

## Research Article

# 90° Peel off Tests of Tissue Engineered Osteochondral Constructs: A New Method to Determine the Osteochondral Integration

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One promising treatment of localized osteochondral defects in the knee joint may be the implantation of in vitro tissue-engineered osteochondral constructs. A crucial aspect of this kind of osteochondral construct is the bonding between the bone-scaffold and scaffold-based chondral layer. Here, a 90° peel off test is proposed as an appropriate method to measure the integration of cartilage to bone in osteochondral constructs for different primary methods of bonding the cartilage scaffold to the bone scaffold, with and without seeded chondroblasts. The method was developed and then tested on tissue-engineered constructs. The force/displacement data obtained allow determination of both the maximum force and the total energy required to separate the two layers. The tests showed good reproducibility and good discrimination between measurements as a function of seeding times. Average maximum peel-off forces varied between 10 mN for fibrin glue only to 575 mN for constructs with cells after four weeks of incubation. Linear regression of the area under the curve (AUC) as a function of maximum force shows a high correlation between the two parameters with  $R^2 = 0.97$ . The main limitation of the test is that the data provide only a modest ability to decide how uniform the bond is over the area between the two layers.

## 1. Introduction

In adolescents and young adults, osteochondritis dissecans is a severe but fortunately not too common problem in which cracks form in the articular cartilage and underlying bone. Both the poor self-healing capacity and progression of such chondral and osteochondral joint lesions to osteoarthritis and the limitations of current methods of treatment are still problems [1]. Quite often, if the replantation of the dissected osteochondral lesion is not possible, techniques like mosaicplasty are performed, even though these treatments are often not entirely effective and can entail complications with the donor site [2]. One of the promising treatments

in the future may be the implantation of in vitro tissue-engineered osteochondral constructs [3].

The osseous layer of an osteochondral implant integrates well with the surrounding bone as Schaefer et al. [4] could demonstrate, with ready ingrowth of trabecular bone into the osseous layer of the graft. But the integration with the surrounding cartilage was poor. Therefore, an important aspect of the treatment of osteochondral joint lesions with osteochondral constructs will be the mechanical quality of the bond between the two layers as it is imperative that no separation occurs.

For this reason, as reported here, a method was developed to measure key aspects of the mechanical quality of the

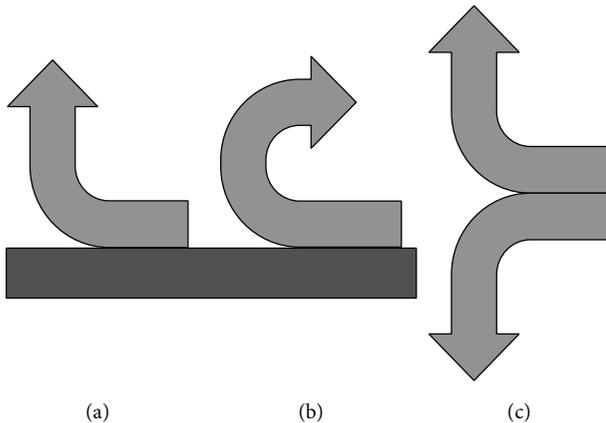


FIGURE 1: (a) 90° peel off test, (b) 180° peel off test, and (c) *t*-peel test.

bond between the osseous layer and the cartilage layer of the constructs. Peel tests are a standard method in industry for evaluating bonding of adhesive materials to surfaces, but they are not a common method in medicine or biology [5]. Peel tests record force during the course of the peeling process, usually conducted at a constant speed. Initial and maximum force can be noted, and the mechanical energy of the peeling process can be computed from the force-distance data. Both parameters can be normalized to specimen dimensions. Various modes are possible (Figure 1). If the bonding of materials is tested by a *t*-peel test, the two layers are pulled apart in the opposite direction each (Figure 1(c)). A peel off test can also be executed with the force perpendicular to the peeled surface in 90° (Figure 1(a)) or parallel to it—that is, at 180° (Figure 1(b)). For this study, the stiffness of the osseous layer made a *t*-peel impractical. Also, the thickness of the chondral layer (close to 1 mm) made the 180° test potentially impractical, as the resultant extreme bending could damage the chondral layer before peeling begins. Therefore a 90° degree test was selected.

