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Comparative study of NMP-preloaded and dip-loaded membranes for guided bone regeneration of rabbit cranial defects

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Abstract

Guided bone regeneration (GBR) has been utilized for several decades for the healing of cranio-maxillofacial bone defects and, particularly in the dental field, by creating space with a barrier membrane to exclude soft tissue and encourage bone growth in the membrane-protected volume. Although the first membranes were non-resorbable, a new generation of GBR membranes aims to biodegrade and provide bioactivity for better overall results. The Inion GTR™ poly(lactide-co-glycolide) (PLGA) membrane is not only resorbable but also bioactive, since it includes *N*-methylpyrrolidone (NMP), which has been shown to promote bone regeneration. In this study, the effects of loading different amounts of NMP onto the membrane through chemical vapour deposition or dipping have been explored. *In vitro* release demonstrated that lower levels of NMP led to lower NMP concentrations and slower release, based on total NMP loaded in the membrane. The dipped membrane released almost all of the NMP within 15 min, leading to a high NMP concentration. For the *in vivo* studies in rabbits, 6 mm calvarial defects were created and left untreated or covered with an ePTFE membrane or PLGA membranes dipped in, or preloaded with, NMP. Evaluation of the bony regeneration revealed that the barrier membranes improved bony healing and that a decrease in NMP content improved the performance. Overall, we have demonstrated the potential of these PLGA membranes with a more favourable NMP release profile and the significance of exploring the effect of NMP on these PLGA membranes with regard to bone ingrowth. Copyright © 2014 John Wiley & Sons, Ltd.

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Keywords guided bone regeneration; PLGA; calvarial defect; NMP; membrane

1. Introduction

The concept of guided bone regeneration (GBR), which was developed into a therapy beginning in the 1980s (Nyman *et al.*, 1982; Dahlin *et al.*, 1988), is based on the formation of space to allow bone ingrowth while excluding soft tissues from entering the created compartment. The slower-growing osteoprogenitor cells can proliferate

without competing with undesired connective tissue. GBR greatly aids the formation of new bone in oral and maxillofacial tissues, which can present complications due to unusual geometries, the necessity for precise application, and oral bacterial flora (Mikos *et al.*, 2006). GBR has contributed to closing bone gaps due to loss from trauma, periodontitis or tumour resection that are non-self-healing and require an intervention. Formerly, the only viable option was utilizing autologous bone, which is effective but requires harvesting bone from another site, a procedure often associated with pain and morbidity of the graft-harboring site (McAllister and Haghighat, 2007; Hughes *et al.*, 2010).

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To apply this therapy, the GBR occlusive pocket is normally achieved by the placement of a membrane to physically seal off the area from non-bony tissue. The first generation of GBR membranes consisted of expanded polytetrafluoroethylene (ePTF), a non-resorbable material (Dahlin *et al.*, 1988; Retzepi and Donos, 2010). This treatment, with the polymer's stability and non-immunogenicity, produced reliable results and has long been held as the 'gold standard' treatment (Hammerle and Jung, 2003; Retzepi and Donos, 2010). However, the risk of disturbing healing with the necessary second surgery to remove the permanent ePTF membrane is a significant drawback. A second generation of resorbable membranes was developed to address this issue. Additionally, degradable membranes can potentially provide better healing, as the material resorption and bone ingrowth occur simultaneously. Synthetic polymers, including polyesters [poly(lactic acid), poly(glycolic acid) and poly(caprolactone) and their co-polymers] (Milella *et al.*, 2001; Geurs *et al.*, 2008; Sculean *et al.*, 2008; Gentile *et al.*, 2011), poly(ethylene glycol) (Wechsler *et al.*, 2008; Ramel *et al.*, 2012) and biologically derived materials such as collagen (Colangelo *et al.*, 1993; Parodi *et al.*, 1998; Wang and Carroll, 2001; Tal *et al.*, 2008; Coic *et al.*, 2010) are the most common GBR membranes.

