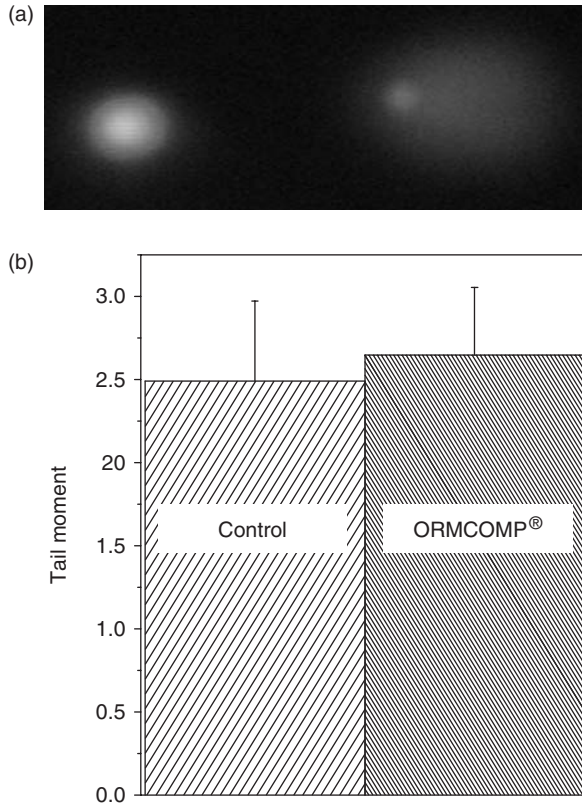


**Figure 1.** Growth behavior of GM-7373 endothelial cells cultivated on cover slips covered with ORMOCOMP® and on control glass cover slips. The presence of ORMOCOMP® did not significantly alter the growth behavior of the cells. Comparable results were obtained for the other cell lines. The results are in agreement with the doubling times shown in Table 1.

**Table 1.** The doubling time of cells cultivated on ORMOCOMP® in comparison to that of cells cultivated under control conditions. The results are given as mean  $\pm$  SEM for five experiments for each cell line.

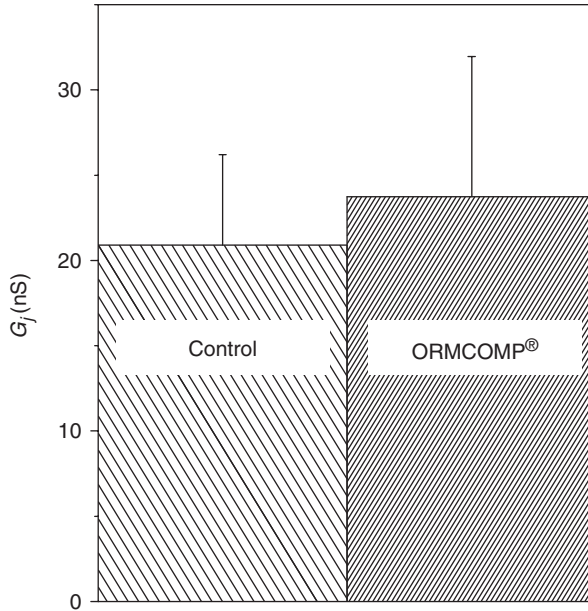
Cell line	Doubling time (h)	
	ORMOCER®	Control
GFSHR-17 granulosa cells	18.8 $\pm$ 2.2	18.1 $\pm$ 0.6
SH-SY5Y neuroblastoma	26.1 $\pm$ 0.7	22.1 $\pm$ 1.9
CHO cells	10.8 $\pm$ 0.3	8.4 $\pm$ 1.5
GM-7373 endothelial cells	32.3 $\pm$ 2.5	28.7 $\pm$ 1.4

The DNA damage effect is characterized by an increase in the sum of cells presenting a tail of the 'comets' (Figure 2a) and thereby by increase in the relative tail moment obtained for these cells [13]. By comparing the relative tail moment using the Student's *t*-test ( $p < 0.05$ ), it could be shown that ORMOCOMP® did not significantly increase the incidence of DNA-damage (Figure 2b).



