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# *In vivo* and *in vitro* degradation of a novel bioactive guided tissue regeneration membrane

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*Abstract.* The aim of this study was to assess degradation of a novel bioactive guided tissue regeneration (GTR) membrane and to quantify the concurrent tissue responses. Pieces of membrane composed of poly-L-lactide, poly-D,L-lactide, trimethylenecarbonate and polyglycolide were dipped into an *N*-methyl-2-pyrroline (NMP) solution and implanted in the mandibles of 10 sheep. The animals were sacrificed at 6–104 weeks. Parallel *in vitro* degradation was analysed by measuring the inherent viscosity, water absorption and remaining mass. One of the 2 *in vitro* sets of membranes was prehandled with NMP. At 6–26 weeks *in vivo*, the gradually more degraded implants were surrounded by a fibrous network. At 52 and 104 weeks, the implants and fibrous networks were non-detectable. Foreign body granulomatous reactions were not observed. *In vitro*, the mass of the NMP-exposed membranes diminished linearly over the 2-year period down to 10%, while the non-NMP-exposed membrane maintained all their mass for the first 16 weeks. The membranes without NMP had absorbed significantly less water at weeks 4 and 8 than the other group. The inherent viscosity decreased relatively uniformly in the *in vitro* groups. In conclusion, the *in vivo* degradation was complete in 12 months with only mild histologic responses; the degradation *in vitro* may be slower. NMP accelerates the degradation.

Key words: degradation; bioactive; guided tissue regeneration; dental surgery.

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The principle of guided tissue regeneration (GTR) based on a barrier membrane has been used in the treatment of periodontal defects for almost 3 decades[11–13,16]. GTR membrane guides the proliferation of various periodontal tissue components after periodontal surgery. More recently, the principle of GTR has also been used in guided bone regeneration (GBR) in the treatment of bony defects in dental implantology as well as in other skeletal locations[7,8,18]. In GBR, a membrane is positioned to prohibit fibroblastic cells from colonizing an intraosseous wound during healing, while more slowly migrating osseous cells fill the defect, resulting in direct bony regeneration and deposition.

Several types of GTR/GBR barrier membranes have been used with varying success. The first generation membranes made of expanded polytetrafluoroethylene (ePTFE) were non-absorbable[3]. To avoid a removal surgery 4–6 weeks after the implantation, bioabsorbable polymer GTR membranes were developed and the first FDA approval for human use

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was received in 1993[10]. This has been followed by several other polylactic (PLA)- and glycolic acid (PGA)-based GTR membranes that have been used successfully in GTR and GBR therapy[4].

A novel GTR/GBR membrane incorporating poly-L-lactide (PLLA), poly-D,L-lactide (PDLA), trimethylenecarbonate (TMC) and polyglycolide (PGA) has been approved by both European authorities and FDA for the treatment of periodontal defects, dental pre- and peri-implant surgery, and covering bone defects and empty sockets. Interestingly, the membrane has been shown to promote healing of bony defects in a rabbit model with a 6-mm drilled hole on the calvarium[21,22]. This effect has been attributed to the *N*-methyl-2-pyrroline (NMP), a pharmaceutical solvent, that is used to temporarily soften the membrane to facilitate shaping and placing during surgery[17,21].

The degradation and tissue biocompatibility of this novel biodegradable system has not been investigated earlier. The present study was designed and conducted to assess the degradation both *in vivo* and *in vitro*. Tissue responses in the course of the degradation were quantified using histological techniques.

## Materials and methods

The study protocol was accepted by the Tampere University Animal Trial Committee and by the Provincial Administrative Board according to Finnish law. The trial was performed according to the Good Clinical Practice (GCP) guidelines as applicable.