Although these bioresorbable GBR membranes have shown some success and obviate the need for a second surgery, on their own they generally do not actively accelerate bone regeneration. As a third generation of GBR membranes, researchers have strived to develop biodegradable, occlusive materials that are also bioactive. The Inion GTR™ membrane is composed of biodegradable co-polymers of polylactic acid (PLA), polyglycolic acid (PGA) and trimethylene carbonate (TMC) and is temporarily softened with *N*-methyl pyrrolidone (NMP), enabling easy handling and placement (Pirhonen *et al.*, 2006). Moreover, it was previously discovered in our laboratory that NMP is a substance capable of accelerating bone formation by enhancing the effect of bone morphogenetic protein-2 (BMP-2) on osteoblasts (Miguel *et al.*, 2009) and inhibiting bone resorption by suppressing osteoclast maturation and activity (Ghayor *et al.*, 2011). Consequently, NMP must be considered as not only a plasticizer but also a drug, rendering the NMP-softened PLGA-based membrane the first GBR membrane that is occlusive, bioresorbable and bioactive.

The NMP-treated PLGA membrane obtains its bioactivity through uncomplicated means, without the use of growth factors or other bioactive molecules that must maintain a proper protein structure to be effective. This simplicity can lead to a more reliable GBR membrane. Moreover, NMP is FDA-approved as an excipient in pharmaceutical formulations and is inexpensive and readily available. However, its timed and sustained delivery is difficult due to the small size of the molecule and its amphiphilic character. In order to optimize the bioactivity of NMP, the concentration and release profile of NMP that is optimal for bone regeneration has to be defined. For that purpose, three different concentrations of NMP were

preloaded into PLGA membranes by chemical vapour deposition and compared to membranes dipped into liquid NMP and the gold standard of GBR membrane, the ePTF membrane produced by Gore (Flagstaff, AR, USA), in *in vitro* release studies and a rabbit cranial defect model. This study demonstrates the significance of varying the NMP loading in PLGA membranes on NMP release and *in vivo* bone regeneration.

2. Materials and methods

2.1. Membranes

Inion GTR™ (Inion Oy, Tampere, Finland), named PLGA 50, and GoreTex ePTFE™ (Gore), named ePTFE, were used in these studies.

2.2. NMP loading of Inion membranes

NMP was loaded onto the Inion GTR membrane by chemical vapour deposition to obtain 10%, 15% and 20% NMP in the membrane, henceforth designated as PLGA 10, PLGA 15, and PLGA 20, respectively. Membranes were placed on top of a piece of filter paper over a Petri dish containing NMP in a desiccator. Deposition took place at room temperature and with the aid of a pump connected to the desiccator over a period of 1–2 days. The membranes were weighed before and after deposition to determine the percentage loading. The 50% NMP loading was obtained by dipping the membrane in NMP for 20 s, followed by air-drying for 15 min; these membranes are henceforth designated PLGA 50. The dipping was performed according to the recommendations of the manufacturer.

2.3. NMP *in vitro* release

Rectangular membranes (20 mm × 15 mm) were placed in 50 ml phosphate-buffered saline (PBS), pH 7.4. The samples were held at 37°C and shaken at 100 rpm. 200 µl samples were removed at each time point up to 16 weeks and stored at 4°C for later analysis. NMP concentration was measured at 220 nm, using 96-well plates coated to enable reading at UV wavelengths (Corning, NY, USA); values were compared to a standard curve of known NMP concentrations. The concentration of released NMP was equated to the equivalent membrane area (two 7 mm diameter membranes) and defect volume (100 µl) used in the *in vivo* rabbit experiments.

2.4. Structural analysis

The structural analysis was performed twice for four test specimens derived from the same Inion GTR membrane by cutting it into four pieces: (a) untreated; (b) vapour-loaded with 20% w/w NMP; (c) vapour-loaded

with 20% w/w NMP exposed to 50 ml PBS, pH 7.4, for 1 h; (d) dip-loaded membrane exposed to 50 ml PBS, pH 7.4, for 1 h. Prior to SEM analysis, the samples exposed to PBS had to be dried overnight by lyophilization (Christ Alpha 2–4, Christ, Osterode, Germany). Next, all membranes were fixed on metallic scanning electron microscopy (SEM) holders with fast glue. Prior to SEM analysis, the membranes were sputtered with gold (SCD 030, Baltec, Balzers, Lichtenstein). Parameters for sputtering leading to a gold layer of 10 nm on the samples were as follows: distance of sample to gold target, 60 mm; sputtering time, 90 s; current, 45 mA. The membranes were analysed using a Zeiss Supra V50 SEM (Carl Zeiss, Oberkochen, Germany) at an acceleration voltage of 5 kV.