It should be mentioned that peel test methods have been employed previously in cartilage-cartilage bonding studies. A *t*-peel test was used to measure the bonding strength of a cartilage-cartilage interface [6]. Englert et al. used a test ring to measure the peel strength of articular cartilage layers from the patellofemoral groove of calf knees. They recorded load versus peeling distance. Their data were analysed in terms of initial stiffness of the chondral laps, load threshold for onset of peeling, and peeling force normalized per incremental line segment as a function of peeling length.

Ahsan and Sah [7] performed a peel off test with slices of tissue from adult cows. Pairs of samples of bovine cartilage were maintained in complete apposition with an applied load on the overlapping area during two weeks. They also tested their specimens using the *t*-peel test and measured a force of  $16 \text{ J/m}^2$ .

The goal of the present study was to demonstrate that the selected 90° peel off test is an appropriate method to measure the integration of the cartilage to bone in osteochondral constructs. This or any method must work even at very low forces as the osteochondral bond is often not strong after

short-time in vivo tissue engineering of such constructs. As described, a testing set-up was developed and then tested on osteochondral constructs produced in vitro.

## 2. Material and Methods

**2.1. Constructs Created and Evaluated.** For this study, a total of 41 osteochondral constructs were tested: 15 with surgical suture for primary fixation, 6 before and 10 after seeding with chondroblasts. Further, in 26 constructs, the primary fixation was accomplished with fibrin glue (Tissucol DuoS DS060601F Baxter, Switzerland). Of these, 10 of the constructs were tested without chondroblasts, 8 were tested before, and 8 are tested after seeding under different conditions and seeding times (see Table 1).

The chondrocytes were obtained from full-thickness human articular cartilage biopsies after mortem (within 24 hours after death) from the lateral knee joint condyle of one individual (age 32) with no history of joint disease, after informed consent by relatives and in accordance with local ethics committee regulations (University Hospital Basel, Switzerland). The chondrocytes were prepared by the Tissue Engineering Group, Department of Research, University Hospital Basel, according to their established method [8].

**2.2. Mechanical Testing of the Osteochondral Bond.** The measurements were performed with an MTS Synergie 100 (MTS Systems, Inc. Eden Prairie, Minnesota, USA) equipped with a 2.5 N load cell (Typ 8432-2.5N, Burster Praezisionsmesstechnik GmbH & Co Kg, Gernsbach, Germany). The 2.5 N load cell was chosen to provide maximum sensitivity without overload. In some preliminary tests, the maximum forces did not exceed 1 N but came close to 1 N. The sampling rate was set to 100 Hz, and identical measurements were omitted to keep the measurement file small. One edge of the cartilage layer was clamped with a small surgical clamp (Figure 2) and attached with a Number 2 surgical suture to the load cell. The osseous layers of the constructs were glued with cyanoacrylate glue onto a glass plate which had been secured in place mechanically on the mechanical testing machine platen. The glue was allowed to set for 5 minutes, and after that period drops of PBS were put on the construct in order to prevent it from drying out. After another 15 min. the measurement was performed. Using the thread hooked to the mechanical testing machine load cell, the cartilage layer clamp was elevated at a constant speed of 50 mm/min (about 0.83 mm/s); that is, the measurements were performed under displacement control. The speed was selected on practical grounds. At substantially lower speeds, the samples tended to dry out during testing. Substantially higher speeds were beyond the performance limits of the available instrument. During the elevation, the load cell force and its upward motion were recorded continuously and simultaneously. type load for debonding (Newton, N) and area under the curve (AUC, unit  $\text{N} \cdot \text{m}$  or J) were calculated from the recorded measurements. The AUC calculation method accounted for the unequal sampling rates in the measurement files.