**Figure 2.** DNA-breaks demonstrated by comet assay. (a) Comparison of comet with DNA breaks and normal DNA. (b) Tail moment of GFSHR-17 granulosa cells grown on ORMOCOMP® and on control cover slips. The presence of ORMOCOMP® did not significantly change the relative tail moment of the cells. The results are given as mean  $\pm$  SEM. At least 1000 cells were evaluated for each treatment. Comparable results were obtained for the other cell lines.

The compatibility of ORMOCOMP® with the formation of cellular junctions appropriate to tissue, such as gap junction coupling, was studied in GFSHR-17 granulosa cells using the double whole patch-clamp technique [11]. Ten out of 12, and 9 out of 12 cell pairs, cultivated on ORMOCOMP® and on control cover slips, respectively showed gap junction coupling. Again the Student's *t*-test ( $p < 0.05$ ) was used to compare the magnitude of the gap junction conductance  $G_j$  of both cell populations. A statistical significant effect of ORMOCOMP® on  $G_j$  was not found (Figure 3).

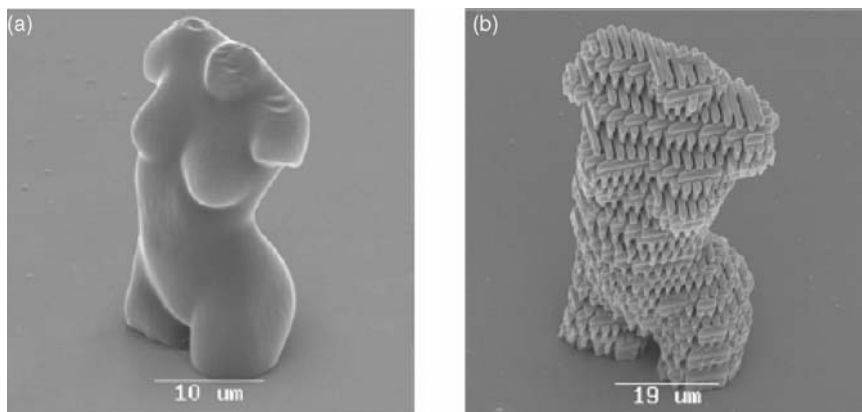


**Figure 3.** Conductance of gap junction  $G_j$  in pairs of GFSHR-17 granulosa cells cultivated on cover slips covered with ORMOCOMP® and on control glass cover slips. ORMOCOMP® does not significantly affect  $G_j$ . The results are given as mean  $\pm$  SEM for 10 and 9 cell pairs, respectively.

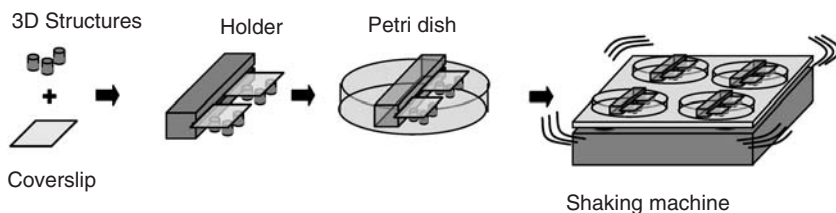
### Cultivation of Cells on 3D Structures Fabricated by Two-Photon Polymerisation Technique

Using 2PP technology it is possible to fabricate any computer-designed 3D structure by direct laser ‘recording’ into the volume of photosensitive material [5]. In our case, computer designed ordinary rapid prototyping software for CAD/CAM fabrication was used to fabricate 3D structures such as the micro-Venus (Figure 4). The 2PP technique allows to control the external shape (Figure 4a), to generate the desired porosity, and to structure the surface with the resolution (structure size) down to 100 nm (Figure 4b). In order to create 3D tissue, CHO cells, GFSHR-17 granulosa cells, GM7373 endothelial cells, and SH-SY5Y neuroblastoma cells, were cultivated on 3D structures standing on the cover slips. To avoid adhesion of the cells to the bottom of the Petri dishes and in order to keep the cells in suspension, the Petri dishes were placed on a shaking table (Figure 5) and the cells were allowed to grow. Microscopic analysis of the cells after 3–4 days culture showed that the cells were able to adhere to the lateral surfaces





**Figure 4.** Examples of 3D structures fabricated by 2PP technique: 2PP allows to control the shape (a) and to structure the surface of the structure in 100 nm range (b) which is important for the fabrication of scaffolds.

