Evaluation of an *in situ* formed synthetic hydrogel as a biodegradable membrane for guided bone regeneration

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**Abstract:** The aim of the present study was to test whether or not the application of an *in situ* formed synthetic hydrogel made of polyethylene glycol (PEG) used as a biodegradable membrane for guided bone regeneration will result in the same amount of bone regeneration as with the use of an expanded polytetrafluoroethylene (ePTFE) membrane. In eight New Zealand White rabbits, four evenly distributed 6 mm diameter defects were drilled into the calvarial bone. Three treatment modalities were evenly distributed among the 32 defects: hydroxyapatite (HA)/tricalciumphosphate (TCP) granules covered at the outer and inner surface with a PEG membrane (test), HA/TCP granules covered at the outer and inner surface with an ePTFE membrane (positive control) and HA/TCP granules alone without membranes (negative control). After 4 weeks, the animals were sacrificed and the calvarial bones were removed. The area fraction of newly formed bone was determined by histomorphometrical analysis of the vertical sections from the middle of the defect and by micro-computed tomography of the entire defect. Multiple regression analysis (SAS® GLM) was used to model the amount of new bone formation. The quantitative histomorphometric analysis clearly revealed higher values of newly formed bone for the two membrane groups compared with the negative control group. The average area fractions of newly formed bone measured within the former defect amounted to 20.3 ± 9.5% for the PEG membrane, 18.9 ± 9.9% for the ePTFE membrane, and 7.3 ± 5.3% for the sites with no membrane. The micro-computed tomography also showed higher values of new bone formation for the PEG and for the ePTFE groups compared with the negative control group. The GLM revealed a highly significant effect of the treatment on the amount of bone formation ($P = 0.0048$). The values for the negative control group were significantly lower than the ones found in the PEG membrane group ($P = 0.0017$), whereas the ePTFE membrane group showed no significant difference from the PEG membrane group. It is concluded that the PEG membrane can be used successfully as a biodegradable barrier membrane in the treatment of non-critical-size defects in the rabbit skull, and leads to similar amounts of bone regeneration as an ePTFE membrane.

**Key words:** bone regeneration, bone transplantation, hydroxyapatite, membranes, rabbits, tricalciumphosphate

Animal experiments and human clinical studies have documented the possibility of regenerating bone by applying the technique of guided bone regeneration (GBR) [Dahlin et al. 1989; Nyman et al. 1990; Becker et al. 1991; Lang et al. 1994]. The initial and successful use of expanded polytetrafluoroethylene membranes (ePTFE) as barriers has made this material the standard for GBR [Dahlin et al. 1991; Davaranah et al. 1991; Nevins & Melloni 1992]. Nevertheless, this membrane has a number of disadvantages: Most importantly, the necessity for a second surgery to remove
the membrane and a high risk for membrane exposure with a subsequent tissue damage (Simion et al. 1994; Machtei 2001). The replacement of non-resorbable by resorbable membranes would, therefore, be desirable (Hämmerle & Karring 1998).

A wide range of resorbable membranes made of either collagen or polyglycolic acid and/or polyactic acid have been investigated in experimental and clinical studies (Lundgren et al. 1994; Mayfield et al. 1997; Simion et al. 1997; Zitzmann et al. 1997). As a consequence of the good results partially based on the low rate of complications, resorbable membranes have become the standard for most clinical situations.

Common to all presently used membranes is the fact that their fabrication is completed before they are delivered for patient use. Consequently, they are made available in standard sizes and forms and need to be adapted to the patient’s individual situation. Alternatively, a membrane could be custom made for an individual defect directly intraoperatively by using a material different from the ones mentioned above.

Hydrogels made of polyethylene glycol (PEG) fulfill a number of criteria required to serve as in situ forming synthetic membranes. PEG has been shown to be highly biocompatible (Pang 1993, Working et al. 1997). It is presently approved for several pharmaceutical applications (Zalipsky & Harris 1997) and as medical devices, e.g. a sprayable adhesion barrier (Metzler et al. 2003).