2.5. Animal model

The ethical approval of the local authorities (Veterinäramt Zurich, Switzerland) was obtained before the start of the experiments. Fifteen New Zealand White rabbits were sedated by ketamin and further anaesthetized by a halothane–N₂O inhalation method. The surgical area was clipped and prepared with iodine for aseptic surgery. A linear incision was made from the nasal bone to the mid-sagittal crest. The soft tissues were reflected and the periosteum was dissected from the site (occipital, frontal and parietal bones). Four 6 mm craniotomy defects were created (two in the parietal and two in the frontal bone) using a 6 mm trephine in a dental handpiece. The surgical area was flushed with saline to remove bone debris. To avoid any dural perforation, the defects marked half-way through the bone by the trephine were finally made by a round burr, creating a 6 mm defect. Since the defect size was exactly 6 mm, membranes on the dural side were chosen to be 7 mm in diameter and fixed by the pressure from the dura. Another rectangular membrane of 10 × 10 mm was placed above the defect. This experimental scenario with membranes on both sides of the cranium was developed by Dahlin *et al.* (1991). To determine the effect of released NMP on an empty defect, each defect in the parietal bone treated with a membrane was paired with a corresponding empty defect in the other parietal bone. The same was done for the frontal bone. The pairs were assigned randomly to the animals. To stabilize their positioning, the two membranes on the top were sutured together and the entire unit sutured to the remaining periosteum. The soft tissues were closed with sutures. After the operation, analgesia was provided by injection of Novalgin (50 mg/kg). Four weeks after operation the rabbits were sacrificed after sedation with barbiturates by an overdose of ketamin, and the calvarial bone was excised.

2.6. Radiography and histology

Specimens were radiographed using a dental radiography unit with ultra-speed dental films (Eastman Kodak Co.,

NY, USA). The radiographs were photographed, scanned and later used to localize the middle section of the defects. After radiography, the samples were first prepared by a sequential water substitution process that involved 48 h in 40% ethanol, 72 h in 70% ethanol (changed every 24 h), 72 h in 96% ethanol and finally 72 h in 100% ethanol. The samples were placed in xylene for 72 h (changed every 24 h) to de-fat the recovered bone. Next, infiltration was performed by placing the samples in methyl methacrylate (MMA) for 72 h (Fluka 64200), followed by 3 days in 100 ml MMA + 2 g di-benzoylperoxide (Fluka 38581, dehydrated in desiccator) at 4°C. Samples were embedded by placing them in 100 ml MMA + 3 g dibenzoylperoxide + 10 ml plastoid N or dibutylphthalate (Merck 800 19.25) and allowing polymerization to occur at 37°C in an airtight water bath. Sections (4.5 µm) were prepared from the middle of the defects and stained with Goldner trichrome. Digital images were taken and processed using an image analysis program (Adobe Photoshop).

2.7. Analysis of data

Digital images of vertical sections from the middles of the defects were taken with a scale (mm) in order to localize the defect margins, which were 6 mm apart. In combination with the calvarial bone thickness of the surrounding bone, the total area of the defect could be reconstructed. Using the Adobe Photoshop Elements program, the total pixel number of the defect area could be determined. The part of the total defect filled by bone was also determined and expressed by pixel number of the bony area. The percentage of bone healing was calculated as: bony regeneration (%) = pixel number of the bone area × 100/total pixel number of defect.

Defect bridging was determined in the middle section to compare the extent of bone coverage across the *x* axis of the defect. First, the areas with bone tissue were projected onto the *x* axis. Next, the stretches of the *x* axis where bone formation had occurred at any level were summed up as described earlier (Kruse *et al.*, 2011). Bone bridging is given as a percentage of the defect width (6 mm) where bone formation has occurred.

2.8. Statistical analysis

Mean values and standard deviations (SDs) were calculated for the percentages of bone regeneration and bone bridging. The significance of differences was evaluated by the Kruskal–Wallis test, followed by the Mann–Whitney test. The significance of differences between membrane-treated and the corresponding untreated defect was determined using the Wilcoxon signed rank test. The limit for significance was set to $p < 0.05$. Statistical analysis was performed by using a statistical software package (IBM SPSS Statistics v. 21 for Windows).