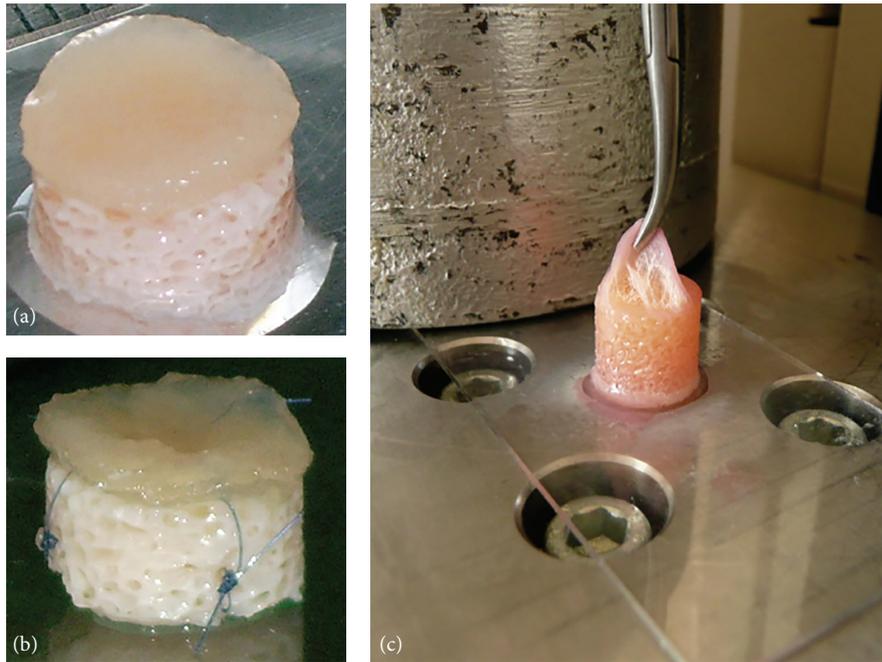


FIGURE 2: (a) Collagen type 2 scaffold. (b) Scaffold with surgical suture. (c) 90° peel off test.

TABLE I: Measured constructs.

Number of repetitions	Primary bonding	Codocytes	Culture	Collagen type	Bone cylinder
6	Suture <sup>1</sup>	no	0	II <sup>2</sup>	Bio-Oss <sup>3</sup>
10	Suture <sup>1</sup>	yes	0	II <sup>2</sup>	Bio-Oss <sup>3</sup>
9	Tissuecol <sup>4</sup>	no	0	II <sup>2</sup>	Skelite <sup>5</sup>
8	Tissuecol <sup>4</sup>	yes	0	II <sup>2</sup>	Skelite <sup>5</sup>
2	Thrombin <sup>6</sup>	yes	0	I/III <sup>7</sup>	Tutobone <sup>8</sup>
3	Thrombin <sup>6</sup>	yes	2 weeks	I/III <sup>7</sup>	Tutobone <sup>8</sup>
3	Thrombin <sup>6</sup>	yes	4 weeks	I/III <sup>7</sup>	Tutobone <sup>8</sup>

<sup>1</sup>Suture: Monolene blue polyester monofil USP6-0 SSC Société sterile catgut Neuhausen, Switzerland <sup>2</sup>Collagen type II: Chondrocell charge 002664 Geistlich Biomaterials <sup>3</sup>Bio-Oss Geistlich Pharma AG, Wollhusen, Switzerland  $d = 8$  mm <sup>4</sup>Tissuecol DuoS DS060601F Baxter, Switzerland <sup>5</sup>Skelite RDK 0610D02 Millenium Biologix Inc. Kingston, Canada  $d = 8$  mm <sup>6</sup>Thrombin solution, Tisseel, Baxter <sup>7</sup>ChondroGide, Geislich, Wohlhusen, Switzerland <sup>8</sup>Tutobone, Tutogen Medical Inc., Florida USA  $d = 11$  mm.

