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Masquelet Technique: the effect of altering implant material and topography on membrane matrix composition, mechanical and barrier properties in a rat defect model

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Abstract

The Masquelet technique is a surgical procedure to regenerate segmental bone defects. The two-phase treatment relies on the production of a vascularized foreign-body membrane to support bone grafts over three times larger than the traditional maximum. Historically, the procedure has always utilized a bone cement spacer to evoke membrane production. However, membrane formation can easily be effected by implant surface properties such as material and topology. This study sought to determine if the membrane's mechanical or barrier properties are affected by changing the spacer material to titanium or roughening the surface finish. Ten-week-old, male Sprague Dawley rats were given an externally stabilized, 6mm femur defect which was filled with a pre-made spacer of bone cement (PMMA) or titanium (TI) with a smooth (~1µm) or roughened (~8µm) finish. After 4 weeks of implantation, the membranes were harvested, and the matrix composition, tensile mechanics, shrinkage, and barrier function was assessed. Roughening the spacers resulted in significantly more compliant membranes. TI spacers created membranes that inhibited solute transport more. There were no differences between groups in collagen or elastin distribution. This suggests that different membrane characteristics can be created by altering the spacer surface properties. Surgeons may unknowingly effecting membrane formation via bone cement preparation techniques.

Keywords

Masquelet Technique; Induced Membrane; Animal Model; Membrane Mechanics; Membrane Barrier Properties

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Conflict of interest statement

One author, Dr. Watson, receives intellectual property royalties from Smith and Nephew, Zimmer Biomet, and AOS, and he serves as a consultant for Nuvasive. None of these present a direct conflict of interest with the presented research. None of the other authors have any conflicts of interest.

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Introduction

The Masquelet or Membrane Directed Bone Formation technique (MDBF) is a newer two-step procedure to address segmental bone defect reconstruction. (Aurégan and Bégué, 2014; Chadayammuri et al., 2015; Giannoudis et al., 2011; Masquelet and Begue, 2010; Taylor et al., 2012) The procedure has shown promise in addressing a wider clinical need while also providing a less arduous treatment regime than distraction osteogenesis. (Aurégan and Bégué, 2014; Giannoudis et al., 2011; Gouron, 2016; Taylor et al., 2012) During the first phase, a bone cement (polymethyl methacrylate, PMMA) spacer is implanted where bone regeneration is desired. Over the following weeks to months a foreign-body or 'induced' membrane encapsulates the spacer. (Aho et al., 2013; Bosemark et al., 2015; Christou et al., 2014; Cuthbert et al., 2013; Fischer et al., 2016; Gouron et al., 2014; Gruber et al., 2016, 2013, 2012; Henrich et al., 2013; Klaue et al., 2009; Liu et al., 2013; Luangphakdy et al., 2017; Nau et al., 2016; Shah et al., 2017; Viateau et al., 2006; Wang et al., 2015) Then a second surgery is performed to remove the spacer leaving the membrane in place. The membrane compartment is filled with morselized bone graft material which mineralizes over the following months independent of defect size. (Karger et al., 2012; Masquelet and Begue, 2010)

There are three main theories for the MDBF technique's success: (i) the membrane's pre-established vascular network helps revascularize the graft quickly preventing necrosis, (ii) the membrane secretes factors to modulate cell behavior and promote regeneration, (iii) the membrane serves a barrier to prevent soft tissue invasion and resorption. (Giannoudis et al., 2011; Taylor et al., 2012) However, none of these hypotheses have been tested nor have the effects of procedural alterations been thoroughly assessed.

Decades of previous implant research has shown that implant surface properties impact foreign-body membrane development. (Franz et al., 2011; Kenneth Ward, 2008; Richards, 2007) Different spacer materials and topologies adsorb different proteins thus changing the original matrix formed around the spacer and thus the cells it attracts. (Richards, 2007) All objects implanted in the body that cannot be degraded will eventually be enveloped in a foreign-body membrane to effectively wall it off from the body. (Franz et al., 2011; Kenneth Ward, 2008)

Titanium (Ti) implants have become the most favorable biomaterial used for orthopaedic implants because Ti induces a relatively thin membrane and promotes osteogenic factor expression and enhances osseointegration. (AOTrauma, 2013; Geetha et al., 2009; Goriainov et al., 2014; Nuss and von Rechenberg, 2008) Plastics, like the PMMA used for the MDBF technique, have been shown to produce thicker membranes when implanted into bone. (Nuss and von Rechenberg, 2008) Perhaps equally important as spacer material in influencing foreign body membrane formation is spacer topography. (Franz et al., 2011; Goriainov et al., 2014; Nuss and von Rechenberg, 2008; Richards, 2007) Roughened implants have been shown to provide more traction, decreasing tissue motion and resulting in the formation of a thinner membrane. (Nuss and von Rechenberg, 2008) Thin membranes are advantageous in the context of orthopaedic implants because they allow better implant integration into surrounding bony tissue, preventing surrounding bone necrosis, resorption, or fracture.