3. Results

3.1. *In vitro* NMP release

PLGA membranes, either preloaded with NMP using chemical vapour deposition or dipped into liquid NMP, were assessed for their *in vitro* release of NMP. As shown in Figure 1A, the method of dipping the PLGA membrane into NMP and then letting it dry, which results in approximately 50% loading, leads to almost immediate loss of all of the NMP; after 4 min 81.7% was released and over 95%

Figure 1. *In vitro* NMP release. (A) Cumulative percentage release of NMP from PLGA 10, PLGA 15, PLGA 20 and PLGA 50 membranes over 7 h. (B) Calculated NMP concentration in the calvarial bone defect at 0–1, 1–4, 4–9 and 9–24 h for PLGA 10, 15, 20 and 50. Fading indicates that the PLGA 50 data point at 0–1 h extends past the y axis. (C) Calculated NMP concentrations in the calvarial bone defect at 0–1 h for PLGA 10, PLGA 15, PLGA 20 and PLGA 50

was released after just 15 min. Moreover, the concentration in the first hour of the equivalent membrane and defect size as used in the *in vivo* experiments reached 130 mM (Figure 1C). In contrast, the preloaded membranes released NMP at a much slower rate. The PLGA 10 and PLGA 15 membranes had similar curves, releasing about 20% after 7 h and 37% after 24 h (Figure 1A). Membranes with 20% NMP released significantly more than the other two preloaded conditions, beginning at 90 min. After 7 and 24 h, PLGA 20 released almost 57% and 73% of the NMP, respectively. In the time intervals 0–1, 1–4, 4–9 and 9–24 h, the PLGA 10 and PLGA 15 never reached an equivalent concentration of > 5 mM (Figure 1B). However, PLGA 20 released NMP to accumulate a concentration of at least 8.7 mM and up to 14.4 mM during these time intervals. The release from the PLGA 20 samples was prolonged over 16 weeks. The NMP release had already started to level out after 1 day and almost reached a plateau by 1 week, with 83% released. Over the course of the 16 weeks, additional NMP was continuously slowly deposited in the supernatant, with a cumulative release of 96.6% after 16 weeks. The amount of loading had a pronounced effect on the release curve and local concentration of NMP.

3.2. Structure of the membranes

The type of loading of the membrane with NMP influences the structure of the membrane. SEM analysis revealed that vapour deposition to 20% w/w NMP (PLGA 20) increases the thickness of the membrane, on average from 236 to 266 μm (Figure 2A, B). Upon exposure to PBS for 1 h and lyophilization, the membrane which underwent NMP loading via vapour deposition (PLGA 20) was on average 249 μm thick, the dip-loaded membrane (PLGA 50) on average 298 μm (Figure 2C, D). This marked increase in thickness of the dip-loaded membranes in comparison to vapour-loaded membranes after exposure to PBS and the release of the majority of NMP is due to an increased porosity of the outer layers of the dip-loaded membranes

Figure 2. SEM images of the Inion GTR membrane at lower and higher magnifications: (A) Inion GTR membrane; (B) PLGA 20; (C) PLGA 20 (vapour deposition) exposed to PBS and lyophilized; (D) PLGA 50 (dip loading) exposed to PBS and lyophilized. White bars = 20 μm (upper panel), 1 μm (lower panel)

(Figure 2C, D, lower panel), which has been described previously (Pirhonen *et al.*, 2006).

3.3. Rabbit *in vivo* studies

During operations on the rabbits it was evident that the ease of handling of PLGA membranes decreased with the amount of NMP. With lower amounts of NMP the membrane was stiffer, interfering with ease of membrane placement. After operation, no adverse reactions to any of the membranes occurred and all the animals remained in good health.

Figure 3 shows examples of some histological sections from the middle of the defect. Although not shown, the PLGA 50 histology appeared similar to that of the other PLGA membranes. More bone ingrowth occurred with the PLGA than the ePTFE membranes. The bone formed during the 4 week repair phase was more of a cancellous type of bone rather than cortical bone. A cellular interaction with the membrane was not observed, nor was bone deposited onto the membrane. A fibrous capsule was detected surrounding all of the membranes (including PLGA 20; not shown to maximize the size of the images included), with its thickness in the range ca. 10–100 μ m (Figure 4). Capsule thickness and NMP content did not appear to correlate either positively or negatively.