The statistical method used to evaluate the data was the Wilcoxon signed-rank test, performed using the R Development Core Team (2010) language and environment for statistical computing. (R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org>) The significance level was set to  $P < 0.05$ .

### 3. Results

All results presented in this are summarized in Table 2 and in Figures 3, 4, and 5.

The process of the individual measurements is shown in Figure 3.

Linear regression of AUC as a function of maximum force of all measurements except the surgical sutures revealed a high correlation between the two parameters ( $R^2$  0.97 when tested with the raw values and  $R^2$  0.98 when tested with the

logarithmic values;  $P < 0.01$  in both cases; see Figure 6). The sutured samples have been excluded from these calculations, because they are mainly fixed at the edge of the specimen and the AUC therefore does not reflect a peel off of two homogeneously bonded surfaces. In Figure 6, they are plotted as dark red triangles. As shown, the correlation for sutured specimens nevertheless fits into the same picture. As a result, the  $R^2$  values are little changed (0.96 and  $R^2$  0.97, resp.) if the sutured values are included.

### 4. Discussion

A method has been evaluated and is proposed to measure the quality of the mechanical bond between the chondral and osseous layer of tissue-engineered osteochondral constructs. This peel off test measures the maximum force and the energy used to separate the two layers.

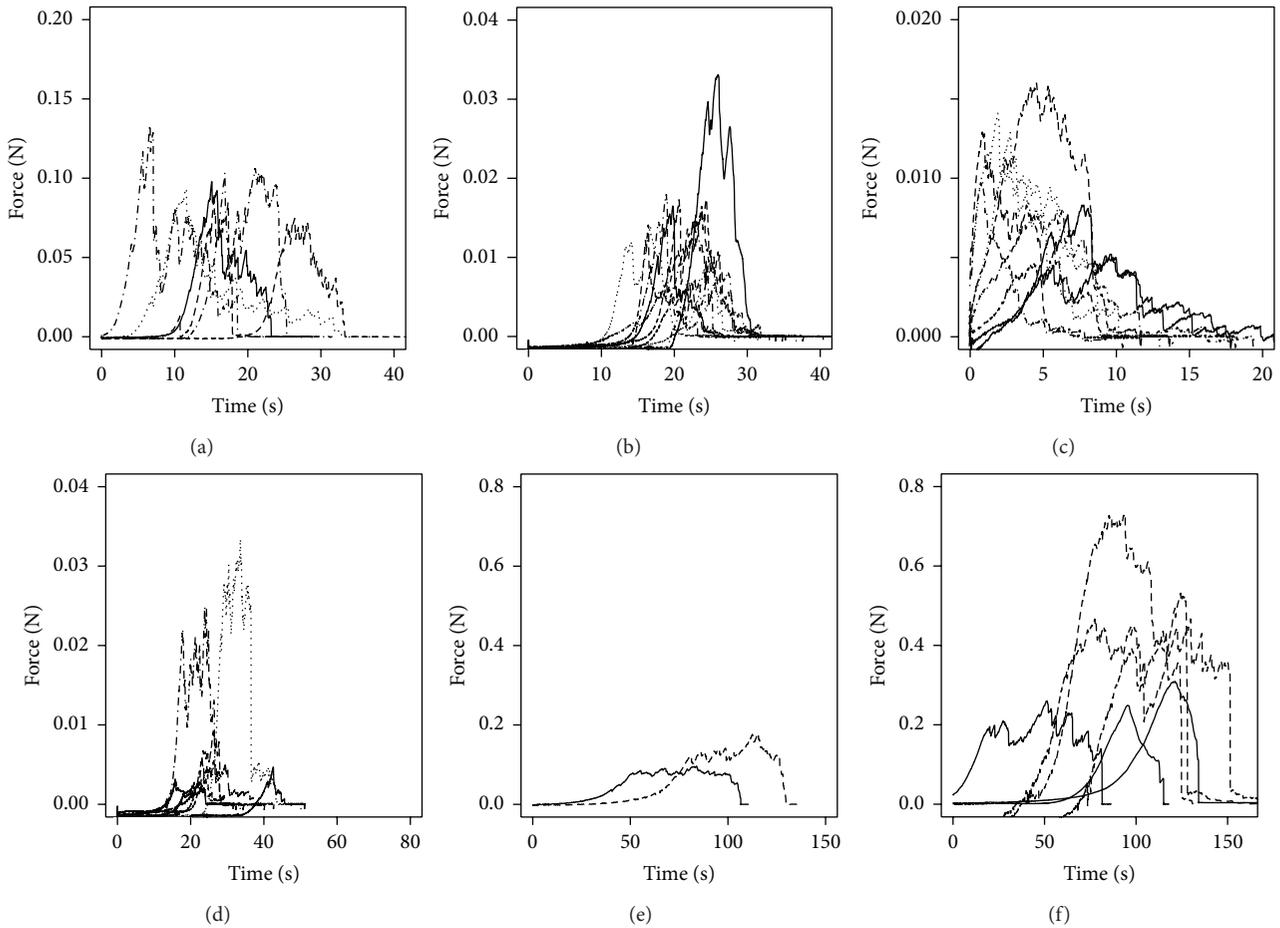