In a recent animal study, the barrier function of this PEG material was examined after subcutaneous placement in rats (Wechsler et al. submitted). Histological analysis revealed prevention of cellular penetration in the membrane group up to 4 months. To date, no information regarding the use of this material in GBR procedures is available.

The aim of the present study was to test whether or not the application of an in situ formed synthetic hydrogel used as a biodegradable membrane for GBR will result in the same amount of bone regeneration as with the use of a standard ePTFE membrane.

Material and methods

Animals

Eight adult (12 months old) New Zealand White rabbits, weighing between 3 and 4 kg, were used in the present study. The animals were kept in a purpose-designed room for experimental animals, and were fed a standard laboratory diet. The study was evaluated and accepted by the responsible Veterinary Authority.

Membranes

In the test sites, PEG hydrogel membranes were used. They were made by mixing a multi-arm PEG with thiol endgroups and a multi-arm PEG with acrylate endgroups in an aqueous buffer system (triethanolamine/HCl) at physiological pH. In this system, the PEG termini connect through a highly self-selective addition reaction, forming a network with hydroly ester linkages. In order to control the flow properties of the material, carboxymethyl cellulose (CMC) was added to the buffer solutions as a viscosity modifier (Wang et al. 2004).

Two different application forms of the same polyethylene glycol membrane were prepared: an in situ gelating form to cover the skin-facing side of the defect and a precast form to be placed between the internal cortical plate of the calvarial bone and the dura. Because the membrane for the internal side of the defects had to be placed underneath the bony borders, it was decided to allow gelation of the membrane before placement.

For the in situ gelating membranes, two sterile filtered PEG components and the corresponding autoclaved buffer solutions were packed in four separate syringes. The amount of PEG and the amount of buffer components were adjusted, such that the two PEG components had equimolar numbers of endgroups. Each PEG component was dissolved in its buffer by a syringe-to-syringe mixing process. Subsequently, the two syringes containing the dissolved PEG solutions were attached to a static mixer. Upon expelling the contents of the two syringes through a static mixer, the gelation process started. For preparation of the pre-cast membranes, equal aliquots from the two PEG solutions in buffer (without CMC) were quickly mixed, and membranes of 0.4 mm thickness were cast between two glass slides. These membranes were kept in sterile 10 mM PBS [pH 7.4] at 37°C overnight. Round pieces of 8 mm diameter were cut with a biopsy punch just before use. They were shrunk to a diameter of 6 mm by drying.

ePTFE (Gore-Tex® Regenerative Material, W.L. Gore & Associates Inc., Flagstaff, AZ, USA) were used as positive controls. The ePTFE membranes were stabilized by using titanium nails (Frios®, Friaden GmbH, Mannheim, Germany).

Surgical procedure

Anesthesia was administrated to the animals by injection of 65 mg/kg of ketamine and 4 mg/kg of xylazine and maintained with isoflurane/O₂. The surgical area was first shaved and disinfected with isophor to allow for aseptic surgical conditions. A straight incision was made from the nasal bone to the midbaggal sagittal crease. The soft tissues were retracted and the periosteum was elevated from the site. In the area of the right and left parietal and frontal bones, four evenly distributed 6 mm diameter craniotomy defects were prepared with a trephine burr under copious irrigation with sterile saline. Care was taken to avoid injury to the dura. Subsequently, the surgical area was thoroughly flushed with saline to remove bone debris.

In order to prevent the periosteal cells from access to the defect area and to avoid herniation of the brain, a pre-cast PEG membrane of 6 mm diameter (test) or an ePTFE membrane with 8 mm diameter (positive control) was placed between the dura and the inner surface of the calvarial bone [Fig. 1]. Following placement of the pre-cast PEG membranes, a certain period of time was allowed to reattain their original diameter of 8 mm by rehydration and thus maintained in position by resting against the defect borders. All sites were filled with highly porous hydroxyapatite.