Evaluation of the level of bony regeneration of the defect revealed that the use of membranes (GBR) improved bony healing and that a decrease in NMP content improved the performance of the respective membrane (Figure 5). All the defects treated with membranes showed significantly more bone growth than their corresponding untreated controls, except for PLGA 50. Both PLGA 10 and PLGA 15 demonstrated significantly higher bony regeneration, both with an average of approximately

65%, than the ePTFE membrane. The determination of bone repair via pixel number is focused on the area with bony structures and, as is illustrated in Figures 3 and 4, the structure of the newly formed bone was more cancellous and enclosed a lot of area not filled with solid bone. The results in empty defect controls located adjacent to NMP-releasing membranes indicated that NMP spill-over did not occur, or alone without the GBR membrane did not improve bone regeneration.

The defect bridging, which evaluates the bone projected onto the *x* axis, was also significantly increased for defects treated with a membrane compared to untreated defects, excluding PLGA 10 and ePTFE (Figure 6). PLGA 10 facilitated significantly greater bone bridging than the ePTFE membrane, and the other preloaded PLGA membranes indicated a higher level of defect bridging, although not significantly. Similar to the bony regeneration data, the empty defect controls did not show an effect of NMP alone on defect bridging.

4. Discussion

Calvarial models are the most selective models for bone regeneration because the poor blood supply and the membranous (cortical) structure of the bone have an adverse effect on bone healing (Schmitz and Hollinger, 1986). In this project, no attempt was made to produce a critical-sized defect that, by definition, would not heal during the lifetime of an animal and whose healing would require osteoinductive agents, such as bone morphogenetic proteins (BMPs). Therefore, four single 6 mm diameter defects were created in each animal instead of one 15 mm diameter critical-sized defect; the latter would put the focus on the mechanical properties of the membrane needed to

Figure 3. Trichrome-stained histological sections of defects created in rabbit calvarial bone: M, membrane; solid black line, length of defect. Trichrome stains bone green and osteoid red; no encapsulation of the membrane was seen and ingrowth of cells did not occur. (A) PLGA 10; (B) PLGA 15; (C) PLGA 20; (D) ePTFE. [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 4. Interaction between different membranes and bone: (A) PLGA 10; (B) PLGA 15; (C) PLGA 50; (D) ePTFE; M, membrane. Trichrome stains bone green and osteoid red; all membranes were surrounded by fibrous tissue of thickness 10–100 μm ; ingrowth of cells or a direct deposition of bone onto the membranes did not occur; red bar = 200 μm . [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 5. Bony regeneration of the defect was determined based on percentage pixel number: (A) defects treated with membranes; (B) the corresponding untreated defects. Compared to the ePTFE membrane, bone regeneration was significantly increased in defects treated with PLGA 10 or PLGA 15; p values are provided. The difference between membrane treated and corresponding empty defect was significant for all conditions except the PLGA 50 pairs. [Colour figure can be viewed at wileyonlinelibrary.com]

stretch the 15 mm, or would demand the use of bone substitute materials, creating additional variables in this model. The size of the defect (6 mm) was selected to resemble the typical magnitude of an osteotomy gap in maxillofacial surgery and defects in dentistry. This strategy provides the possibility of evaluating the interaction between spontaneous bone regeneration and the influence of different GBR membranes on the healing response, including the influence of release profiles of bioactive substances from the membranes.

The overall results of this rabbit cranial defect study demonstrate that GBR is very effective in promoting the bony healing of such defects. Although ePTFE is non-degradable, this membrane was used as a control because

it is the original gold standard membrane for guided bone regeneration as well as because of its reliability, synthetic nature and purity. The use of a degradable collagen-based membrane as a control would have shifted the focus of our study on NMP release to differences in mechanical properties, and would demand the use of a bone-substitute material. The clinical drawbacks of needing to remove the ePTFE membrane are inconsequential in this study, due to the time frame of the experiment. Compared to the gold standard ePTFE (Gore), all PLGA-based membranes performed at least equally well, if not better, depending on the analysis type. Moreover, in a clinical setting, the non-degradable ePTFE membrane necessitates additional surgery to remove it, while the PLGA membranes will resorb.