FIGURE 3: Force versus time diagram of the peel off tests of (a) collagen type II cartilage scaffold constructs without seeded chondroblasts created with fibrin glue, (b) collagen type II cartilage scaffold constructs with seeded chondroblasts created with fibrin glue, (c) constructs with a collagen type I/III cartilage scaffold created with eight units of fibrin glue tested directly after the bonding, (d) constructs with a collagen type I/III cartilage scaffold tested after two weeks of culture time, and (e) constructs with a collagen type I/III cartilage scaffold tested after four weeks of culture time.

TABLE 2: Mean and standard deviation or extremes (if less than 8 repetitions) of all measurements.

Number of repetitions	Primary bonding	Maximum force (mN)	Standard deviation or extremes (mN)	AUC (N*s)	Standard deviation or extremes (N*s)
6	Suture w/o cells	99*	24	0.48	0.17
10	Suture with cells	14	8	0.058	0.039
9	Tissuecol w/o cells	10	3	0.042	0.018
8	Tissuecol with cells	11	11	0.062	0.081
2	Thrombin, no culture	137	96–179	1.07	0.87–1.28
3	Thrombin, 2 weeks culture	271	248–305	1.62	1.10–1.57
3	Thrombin, 4 weeks culture	574	467–728	5.03	4.14–5.05

\*The  $t$ -test between the two suture groups revealed a significant difference, between the two Tissuecol groups not.

There are published international standards for peel tests (ASTM, West Conshohocken, Pennsylvania, USA) which determine the method of test execution for different industrial materials. ASTM D 3330 was the starting point for the tests developed and used here [5]. Until now there have been two published papers which analysed with a  $t$ -peel test

the mechanical quality of the cartilage-cartilage bond for specially prepared articular cartilage specimens. For example, Ahsan and Englert describe a  $t$ -peel for testing cartilage slices [6, 7]. But so far, to our knowledge, the biomechanical qualities of the integration between the two layers of an osteochondral construct have not been investigated. The