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Fig 1. A pre-cast and dried polyethylene glycol membrane with a 6 mm diameter before it is placed between the dura and the inner surface of the calvarial bone.
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Fig. 2. After filling of all four craniotomy defects with a similar amount of porous hydroxyapatite [HA]/tricalciumphosphate [TCP] granules, the in situ gelating polyethylene glycol membrane is applied in a viscous form over the granules.

(HA)/tricalciumphosphate (TCP) granules (Institut Straumann AG, Basel, Switzerland) in a size range of 100–1000 μm mixed in a ratio of 60:40.

The three treatment modalities were evenly distributed among the eight animals in the four defects. The two remaining sites in the last animal, however, were used for test group treatments. From the total of 32 sites, the following treatments were performed:

Positive control: 10 sites: HA/TCP granules and ePTFE membranes;
Negative control: 10 sites: HA/TCP granules alone without membranes; and
Test: 12 sites: HA/TCP granules and PEG membranes.

For the test sites, the in situ gelating PEG membrane was applied in a viscous form over the HA/TCP granules and the adjacent bone in order to cover the defects completely (Fig. 2). Within 60 s, the PEG gels had set and thus stabilized the grafted area. Because of the good adaptation, no further stabilization by tacking of the membrane was necessary. A transformation to a more opaque appearance of the gel indicated the completed setting process.

At the positive control site, the ePTFE membranes were trimmed and draped over the bone substitute material and the adjacent bone in order to overlap the defect margins by at least 2 mm. The ePTFE membranes were stabilized by using titanium nails (Frios®, Friadent Gmbh). Subsequently, the soft tissues were closed with interrupted sures.

Four weeks later, the rabbits were sedated with barbiturates and sacrificed by an overdose of Ketamin. The skull containing all four craniotomy sites was removed and placed in 40% ethanol.

**Micro-computed tomography (μCT)**

Before histological preparation, the intact specimens were subjected to μCT 40 (Scanco Medical AG, Bassersdorf, Switzerland) as described previously [Muller et al. 1998]. In brief, μCT was performed using an isotropic resolution of 30 μm. A constrained Gaussian filter ($\sigma = 1.2$ and width $= 1$) was used to suppress the noise in the volume parts. Mineralized bone tissue was segmented from non-mineralized tissue and graft material using two global thresholds of 19% and 50% of the maximum possible grey-scale value, respectively. A one-voxel thick dilation was then applied to the graft material, to remove partial volume elements resulting from digitization and filtration. At the initial defect location, a cylindrical volume of interest (VOI) was identified for quantitative analyses, allowing for a three-dimensional assessment of bone volume and graft volume.

**Histologic preparation**

The samples were dehydrated in a graded series of increasing ethanol concentrations. Thereafter, they were embedded in methylmethacrylate without being decalcified according to standard procedures [Schenk et al. 1984]. The specimens were sectioned in the frontal plane through the middle of the defects. Sections of 200 μm were obtained, ground and polished to a uniform thickness of 60 μm. The specimens were stained with Goldner (1938). Digital images were taken and processed with an image analysis program (Adobe® Photoshop® 7.0.1).

**Histomorphometry**

Quantitative evaluation of bone regeneration was assessed by applying standard morphometrical techniques [Weibel 1980; Gundersen et al. 1988]. Measurements were carried out directly under a light microscope at a magnification of $\times 160$, using an optically superimposed eyepiece test grid composed of 100 points and 100 cyclid lines [Schenk & Olah 1980; Weibel 1980]. The number of test points overlying the profiles of the different components within the former defects (mineralized bone tissue, non-mineralized tissue and graft particles) was counted. They are defined and symbolized according to the standard nomenclature of the International Society for Stereology (Exner 1987).

**Statistics**

All data for the statistically variables of new bone formation, amount of graft material and non-mineralized tissue were first analyzed by descriptive methods (QQ-plots, box-plots), and mean values and standard deviations were calculated (SAS/STAT, Version 8, SAS Institute Inc., Cary, NC, USA). General linear model procedures (SAS/STAT) were performed in order to assess critical parameters explaining the amount of new bone formation (outcome variable) within the prepared defect area assessed by histological methods. Prior to the statistical analysis, a normal distribution of the data was verified. The parameters tested with respect to their influence on the outcome variables were ‘treatment group’, ‘animal’ and ‘defect location’. The level of significance chosen in all statistical tests was set at $\alpha = 0.05$.