Figure 6. Defect bridging as a percentage of the 6 mm defect bridged by bone in: (A) samples treated with membranes; and (B) the corresponding empty defects. Compared to the ePTFE membrane, bone bridging was significantly increased in defects treated with PLGA 10; p value is provided. The difference between membrane treated and corresponding empty defect was significant for PLGA 15, 20 and 50. [Colour figure can be viewed at wileyonlinelibrary.com]

Although the PLGA membranes overall result in greater bone formation, it is evident that the NMP loading on the PLGA membrane influences its performance. PLGA 10 and 15 demonstrated significantly more bony regeneration than ePTFE (Figure 5). Additionally, PLGA 10 achieved significantly better defect bridging (Figure 6). From these results, it appears that lower NMP loading linked with a slower NMP release produces greater bone regeneration. Although the lower NMP loading leads to greater regeneration, it is not entirely possible to determine whether the loading method and the respective NMP release profile or the amount of NMP leads to differences.

As clearly demonstrated with the *in vitro* results (Figure 1), the NMP release profile from the PLGA membranes varied, based on the loaded amount and the loading methodology: dipping or vapour deposition. The vapour-deposited membranes retained NMP to a much greater extent than the dip-loaded membranes. As demonstrated by the long-term data of PLGA 20, a majority of the NMP was released over the course of a week, whereas the dip-loaded membranes lost all NMP within minutes. This result is most likely due to the fact that the majority of NMP remains in the outer layer of the membrane with dipping, since NMP is only allowed 15 min to diffuse into the PLGA. Vapour deposition, however, allows the NMP time to diffuse throughout the membrane, during the 1–2 day loading period. PLGA 20, which had the highest amount of NMP loaded by chemical vapour deposition, released a significantly higher percentage of NMP than PLGA 10 and 15, as well as having a higher NMP concentration at every interval, although not always significantly. The fast-burst release of NMP from the dipped PLGA membrane, PLGA 50, led to an extremely high concentration of 130 mM in the first hour, which is potentially toxic to cells. Our previous experience has indicated that C2C12 cells are not viable with NMP concentrations > 10 mM (data not shown). The vapour-deposited membranes, PLGA 10, PLGA 15 and PLGA 20, all maintained NMP concentrations < 10 mM at all calculated time intervals except 1–4 h for PLGA 20. Although the *in vivo* situation is greatly

complicated, these data illustrate the possibility of damagingly high NMP levels with PLGA 50. Despite the fact that NMP is safe and approved in humans by the FDA as an excipient, it can be locally toxic at high levels (Davies *et al.*, 2013; Pelaez-Vargas *et al.*, 2013). Moreover, a longer release period, as in the case with the vapour-deposited membranes and particularly PLGA 10 and 15, may promote NMP's BMP-enhancing effects over an extended time period, possibly inducing greater bone growth. It is possible that both of these factors contributed to the improvement with lower NMP loading. The mechanical properties of the NMP membrane also changed with the loading and release of NMP, which could contribute to differences in bone regeneration. However, the 6 mm cranial defect model was not sensitive to differences in the mechanical properties so long as they were sufficiently stiff that a properly fixed membrane covered the defect without collapsing. Therefore, we believe that differences in bone regeneration were mainly due to differences in the release kinetics of NMP, which was shown in previous work to have bone-enhancing effects (Miguel *et al.*, 2009).

Although the membranes with the lowest amounts of NMP performed best, there are practical considerations that are important in a clinical setting that could favour other membrane formulations. From the handling characteristics, only the preloaded PLGA 20 was suitable for GBR in dentistry; when the NMP content is $< 20\%$ the membrane is too stiff for this application. As mentioned previously, reducing the NMP content below 20% led to better results. In order to develop a second generation of PLGA membrane, we have to balance the NMP effect as a plasticizer and the pharmaceutical effect of NMP, which is lethal to cells at higher concentrations.