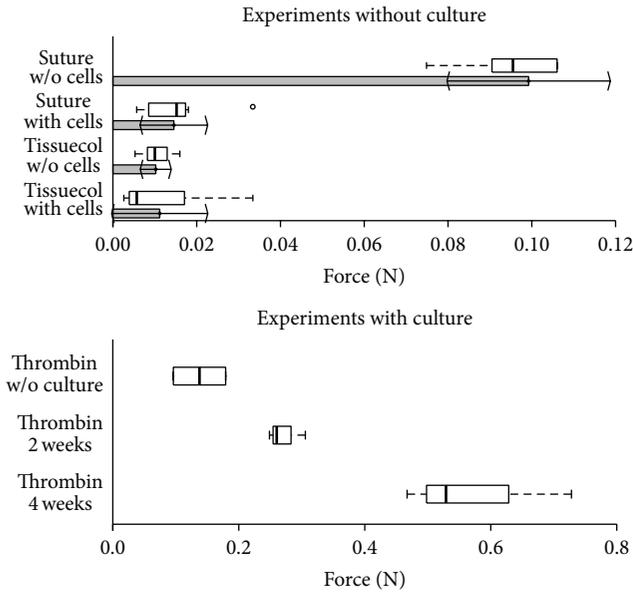


FIGURE 4: Maximum forces for a series of peel off tests (box and whisker-plots showing median and quartiles above and below with nonrobust mean and standard deviation in gray).

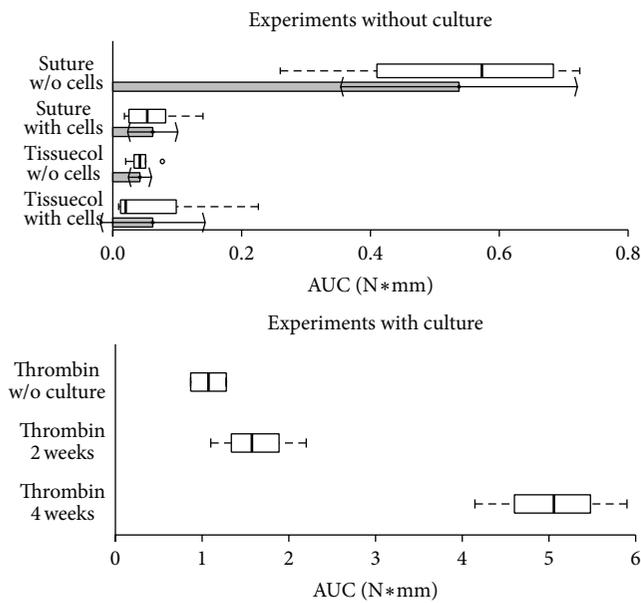


FIGURE 5: Area under the curve (AUC) for a series of peel off tests (box and whisker plots showing median and quartiles above and below with nonrobust mean and standard deviation in gray). AUC is a measure of energy expended during pull off.

test method developed and used here is proposed as a new method in the field of cartilage testing, specifically for mechanical evaluation of osteochondral integration. It should be noted, however, that the method is best suited to evaluating specimens in which the degree of bonding is modest. It may not be applicable to completely mature (i.e., better-bonded) osteochondral samples and especially specimens

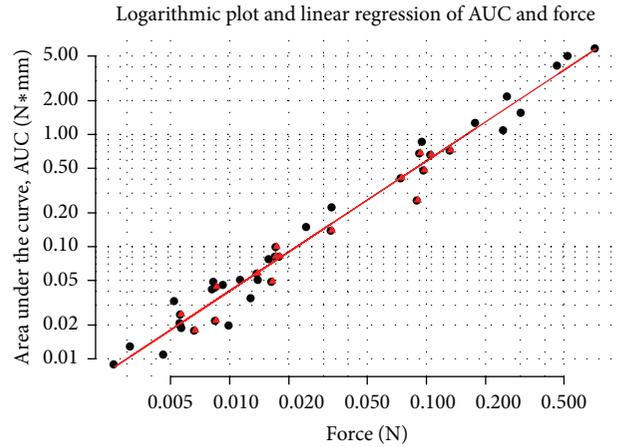


FIGURE 6: Logarithmic plot and linear regression of AUC as a function of the maximum peel-off force of all measurements except for the surgical sutures. The surgical suture results are marked as dark-red triangles.

from a natural joint because of the strength of the natural bond.

It has to be assured that any new test procedure is precise and reproducible. The test set-up should possess few possibilities of interference and if possible the test should be easy to perform. The last point is not only a convenience for the person who runs the test but also reduces the likelihood of errors and increases the reproducibility. Finally it has to be made sure that the recorded measurements are from essentially identically-performed tests. Ideally a test method is also independent of the kind of the samples tested.