**Results**

During the experiment, all animals showed an uneventful healing of the area of surgery. No significant reductions in body weights were noted, and no postoperative infections were observed. All animals behaved normally during the healing phase.

**Descriptive histology**

Microscopic examination demonstrated new bone formation originating from the bony borders directed toward the center of the defect. Complete bridging of the former defects with mineralized bone occurred only in the two membrane groups (Fig. 3a-c). For the PEG membrane group, eight out of 12 and, for the ePTFE membrane group, six out of 10 defects revealed a bone bridging after 4 weeks of healing. In both membrane groups, bone formation could occasionally be observed on the outside surface of the membranes. No adverse reactions could be seen in any of the treatment groups. The biodegradable PEG membrane could still be identified after 4 weeks of healing. The PEG membrane appeared to be thicker at 4 weeks compared with the time of membrane application. The indivi-
dual particles of the grafting material were identifiable and they were found to be surrounded by varying amounts of newly formed bone. In the negative control group where no membrane was used, the amount of grafting particles seemed to be reduced but no particles could be detected in the neighboring area.

**Histomorphometry**

The quantitative histomorphometric analysis clearly revealed higher values of newly formed bone for the two membrane groups compared with the control group. Average area fractions of newly formed bone measured within the former defect amounted to 20.3 ± 9.5% for the PEG membrane, 18.9 ± 9.9% for the ePTFE membrane and 7.3 ± 5.3% for the control (Table 1 and Fig. 4). The area fraction occupied by the grafting material was again higher for the PEG membrane group (23.7 ± 6%), and the ePTFE group (20 ± 5.8%) compared with the control group (13.5 ± 7%), where no membrane was applied.

**Quantitative μCT**

X-ray attenuation of the graft material was markedly larger than that of bone tissue, such that segmentation using global thresholds could be used to distinguish graft material from bone. Substantial amounts of graft material were still present at 4 weeks; hence, the volume of interest (VOI) representing the initial defect location could be identified.

In agreement with the results obtained by histomorphometry, the μCT showed higher values for new bone formation for the two membrane groups compared with the control group. This difference, however, was not as pronounced as the one obtained by the histomorphometrical analysis. The CT-derived values were 23.3 ± 4.6% for the PEG membrane group, 24.8 ± 4.7% for the ePTFE group and 16.5 ± 5.6% for the control group (Table 1, Figs 5 and 6a–c).

Similarly, the assessment of the volume fraction of the grafting material revealed values very similar to the ones obtained by the histomorphometry. Thus, the mean value for the PEG membrane group reached 17.9 ± 3.5%, 18.2 ± 4.2% for the ePTFE group and 13.9 ± 5.6% for the control group.

**Statistical analysis**

Several general linear model procedures were used in order to explain the dependent variable ‘new bone formation’ assessed by the histological analysis (Table 2). The model including the explanatory variables ‘treatment group’ (PEG membrane, ePTFE membrane, control), ‘animal’ (rabbits 1–8) and ‘defect location’ (left, right, anterior, posterior) was able to explain 66% of the variability in new bone formation and was highly significant ($P = 0.0128$). The explanatory variables ‘treatment’ had a highly significant effect on the amount of bone formation ($P = 0.0048$). In detail, the negative control group values were significantly lower than the ones found in the PEG membrane group ($P = 0.0017$), whereas the ePTFE membrane group was not significantly different from the PEG membrane group. The explanatory variable ‘defect location’ was borderline significant ($P = 0.048$). Defects located on the left side of the skull showed a tendency for higher bone formation. The individual animals did not show a significant influence on the dependent variable ‘new bone formation’.

**Discussion**

The present study demonstrated that the new PEG membrane can successfully be used as a biodegradable barrier membrane for GBR in non-critical-size defects in the rabbit skull. This was documented by a similar amount of bone regeneration compared with defects treated with ePTFE membranes. The ePTFE and the PEG membranes revealed statistically significantly more bone formation compared with defects treated with no membrane.