(Geetha et al., 2009) However, in the context of MDBF, membrane properties that may be advantageous to bone regeneration and healing have not been identified. It is possible that altered spacer surface properties could positively or negatively affect membrane formation and ultimate healing outcomes.

Controlling for variables such as implant material and topography may be important, as these factors both affect the initial protein matrix formed around the implant, which in turn affects cellular adhesion and matrix formation.(Nuss and von Rechenberg, 2008; Richards, 2007) The matrix composition affects tissue mechanics which could in turn mediate cellular behavior on both the cell and tissue length-scales.(Green et al., 2014) At the cell length-scale, the cellular matrix's elastic properties impact stem cell lineage differentiation and phenotypic expression.(Discher et al., 2005; Engler et al., 2006; Sharma and Snedeker, 2010; Shin et al., 2013) At the tissue length-scale, exogenous mechanical forces have been shown to modulate cellular behavior.(Califano and Reinhart-King, 2010; Chan et al., 2010) This is especially true for cells related to chondro- and osteogenic processes.(Bonewald and Johnson, 2008; Chan et al., 2010; Galli et al., 2010; Glatt et al., 2016; McBride et al., 2008; McBride and Silva, 2012) Changes in membrane compliance could alter how whole bone forces are transduced to individual residing cells and indirectly modulate bone repair.

The induced membrane is also theorized to affect the cells within the defect by providing a barrier between the graft and surrounding soft tissues.(Dimitriou et al., 2012; Giannoudis et al., 2011; Taylor et al., 2012) Thus, by altering the implant material and surface topography, diffusion could be varied. Thinner or less collagenous membranes may decrease diffusion time and allow movement of more/larger particles. This would impact both factor influx to the graft from surrounding soft tissues as well as factor efflux from the tissue compartment. Thus, local concentrations of positive and negative biochemical regulators could differ during the second treatment phase based on the membrane environment established during the first treatment phase.

Based on the understanding that implant material and topography can alter membrane morphology, we hypothesized that altering spacer material and topography will alter the matrix composition of the membrane in the MDBF milieu. In turn, the altered matrix composition will likely impact the mechanical properties of the induced membrane, including tensile and shrinkage properties, as well as barrier properties. Since it has been shown that TI and roughened implants produce thinner membranes, we expect these membranes to be inferior in matrix composition, mechanical properties, and barrier function.

Materials & Methods

Animal Model

10-week-old, male Sprague Dawley rats (Charles River, Wilmington, MA) were used for all experiments. Our Institutional Animal Care and Use Committee approved all procedures (protocol #2451). Euthanasia via carbon dioxide asphyxiation was performed following American Veterinary Medical Association 2013 guidelines (20–30% gradual replacement).

Surgical Procedure

Phase one of the Masquelet Technique (implantation of external fixator and spacer) was performed on all animals (Figure 1, N=120 for all studies). After installing an external fixation device in the right femur, a 6mm long defect was created at approximately the bone mid-shaft. Animals were then randomly assigned to one of 4 spacer groups (PMMA Smooth, PMMA Rough, TI Smooth, or TI Rough) (For spacer fabrication and surgical details see supplemental section). Smooth spacers had surface roughness of approximately 1 μ m while rough spacers had an estimated 8 μ m surface roughness. These values were chosen based on previous studies (Goriainov et al., 2014) and the relative size of osteoblasts and macrophages (10–20 μ m)(Krombach et al., 1997). If the larger texture is too big, the cells perceive it as a flat surface, and thus may not behavior differently.

Immunohistochemistry (IHC)

In order to better understand the matrix protein composition, semi-quantitative IHC assays for collagen Type 1 and elastin (n = 7–11/group, N=36) were performed (details in supplemental section). Four weeks after implantation, the operated limb was harvested, fixed, and processed for cryosectioning. Two serial sections per animal were processed for IHC for collagen type 1 (ab34710, Abcam, Cambridge, MA) or elastin (ab21610, Abcam). A third section served as a no primary antibody negative control. A fourth section was stained with picrosirius red and alcian blue and imaged under polarized light to assist in distinction of each membrane layer.

IHC sections were imaged under fluorescent light (Leica DMI4000B, Leica Microsystems, Buffalo Grove, IL). ImageJ (NIH, Bethesda, MD) was used to segment the non-birefringent and birefringent layers, and a MATLAB code was used to find the average green fluorescent intensity in each region. Then, the average green fluorescent intensity of the corresponding region in each animal's negative control was subtracted from the experimental sections' values to control for tissue auto-fluorescence and non-specific binding.