### Animals and implants

The experimental animals were skeletally mature female Finnish landrace sheep. The animals were clinically examined by a veterinarian to confirm the healthy condition before the study initiation, and these examinations were continued on a daily basis for the entire preoperative week. At first check, *Clostridium*-vaccination (Heptavac, Intervet Ltd, Milton Keynes, UK; immunization against *Cl. chauvoei*, *Cl. perfringens* B, C and D, *Cl. septicum*, *Cl. tetani*, *Cl. novyi*) was given to avoid these common infections; the vaccine does not have any known effects on the bone healing or biodegradation of polymer implants. A total of 10 sheep was used. Two animals were sacrificed at each check point: 6, 12, 26, 52 and 104 weeks postoperatively.

The implants used were membranes and tacks of Inion GTR™ product range (Inion Ltd, Tampere, Finland). The implants were manufactured and quality checked according to the manufacturer's standard methods and requirements. All the implants were sterilized with γ-irradiation (minimum dosage 25 kGy). The implants used in the *in vivo* and *in vitro* experiments are shown in Table 1. The manufacturer supplied all the required instrumentation, i.e. drill bits and tack applicators. All the instruments were provided sterile (γ-irradiation or autoclave).

### Preoperative procedure

The condition of each animal was clinically assessed daily 7 days before operations. Preoperatively, water was given *ad libitum*, while feed was withheld for 24 h prior to the surgery.

Preoperatively, the sheep were given 1 mg atropine s.c. and benzylpenicillinprocaine 15 mg/kg i.m. The sheep were anaesthetized with medetomidine 0.030 ml/kg i.m. and ketamine hydrochloride 1.5 mg/kg i.m. Every 30 min the sheep received intravenously 50% of the original amount of medetomidine and ketamine hydrochloride, as necessary. Lidocain/epinephrine (2%) was used for local anaesthesia.

Postoperatively, benzylpenicillinn procaine 15 mg/kg was administered for 5 days s.c. and ketoprophen 5 mg/kg i.m. once a day for 3 days.

### Surgical procedure

The right mandible of the sheep was shaved, washed and scrubbed with chlorhexidine gluconate solution. A skin incision was made below the lower border of the mandible in the region of the diastema and carried through the periosteum. The periosteum on the buccal side of the mandible was elevated. The membranes were plasticized by 'dipping' them in an NMP solution for 20–30 s, as advised in the manufacturer's instructions for use. Thereafter, the membranes were kept on the table approximately 5–10 min before implanting. A 10 mm × 10 mm piece of the membrane was implanted on the buccal side of the mandible and secured with two tacks. The wound was closed in layers using absorbable sutures.

*Table 1*. The implants used in the study

| Implant type | Number of implants | Purpose of implants | Remarks |
|---|---|---|---|
| Membrane | 10 | Histology, biodegradation | Right mandible |
| Tack | 20 | Histology, biodegradation | Fastens the membrane |
| Membrane | 24 | Inherent viscosity | *In vitro* tests |
| Membrane | 66 | Mass loss | *In vitro* tests |
| Membrane | 54 | Water absorption | *In vitro* tests |

### Postoperative measures

After the operations, the sheep were kept in indoor pens for 7 days before moving them outdoors. All sheep were given soft food for 3 days postoperatively and hay thereafter. For the first 7 postoperative days, the sheep were observed for neurological symptoms, movement and appetite. The operated areas were scrutinized for infection, inflammation, swelling and wound dehiscence. Temperature of each animal was measured daily for the 1st postoperative week.

### Histological specimen preparation

After sacrifice, the mandibles were carefully dissected and macroscopically inspected. For histology, the regions of the mandibles containing the implants and the overlying soft tissue were removed and fixed in 4% phosphate-buffered formaldehyde (pH 7.4). After washing and dehydration, the specimens were embedded in methylmethacrylate. Sections of 150 μm were cut perpendicular to the mandible using a low-speed diamond saw (Isomet, Buehler Inc., Illinois, USA). All samples were ground flat and polished by hand (SiC papers from 800 to 4000 grit size). The sections were mounted on objective slides and stained with hematoxylin and eosin (HE). Tissue-implant interface was evaluated with a light microscope to observe tissue reactions and implant degradation.