The newly developed PEG membrane was designed to meet specific requirements for GBR. The membrane was formed in situ by gelation of a multifunctional thiol-modified PEG with a multifunctional acrylate-modified PEG. It has been demonstrated that at the end of the gelation process, this PEG gel is cell occlusive, because of the fact that the distances

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**Table 1. Percentages of newly formed bone after 4 weeks, including mean values, standard deviations and number of sites**

<table>
<thead>
<tr>
<th>Membrane Type</th>
<th>Treatment</th>
<th>Mean (%)</th>
<th>SD (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG</td>
<td>Control</td>
<td>7.3</td>
<td>5.3</td>
<td>1</td>
</tr>
<tr>
<td>ePTFE</td>
<td>Control</td>
<td>16.5</td>
<td>5.6</td>
<td>1</td>
</tr>
<tr>
<td>PEG</td>
<td>Treatment</td>
<td>23.7</td>
<td>6.0</td>
<td>1</td>
</tr>
<tr>
<td>ePTFE</td>
<td>Treatment</td>
<td>20.0</td>
<td>5.8</td>
<td>1</td>
</tr>
</tbody>
</table>

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in a reduction of the swelling properties under physiological conditions.

In recent human and animal studies, a membrane composed of a liquid polymer of lactic acid and N-methyl-pyrrolidone has been investigated as a barrier in procedures aimed at regeneration of lost periodontal tissues [Bogle et al. 1997, Garrett et al. 1997]. Although this material is available as a fluid that can precipitate in situ by adding water [Rosen & Reynolds 1999], in the majority of the studies a membrane made of the polymer was formed extraneously, before it was applied to cover the periodontal defect [Garrett et al. 1997, Jepsen et al. 2000]. Generally speaking, significant amounts of periodontal tissue regeneration have been reported, when using this membrane [Polson et al. 1995, Hou et al. 2004].

It has been shown that stability of the grafted area is a prerequisite for bone regeneration [Wikesjö & Nilveus 1990]. In the present study, both ePTFE and PEG membranes lead to similar amounts of new bone formation. While the ePTFE membrane was secured with titanium pins, the PEG membrane was only closely adapted to the bone surrounding the defects during the application process. This indicates that the PEG fulfills the requirements for GBR membranes with respect to cell stabilization of the area of regeneration.

A common problem associated with resorbable membranes is their inability to maintain space and therefore the risk of membrane collapse [Levy et al. 1994, Ito et al. 1998]. In a recent animal study using a rabbit cranial defect model, it could be demonstrated that a resorbable membrane without grafting materials collapsed into the defects. This resulted in a sparse amount of newly formed bone, confined to the edges of the defects [Aaboe et al. 1998]. In order to compensate for the insufficient physical strength, the use of membrane-supporting materials in conjunction with resorbable membranes has been proposed (for a review, see Hämmerle & Karring). The in situ formed PEG hydrogel is also not able to provide space for regeneration. Hence, the defect must be filled with a grafting material prior to membrane application. A highly porous, HA/TCP graft material was used in this experiment. In previous studies, successful bone formation has been demonstrated, when similar materials were used in clinical trials in orthopedic indications [Ransford et al. 1998, Cavagna et al. 1999] and in animal studies in periodontal indications [Nery et al. 1992].
In the present study, HA/TCP granules without any membrane revealed 7.3% new bone formation within the defect. With the use of either the ePTFE or the PEG membrane, however, the amount of newly formed bone significantly increased to 18.9% for the ePTFE membrane and to 20.3% for the PEG membrane. In the defects with no membranes, less HA/TCP granules could be detected after 4 weeks (15.5% in the control, 23.7% in the PEG and 20% in the ePTFE group). In the negative control group, some granules may have been displaced because of lack of stabilization resulting from a membrane. Therefore, some granules could be macro- and microscopically detected outside of the former defect in sites with no membrane protection.