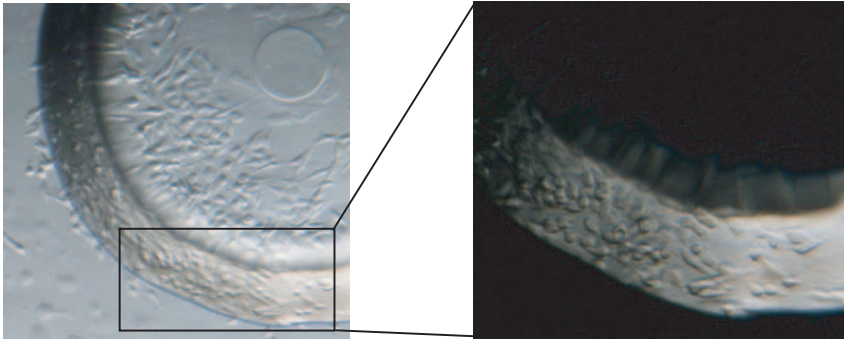


**Figure 5.** System developed for cell growth on 3D structures composed of ORMOCOMP®. The structures were fabricated on the cover slips. The cover slips were inserted in the holder made of Plexiglas, which was placed in a Petri dish containing cell culture medium. The structures were placed upside down. After addition of cells, the Petri dish was mounted on a shaking table in cell incubator.

and to form layers, which spread from the bottom to the top of the 3D structures (Figure 6). These results demonstrate that 2PP technique applied on ORMOCOMP® in combination with cultivation of cells allows the design of 3D tissue with a well controlled shape.

## DISCUSSION

The possibility to create scaffolds with a desired topology in 3D is very appealing for tissue engineering [2]. It is well known that scaffold material properties, mechanical, as well as chemical, have a great influence on cell proliferation and acquired functionality [2]. The family



**Figure 6.** Specimen of SH-YY5Y neuroblastoma cells grown on lateral surfaces of 3D structures composed of ORMOCOMP<sup>®</sup>. Cells are seen as growing on the lateral surface of 3D structures generated by application of 2PP on ORMOCOMP<sup>®</sup>. Comparable results were obtained for the other cell lines.

of ORMOCER<sup>®</sup> materials offers the advantage of malleability and elasticity control [3,5]. Moreover, 3D structures with resolution down to 100 nm can be generated by application of 2PP technique on ORMOCER<sup>®</sup> [3,4,6–8] (Figure 1). In this report, using different cell lines, we show that one important representative of this class of polymer ORMOCOMP<sup>®</sup> supports cell growth. Not only the cells are able to grow on ORMOCOMP<sup>®</sup> (Figure 1, Table 1), they are also able to form tissue related cell-to-cell junctions, such as gap junctions (Figure 3). Furthermore, it is shown that cells can be grown of the vertical surfaces of ORMOCOMP<sup>®</sup> structures generated by 2PP technique. These results demonstrate the great potential of 2PP technology applied to appropriate material like ORMOCER<sup>®</sup> for tissue engineering.