### *In vitro* degradation

As a comparison to the *in vivo* samples, 2 sets of *in vitro* degradation data were gathered under the same conditions as the *in vivo* implantation. The 1st *in vitro* set consisted of GTR membranes prepared with NMP as described earlier, while the other set of membranes was not exposed to NMP at all.

The *in vitro* samples were placed in glass vials with approximately 10 ml phosphate-buffered saline (PBS) of pH 7.4 ± 0.2. The samples were subjected to ageing up to 104 weeks at 37 ± 1 °C in an incubator. The PBS was changed

every 2 weeks, with the pH of the solution checked every time.

The follow-up of the *in vitro* samples comprised of 14 checkpoints: 0, 2, 4, 8, 12, 16, 20, 26, 34, 42, 52, 64, 80 and 104 weeks. The baseline (0 week) measurements were performed after keeping the samples in water for 24 h. Degradation of polymer devices was analysed by measuring the inherent viscosity of polymer, water absorption and remaining mass of devices according to the *in vitro* experiment recommendations by the FDA. Inherent viscosity, a measure of molecular weight, was assessed with a Lauda capillary viscometer (Lauda, Lauda-Königshofen, Germany) at weeks 0–52. The apparatus measured 3 inherent viscosity values (mean is shown) of 1 membrane of both of the *in vitro* series at each checkpoint.

Water absorption was assessed at weeks 0–42 and the remaining mass at weeks 0–104. For remaining mass, 3 samples of the 2 series were vacuum-dried at each follow-up point, and thereafter, the mass was compared to the initial mass. Water absorption is the dry mass subtracted from the mass immediately after taking the implant from the PBS vial. Three membranes of the 2 series were analysed at each follow-up point.

#### Data analysis

For the *in vitro* membranes, mean ± stanstandard deviation (SD) of remaining mass and water absorption at each follow-up point was calculated separately for both of the membrane sets (Figs 1 and 2). The inherent viscosity values are means at each follow-up point (Fig. 3). For remaining masses and water absorption, the differences between the 2 membrane sets at each checkpoint were evaluated with Student's *t*-test for independent samples. Differences were considered statistically significant when $P < 0.05$.

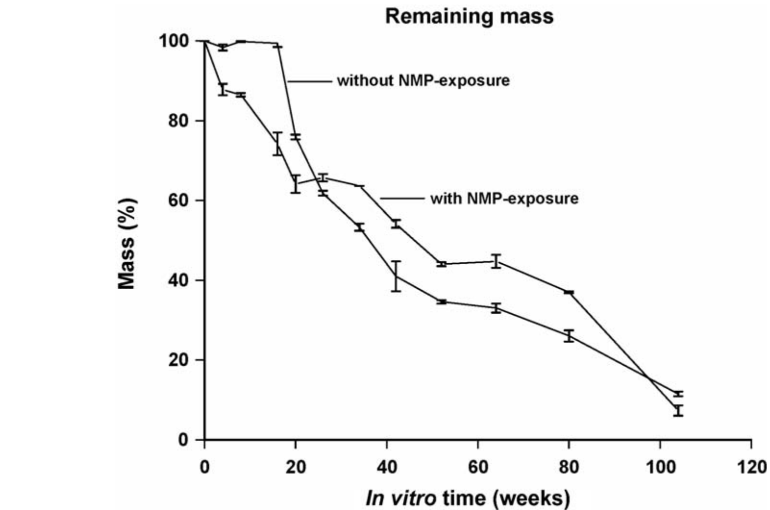


*Fig. 1.* Remaining mass (mean ± SD) of the GTR membranes with and without NMP-exposure as a function of time. The membranes were immersed in phosphate-buffered saline for the entire follow-up at pH 7.4, 37 °C.

## Results

### *In vivo* experiments

All 10 animals recovered well from the operations and the wounds healed without infections. The sheep remained healthy for the entire course of the study, and none of them died prematurely.