Another aspect that should not be overlooked is that with the use of a preloaded membrane we lose the capability of tissue integration (Figure 4). As previously demonstrated, the PLGA membranes that are dipped into NMP integrate with the tissue, with extensive ingrowth of cells and osteoid and new bone formation adjacent to the membrane (Pirhonen *et al.*, 2006). It is suspected that the surface roughness plays a crucial role in tissue integration

(Figure 2). Before being placed in NMP, the polymer surface is dense, with a smooth surface, but upon dipping in NMP it obtains a three-part structure with a dense inner layer and outer rough porous layers (Pirhonen *et al.*, 2006). It was previously shown that this smooth PLA/PGA/TMC film does not allow tissue integration into the membrane, while the roughened surface does promote incorporation. Simion *et al.* (1999) showed that titanium-coated ePTFE membranes with high surface roughness integrated into the host tissue to a much greater extent in a GBR model. Additionally, other studies have described the positive relationship between surface roughness and osseointegration (Hayes and Richards, 2010; Choong *et al.*, 2012; Davies *et al.*, 2013; Pelaez-Vargas *et al.*, 2013). This topography mimics the natural bone remodelling situation, where active osteoclasts create carved-out pits and lacunae. Therefore, the membrane surface needs to be roughened prior to loading with NMP. If the roughening leads to an increased flexibility of the membrane, the NMP content could also be reduced and optimized for its pharmaceutical action. Moreover, since the PLGA membranes are resorbable, long-term cell integration of the membrane may not be important for the clinical outcome.

Another cellular response that may not have long-term consequences is the formation of a fibrous capsule, which was found with all of the membranes (Figure 4). Since the PLGA membranes degrade, the fibrous capsule may break down once the material is no longer present, imparting no extended negative effects.

5. Conclusion

PLGA membranes loaded with various amounts of NMP all demonstrated bone regeneration that was equal to, or better than, the gold standard ePTFE membrane in a rabbit calvarial defect model. By utilizing vapour deposition for loading, we developed a slow-release system of NMP from PLGA. Differences between the PLGA membranes with different NMP loadings were detected in *in vivo* bone regeneration as well as *in vitro* NMP release profiles. The release curve of NMP may influence its performance. These results indicate the importance of analysing and optimizing NMP release. Overall, we have demonstrated the potential of these PLGA membranes and the significance of exploring the effect of NMP on these PLGA membranes with regard to bone ingrowth.

Conflict of interest

The authors have declared that there is no conflict of interest.