### **3D Structures Fabricated by Two-Photon Polymerization Technique**

The 3D polymeric structures were generated by application of technique 2PP on ORMOCOMP<sup>®</sup>. The 2PP technique is a very attractive 3D rapid micro-structuring technology [6,7]. Its principle is similar to that of well-known stereo-lithography (SL) technique, but 2PP provides much better structural resolution and quality (Figure 4). Both techniques use light to induce a photochemical reaction leading to a formation of polymolecules. For organic–inorganic hybrid polymers of the ORMOCER<sup>®</sup> family, this implies a localized transition from the liquid to the solid state [3,6,7]. The very important distinction between the 2PP and SL technologies is that in

case of 2PP, near-infrared (IR) laser pulses, and in case of SL ultraviolet (UV), laser radiation are used for curing of UV photosensitive materials. The 2PP technique, employs a two-photon absorption phenomenon, allowing energy deposition within the volume. The volume in which light-material interaction takes place is limited to the laser focus region. Due to nonlinear nature of this process, resolutions well beyond the diffraction limit can be achieved down to 100 nm (Figure 4b).

### **Biocompatibility of ORMOCOMP®**

For adherent cells, the proliferation is only possible if the cells adhere to the cultivation surface. Different properties of the culture material affect the adherence of the cells onto the surface. It is known that a favorable distribution of charges influences the interaction between cellular adhesion molecules, such as integrins, and material [14]. Glass is known to present favorable charge distribution on its surface, therefore cells grow efficiently on glass [14]. On the contrary, plastic materials are known to inhibit cell adhesion, and thereby, the cell growth. To improve the adhesion on plastic, proteins of the extracellular matrix (ECM), like fibronectin or collagen, are generally applied onto the plastic surface [15]. The ECM proteins use their charges, as well as the other surface structures, to stabilize the cells [15]. Whatever regulates the adhesion of cells on the surface, if the adherent cells used in this study are not allowed to adhere, they react by reduction of the proliferation and even by inducing cell death which correlates with increase of the relative tail moment (results not shown). Since, the presence of ORMOCOMP® did not alter the proliferation parameter of (Figure 1, Table 1) and did not increase the tail moment (Figure 2), it is assumed that ORMOCOMP® sustains the adhesion of the cells. Whether the cell adhesion onto ORMOCOMP® is conditioned by the favorable charge distribution on the surface, is not known. Further studies of ORMOCOMP® are needed to clarify this specific topic.

As the intercellular junctions of a new tissue are formed, the gap junctions, composed of connexins, are the last junctions to be formed. Their formation is conditioned by cell-to-cell adhesion structures, composed by other molecules such as cadherins and cadherin-associated molecules [16]. The observation that the presence of ORMOCOMP® did not alter the formation of gap junctions (Figure 3) indicates that cultivation of cells on ORMOCOMP® does not affect the formation of other tissue-related cell-to-cell junctions.

## Cell Culture onto 3D Structure Composed of ORMOCOMP®

To cultivate cells on vertical/steep surface, the first challenge is to avoid the sedimentation of the cells, so that they are allowed to adhere on the whole surface. In our case this was achieved by keeping the cells on a shaking table (Figure 5). Under these conditions, the cells could adhere on the steep surfaces and began to proliferate along the surface (Figure 6). The shaking table, however, presents some issues related to the hydromechanics, which are automatically introduced into the culture system. This is the reason why some of the structures were not covered with cells, even though they were on the same cover slip and in the same environment. As formulated by Skalak and Fox [17] 15 years ago, combination of physical analysis, simulation technology, and mechanical engineering is still needed, to predict formation of 3D tissue substitutes.

### CONCLUSION

In this report, it is demonstrated that ORMOCOMP® can be used to fabricate 3D structures by application of 2PP technique. The structures generated by 2PP can be seeded with cells in order to grow 3D tissues. Since ORMOCOMP® does not alter the viability of the cells and the formation of tissue related cell-to cell junctions such as  $G_i$ , 2PP technique applied on ORMOCOMP® is a suitable technology for design of implants, which could sustain cell regeneration within the organism.

### ACKNOWLEDGMENTS

This article was partly supported by the Deutsche Forschungsgemeinschaft (DFG); Grant SPP 599. The authors thank Mr Volker Stark of the Nikon Company (Düsseldorf; Germany) for providing the stereo microscope.

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