#### *Macroscopic inspection*

On macroscopic examination after sacrifice, the wounds were barely visible at 6 weeks and could not be detected thereafter. At 6, 12 and 26 weeks, no signs of inflammation or discharge in the operation sites were observed. None of the membranes or tacks had migrated from the sites they had been implanted to. The implants were covered with normal soft tissue. At 52 and 104 weeks, no implant remnants or foreign body reactions were found.

#### *Histology*

Six weeks postoperatively, the HE staining disclosed the implants (Fig. 4) on the buccal side of the mandibles. The implant material formed more or less globular structures surrounded by a fibrous layer. In some areas, the fibroblasts and connective tissue fibers invaded into the surface of the implant. Mild histiocytic reaction was seen, but no signs of foreign body granulomatous reaction, or infiltration of neutrophilic or lymphocytic inflammatory cells were observed.

Twelve and 26 weeks postoperatively, the implants were localized by measuring the distance from diastema and other anatomic landmarks. The implants were markedly degraded, but globular appearance was still visible (Fig. 5). The implant areas were surrounded by a fibrous capsule with fibroblasts and connective tissue fibers invading into the implants in some spots. No signs of foreign body granulomatous reaction or infiltration of neutrophils or lymphocytes were present in the implant areas. At 52 and 104 weeks, the implants were non-detectable, the fibrous

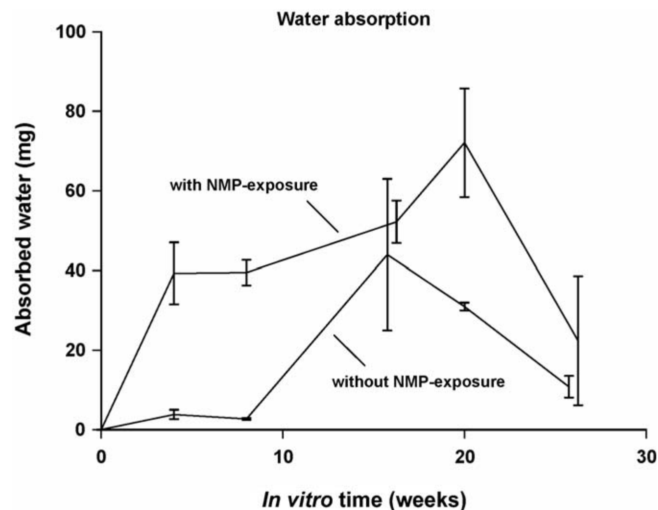


*Fig. 2.* Water absorbed (mean ± SD) by the GTR membranes with and without NMP-exposure in the course of weeks. For the follow-up, the membranes were immersed in phosphate-buffered saline at pH 7.4, 37 °C.

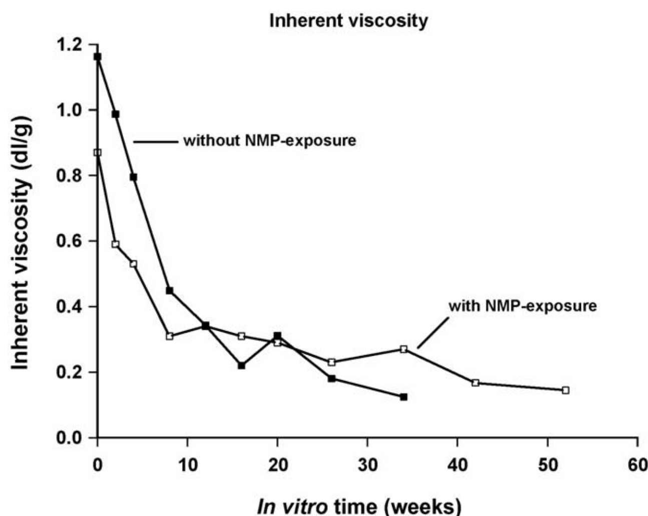


*Fig. 3*. Inherent viscosity of the polymers in the GTR membranes with and without NMP-exposure in the course of weeks. During the follow-up, the membrane was immersed in phosphate-buffered saline at pH 7.4, 37 °C.

network had disappeared, and no signs of inflammation or foreign body reaction were observed.