In the test described here, the fixation of the clamp to the chondral layer without destroying the osteochondral bond demands some manual skills and practice. This is especially the case if the strength of the bond is at the low end of the measurement range encountered here. Also, it is likely that modifications in the experiment protocol, that is, alteration of the velocity at which the top layer is peeled off, will influence the results. The effect of velocity was not explored with this study. Therefore, other measurements made later can only be compared if made under exactly the same measurement conditions employed here, especially the same velocity.

The test developed must be closely observed to ensure that the clamp does not slip. If it does, this will in particular alter the displacement data. If the clamp does not slip and the bond created between the two layers exceeds the tear strength of the top (cartilaginous) layer, failure may occur within the layer rather than at the osteochondral interface. At the testing velocity used (ca. 0.83 mm/s) the authors found it easy to detect slippage by simple visual inspection during the test.

One limitation of the test is that there is not any means to quantitate whether the bond between the two layers is uniform over the whole area. Either a detached edge or a loosely bonded area in the construct affects its integrity. Inequalities can be seen to some extent in the upward and downward force fluctuations observed during testing, but no attempt was made (yet) to see if it is possible to quantify

these fluctuations as a measure of uniformity of bonding. This may be worth exploring in the future. However, a high correlation was found between AUC and maximum peel-off force. This indicates that both calculations revealed credible and consistent results. And in addition, if the results do not correspond, this indicates that most probably the bonding of the specimen is not uniform. A disagreement between AUC and maximum force indicates that the specimen should be further analyzed. And, vice versa, a good correlation between maximum force and AUC shows evidence for an appropriate test protocol and the reliability of the proposed test.

From a theoretical standpoint it seems obvious that, on a circular specimen, the maximal force should occur when the peel front is longest, that is, half way through the sample if the specimen is homogeneously bonded. In some of the measurements, two peaks can be observed, for example, specimen number 02 Thrombin after 2 weeks or specimen number 02 Thrombin after 4 weeks. In these cases, the maximum force might be underestimated.

The construct is destroyed during the measurement process since the chondral layer is separated from the osseous layer. This testing method is thus not suited to testing the quality of a construct before implantation *in vivo*.

During a peel off test of this type, the forces resulting from peeling off one layer from another are continuously recorded. The maximal force reached when peeling off one layer from another is of special interest. It indicates the force required to overcome the bond between the two layers in a worst-case situation. That is, where the destructive force is concentrated roughly along a line rather than being applied to the entire area of the interfacial bond. It is also possible to calculate from the recorded data the energy to detach the top layer entirely, and this provides a relative measure of the quality of the entire interfacial bond, rather than just the maximum resistance encountered. Also it is possible to describe the total energy per area, since the size of the constructs is known (radius 4 mm or 5.5 mm depending on the osseous layer used). Furthermore it would be possible to calculate the power needed for peel off since besides providing the total energy, the time of the measurement in seconds is known. However, power measurement results would likely vary if another testing speed was used.

As an indication of the delicacy of the constructs evaluated here, the measured forces of the collagen type II constructs (except the results of the constructs without seeded cells in the chondral layer united by surgical suture) were about ten times lower than the forces needed to peel off a "sticky notes" paper from a glass plate. The higher forces measured on average for the constructs without seeded chondroblasts created by surgical suture are due to the suture itself. The suture could not be removed before testing as otherwise there would have been no bond at all between the two layers. But for the measurements with seeded chondroblasts created by surgical suture, the sutures were clipped off before testing.

Part of the proposed test protocols has already been applied to *in vitro* studies [9] and showed reliable results as well. In the cited study, the test was only described cursorily, and the evaluation of the AUC and its comparison to the maximum force were not done. However, these results

showed already a significant difference between two and four weeks of seeding.

## 5. Conclusions

Overall the results of this study show that if mechanical integration at the engineered osteochondral interface happens then it is possible to evaluate it quantitatively with the peel off test presented here. This is true even if the bond is immature and thus delicate.

## Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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