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References

- Choong C, Yuan S, Thian ES *et al.* 2012; Optimization of poly(ϵ -caprolactone) surface properties for apatite formation and improved osteogenic stimulation. *J Biomed Mater Res A* **100A**(2): 353–361.
- Coic M, Placet V, Jacquet E *et al.* 2010; Mechanical properties of collagen membranes used in guided bone regeneration: a comparative study of three models. *Rev Stomatol Chirurg Maxillofac* **111**(5–6): 286–290.
- Colangelo P, Piattelli A, Barrucci S *et al.* 1993; Bone regeneration guided by resorbable collagen membranes in rabbits: a pilot study. *Implant Dent* **2**(2): 101–105.
- Dahlin C, Alberius P, Linde A. 1991; Osteopromotion for cranioplasty. An experimental study in rats using a membrane technique. *J Neurosurg* **74**(3): 487–491.
- Dahlin C, Linde A, Gottlow J *et al.* 1988; Healing of bone defects by guided tissue regeneration. *Plastic Reconstruct Surg* **81**(5): 672–676.
- Davies JE, Ajami E, Moineddin R *et al.* 2013; The roles of different scale ranges of surface implant topography on the stability of the bone-implant interface. *Biomaterials* **34**(14): 3535–3546.
- Gentile P, Chiono V, Tonda-Turo C *et al.* 2011; Polymeric membranes for guided bone regeneration. *Biotechnol J* **6**(10): 1187–1197.
- Geurs NC, Korostoff JM, Vassilopoulos PJ *et al.* 2008; Clinical and histologic assessment of lateral alveolar ridge augmentation using a synthetic long-term bioabsorbable membrane and an allograft. *J Periodontol* **79**(7): 1133–1140.
- Ghayor C, Corroero RM, Lange K *et al.* 2011; Inhibition of osteoclast differentiation and bone resorption by *N*-methylpyrrolidone. *J Biol Chem* **286**(27): 24458–24466.
- Hammerle CHF, Jung RE. 2003; Bone augmentation by means of barrier membranes. *Periodontol* **2000** **33**: 36–53.
- Hayes JS, Richards RG. 2010; Surfaces to control tissue adhesion for osteosynthesis with metal implants: *in vitro* and *in vivo* studies to bring solutions to the patient. *Expert Rev Med Dev* **7**(1): 131–142.
- Hughes FJ, Ghuman M, Talal A. 2010; Periodontal regeneration: a challenge for the tissue engineer? *Proc Inst Mech Eng H J Eng Med* **224**(H12): 1345–1358.
- Kruse A, Jung RE, Nicholls F *et al.* 2011; Bone regeneration in the presence of a synthetic hydroxyapatite/silica oxide-based and a xenogenic hydroxyapatite-based bone substitute material. *Clin Oral Implants Res* **22**(5): 506–511.
- McAllister BS, Haghighat K. 2007; Bone augmentation techniques. *J Periodontol* **78**(3): 377–396.
- Miguel BS, Ghayor C, Ehrbar M *et al.* 2009; *N*-Methyl pyrrolidone as a potent bone morphogenetic protein enhancer for bone tissue regeneration. *Tissue Eng A* **15**(10): 2955–2963.
- Mikos AG, Herring SW, Ochareon P *et al.* 2006; Engineering complex tissues. *Tissue Eng* **12**(12): 3307–3339.
- Milella E, Barra G, Ramires PA *et al.* 2001; Poly(L-lactide)acid/alginate composite membranes for guided tissue regeneration. *J Biomed Mater Res* **57**(2): 248–257.
- Nyman S, Lindhe J, Karring T *et al.* 1982; New attachment following surgical treatment of human periodontal disease. *J Clin Periodontol* **9**(4): 290–296.
- Parodi R, Carusi G, Santarelli G *et al.* 1998; Implant placement in large edentulous ridges expanded by GBR using a bioresorbable collagen membrane. *Int J Periodont Restor Dent* **18**(3): 266–275.
- Pelaez-Vargas A, Gallego-Perez D, Carvalho A *et al.* 2013; Effects of density of anisotropic microstamped silica thin films on guided bone tissue regeneration – *in vitro* study. *J Biomed Mater Res B Appl Biomater* **101**(5): 762–769.
- Pirhonen EM, Pohjonen TH, Weber FE. 2006; Novel membrane for guided bone regeneration. *Int J Artif Organs* **29**(9): 834–840.
- Ramel CF, Wismeijer DA, Hammerle CHF *et al.* 2012; A randomized, controlled clinical evaluation of a synthetic gel membrane for guided bone regeneration around dental implants: clinical and

- radiologic 1- and 3-year results. *Int J Oral Maxillofac Implants* **27**(2): 435–441.
- Retzepi M, Donos N. 2010; Guided bone regeneration: biological principles and therapeutic applications. *Clin Oral Implants Res* **21**(6): 567–576.
- Schmitz JP, Hollinger JO. 1986; The critical size defect as an experimental model for craniomandibulofacial nonunions. *Clin Orthop Relat Res* **205**: 299–308.
- Sculean A, Nikolidakis D, Schwarz F. 2008; Regeneration of periodontal tissues: combinations of barrier membranes and grafting materials – biological foundation and preclinical evidence: a systematic review. *J Clin Periodontol* **35**: 106–116.
- Simion M, Dahlin C, Blair K *et al.* 1999; Effect of different microstructures of e-PTFE membranes on bone regeneration and soft tissue response: a histologic study in canine mandible. *Clin Oral Implants Res* **10**(2): 73–84.
- Tal H, Kozlovsky A, Artzi Z *et al.* 2008; Cross-linked and non-cross-linked collagen barrier membranes disintegrate following surgical exposure to the oral environment: a histological study in the cat. *Clin Oral Implants Res* **19**(8): 760–766.
- Wang HL, Carroll WJ. 2001; Guided bone regeneration using bone grafts and collagen membranes. *Quintessence Int* **32**(7): 504–515.
- Wechsler S, Fehr D, Molenberg A *et al.* 2008; A novel, tissue occlusive poly(ethylene glycol) hydrogel material. *J Biomed Mater Res A* **85A**(2): 285–292.