### *In vitro* experiments

The mass of the NMP-exposed membranes diminished linearly over the 2-year period from 100 to 10% (Fig. 1), while the membrane without the prior NMP-handling maintained all of the mass for the first 16 weeks. The two membrane sets differed from each other at every checkpoint (*t*-test, $P < 0.01$, except weeks 42 and 104 when $P = 0.02$).

Water absorption by the NMP-prehandled membrane increased rapidly to week 20 (Fig. 2). The membranes without NMP absorbed significantly (*t*-test, $P < 0.01$) less water at weeks 4 and 8 than the other group. The values after week 26 are neglected because of problems in measuring technique due to the degradation.

Initially, both sets of membranes showed a quick and rather linear decrease in inherent viscosity; the trend ceased at weeks 8 and 16 for the membranes with and without the prior NMP-handling, respectively (Fig. 3). Thereafter, another linear period of decline with a flatter slope followed until the end of the follow-up.

## Discussion

Clinical healing of the animals was uneventful and the observed histologic responses were mild – encompassing only mild histiocytic reaction – over the entire course of the degradation; this is in agreement with many recent studies with minimal to modest aggregation of histiocytes and foreign body reaction cells such as macrophages or multinucleated giant cells[5,6,14,20]. The formerly reported sterile sinus formation, osteolysis or other remarkable adverse reactions have been caused almost solely by older generation implants with high PGA content, bulky size or high initial degree of crystallinity[1,2]. Initial formation of a fibrous capsule or network around the implant is probably a consequence of the body's attempt to confine the implant.

The GTR membranes in the present study were totally fragmented at 6 months and could not be detected at 12 months. These results fall within the boundaries of published degradation profiles of other GTR membranes. Postoperative clinical healing progressed uneventfully in a study with PGA-PDLA membranes implanted to rhesus monkeys with intrabony periodontal defects[14]. Five months after surgery, the barriers had been completely resorbed with no apparent adverse effect on periodontal wound healing. In another study, the same PGA-PDLA membranes were used in the treatment of Class II buccal furcation defects of dogs[5]. Membrane degradation had started at 1 month postoperatively, but the membranes were still detectable in small fragments at the last follow-up point, 6 months. In addition, naturally occurring buccal dehiscence defects of dogs have been repaired with PDLA membranes dissolved in NMP[6]. At the 6-month termination point, 13 of the 16 defect sites still had some remaining polymer.

NMP increases porosity of the GTR membrane and, hence, water absorption by the membranes (Fig. 2). The increased water content and increased total surface area exposed to water accelerate hydrolysis of the ester linkages, which is clearly seen in Fig. 3, where the inherent viscosity or molecular mass of the NMP-prehandled membrane has already decreased at week 0. NMP is promptly diffused from the membranes to the plasma, which explains why the inherent viscosity of the membranes with the NMP exposure does not fall more rapidly than the other membranes after the initial phase. As the degradation progresses beyond week 20, the fragmented membrane does not bind as much water as at earlier stages; this is evident as a drop in water absorption.

The mass loss profile of the membranes without the NMP exposure agrees well

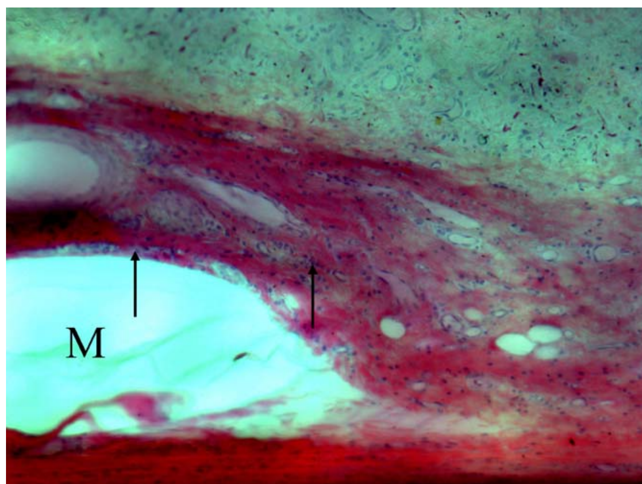


*Fig. 4*. HE staining of a histological section after 6 weeks follow-up. Around the membrane (M), surrounded by a fibrous capsule, mild histiocytic reaction (arrows) is seen. No signs of inflammation are present. Original magnification was ×70. Mandible is right below the lower border of the image.