The model used in the present study has been applied before with a number of variations. Thus, it could be demonstrated that a complete bony bridging of rabbit calvarial defects could successfully be achieved by placing an ePTFE membrane on the external surface of the defects (Hämmerle et al. 1993). No bony bridging was observed in the controls, where no membranes had been used. In a different study, defects with a diameter of 15 mm covered by just one ePTFE membrane facing the skin flap revealed only a sparse amount of newly formed bone (Aabo et al. 1998). The defect area was mainly occupied by herniated brain tissue. In contrast, defects covered by two ePTFE membranes, one placed externally and one placed internally, clearly showed more new bone formation. However, complete bone healing of the 15 mm defects was never observed (Aabo et al. 1998). In the present study, either an ePTFE membrane (positive control) or a pre-cast PEG membrane (test) was used on the internal side of the defects. This allowed to delimit the area of regeneration precisely and to avoid obstruction of the defects by herniating brain tissue. Hence, in the present study the majority of the membrane-treated groups revealed a complete bony bridging after 4 weeks of healing. In another animal study, it was demonstrated that after 18 weeks of spontaneous healing, two-thirds of the 5 mm defects in the rabbit skull showed complete bony bridging (Dodde et al. 2000). This indicates that the use of HA/TCP granules together with either an ePTFE or a PEG membrane revealed a favorable healing of non-critical-size-rabbit cranial defects after only 4 weeks.

In the present study, defects located on the left side of the skull showed a tendency toward higher bone formation, rendering the defect location borderline significant in the GLM. No explanation could be found for this trend.

In agreement with the histological data, μCT also showed higher values of new bone formation for the PEG and for the ePTFE groups compared with the control group. Compared with the histomorphometric analysis, the differences between the treatment groups were smaller. For the histological analysis, only one section taken from the center of the defect was used as the basis for data collection. The radiographical method, in contrast, utilized a series of sections taken from the entire defect area. In the negative control group, only minor bone formation took place, mainly along the defect borders. Taking only one section for histomorphometric analysis may lead to an underestimation of the total amount of bone tissue. In addition, the smaller amount of grafting materials in the negative control group lead to more difficulties in identifying the cylindrical VOI for tomographical analysis. As a result, the μCT may have the tendency to overestimate the amount of bone in the negative control groups. These might be explanations for the higher values of newly formed bone when they were analyzed by μCT.

Both the histomorphometrical and the radiographical analyses were able to detect the increased amounts of bone formed in the membrane groups. Further studies should investigate whether or not μCT can render numerical data that will allow substituting the tedious process of histological processing of tissue samples and subsequent histomorphometrical analysis. A shorter time to obtain the desired data coupled with decreased costs may ultimately result by using μCT (Verna et al. 2002). Clearly, the information obtained from descriptive histology cannot be replaced by radiographical methods.

It is concluded that the newly developed in situ forming synthetic membrane made of PEG can successfully be used as a biodegradable barrier membrane in the treatment of non-critical-size defects in the rabbit skull. After 4 weeks of healing, histomorphometrical analysis and μCT demonstrated similar amounts of newly formed bone for defects treated with the PEG membranes compared with defects treated with standard ePTFE membranes. The ePTFE and the PEG membranes revealed statistically significantly more bone formation compared with defects treated with no membrane. This in situ forming PEG membrane has the potential to meet specific requirements for GBR procedures.

Acknowledgements: The authors especially thank W. Bürgin (University of Berne, Switzerland) for providing expertise and support in the statistical analysis. The constructive discussion and support of Prof. Dr. R. Müller (Institute for Biomedical Engineering, Swiss Federal Institute of Technology (ETH) Zurich and University of Zurich, Switzerland) are greatly acknowledged. The animal care by Dr. H. Christina and F. Nichols (University Hospital, Zurich, Switzerland) and the technical assistance by Y. Bleemhard and A. Tchouboukov are also greatly acknowledged.
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ePTFE メンブレン群が 18.9 ± 9.9，
9.9，メンブレンなしの部位では 7.3 ±
3.5 ± 3.5%であった。

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