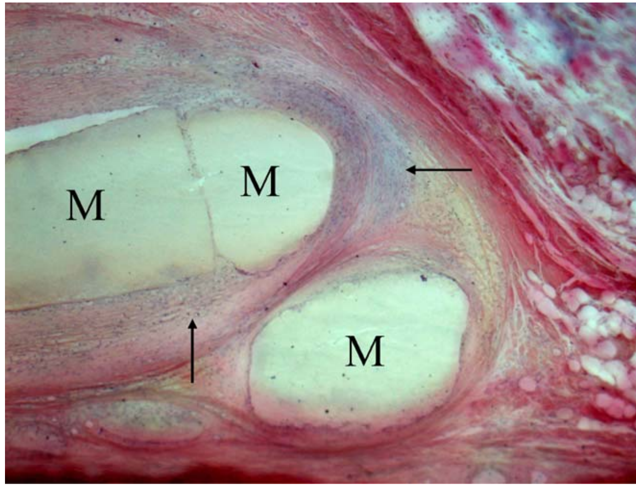


*Fig. 5*. HE staining of a histological section 26 weeks postoperatively. The fragmented membrane (M) is surrounded by a fibrous network and mild histiocytic reaction is seen in areas (arrows). No inflammation is present. Original magnification was ×70. Bone is located below the image.

with the published results on several biodegradable polymers[15]: the mass starts declining only after 16 weeks (Fig. 1). On the other hand, the membranes dipped in NMP lost mass at a strikingly constant rate over the whole period of 2 years. Again, it may be due to enhanced initial hydrolysis caused by NMP. This NMP-induced change in the degradation profile was not manifested in the study where PDLA membranes dissolved in NMP were implanted to the rabbit subcutis[6]; interestingly, those membranes showed remarkably similar inherent viscosity and mass profile with the membranes used in the present study that were not exposed to NMP at all. Since there is a variety of factors contributing to the speed and profile of the degradation, it is difficult to state the cause of this phenomenon without a more exact knowledge of the polymer structure of these PDLA + NMP membranes. Sterilization method is yet another factor affecting molecular mass and degradation times[14]. All the present implants were γ-irradiated according to the standardized routine of the manufacturer and, therefore, sterilization was not a differentiating factor between the *in vivo* and *in vitro* data.

No direct comparison data of remaining mass and inherent viscosity between the *in vivo* and *in vitro* samples were collected in the present study, but the membranes *in vivo* were not detectable at 12 months after the implantation, while the *in vitro* samples still maintained 44 ± 1% of the mass. The finding indicates that the rate of degradation *in vivo* may outweigh the rate *in vitro*. The previous data on the degradation differences between *in vivo* and *in vitro* are somewhat inconsistent: some studies show accelerated *in vivo* degradation[9,19], while other studies fail to show a difference between *in vivo* and *in vitro* series[23]. Accelerated degradation could be explained by the involvement of enzymatic degradation *in vivo*, in addition to the passive hydrolysis and presence of histiocytes. More research is warranted to explore the role of tissue reactions in the biodegradation of polymer implants of different compositions.

The low number of sheep is a limitation of the present study. Nevertheless the current *in vivo* findings form a consistent time series, suggesting that the results are probably not distorted by small sample size.

In conclusion, the studied GTR membrane composed of PLLA, PDLA, TMC and PGA degrades completely *in vivo* in 12 months with only mild histologic responses, being in concert with the results of the other marketed GTR membranes. The degradation *in vitro* seems to be somewhat slower than *in vivo*, suggesting the involvement of enzymatic degradation *in vivo*. NMP accelerates the initial phase of the degradation due to increased hydrolysis.

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