Tensile Testing

Four weeks post-operatively, membranes were harvested for mechanical tensile testing (n=7–9/group, N=34, details in supplemental section). Briefly, as much overlying muscle as possible was removed and the membrane was incised longitudinally to create a flat sheet approximately 6mm tall (axial direction) and 10mm wide (circumferential direction) (Figure 2A–B). Each membrane was split into two pieces (Figure 2B). One piece was stretched in the axial direction while the other was stretched in the circumferential direction (1mm/min, MTS Criterion 42 with 100lb load cell, 10Hz, Figure 2C). Each sample's initial dimensions in the testing grips were measured by foil gauge (thickness) or calibrated images (minimum width and gauge length).

After converting the measured force and displacement to stress and strain, MATLAB was used to create a smoothed stress-strain curves for each sample. The smoothed curves were used to identify the points of yield, ultimate force, and failure. The stress and strain at each point as well as elastic modulus and toughness were calculated for each sample.

Shrinkage Studies

Shrinkage studies were carried out to assess induced membrane pre-stress *in situ* following spacer removal in a separate animal cohort (n=6/group, N=24). Membrane harvesting was carried in the exact same manner as for tensile testing (Figure 2A–B). However, after incising it in the axial direction along the lateral surface to open the membrane tube, it was immediately submerged and flattened in 100 centistoke silicon oil (Sigma Aldrich, St Louis, MO, USA). Silicon oil minimizes friction while reducing tissue swelling, allowing for the free shrinkage of tissues (Figure 2D) and has been used in similar studies using ovine periosteum.(McBride et al., 2011) The membranes were then imaged at a 5X magnification (Leica DMI4000B), and the images measured to determine the percent change in area, axial length, and circumferential length.

Barrier Testing

In a separate cohort of animals, the barrier properties for each group were tested using different-sized, fluorescently tagged dextrans (n=6–8/group, N=26) (Figure 3, details in supplemental section). Briefly, immediately after euthanasia the spacer was carefully removed and the tissue compartment sealed around a small catheter. Then the compartment was filled with a dextran solution containing 10kDa dextrans conjugated to Cascade Blue, 70kDa dextrans conjugated to Texas Red, 500 kDa dextrans conjugated to fluorescein (all dextrans – 100uL at 2ug/uL, Thermo Fisher, Waltham, MA), and 200 μ L of PBS (Total Volume = 500uL). The solution was delivered via a 1.0mL syringe fitted with a 25-gauge needle (BD Biosciences) which was placed in the tubing's open end and sealed with gel cyanoacrylate. The syringe was then placed vertically in a tube stand. A 200g weight was placed on top of the syringe plunger to inject the dextran solution into the membrane compartment at a constant injection pressure. After all the solution was injected and allowing 5 extra minutes for diffusion, the limbs were harvested and processed for cryosectioning. The directly mounted samples were imaged under fluorescent light (Figure 3D). Similar to IHC the amount of each dextran in each region was semi-quantified by the average fluorescent intensity. For this experiment four regions were considered: non-birefringent, birefringent, secondary fibrous tissue (when present n=3–6/group), and a small area of adjacent muscle. Since there was no way to control for background (i.e. tissue auto fluorescence) within each animal, an additional 3 control animals were included in this study. They were implanted with PMMA smooth spacers and underwent identical procedures and tissue processing except the 500uL of injected PBS solution lacked dextrans.

Statistics

All data is displayed as mean and standard deviation. All outcomes were compared with multivariate ANOVA (factors: Material – PMMA vs. TI, Finish – Smooth vs. Rough) with additional factors or repeated measures as appropriate. Fisher's Least Square Difference was used for post-hoc analysis when ANOVA showed significant differences (StatView v. 5.0, SAS Institute, Cary, NC).

Results

IHC

To determine if there were any matrix composition differences between the groups that could affect tissue mechanics, the relative amounts of collagen Type I and elastin, two matrix proteins, were compared semi-quantitatively using IHC for the two regions directly apposed to the spacer, the inner, non-birefringent layer and the outer, birefringent layer, which appeared in all samples (Figure 4). Type I collagen levels were not significantly different between the two regions. In contrast, elastin levels were significantly higher in the non-birefringent layer ($p=0.0001$). On average, there was 34% more elastin in the non-birefringent layer. There were no differences between the four spacer groups.

Tensile Testing & Shrinkage

To determine if there were any differences in the membrane material properties, tensile testing and shrinkage measurements in both the axial and circumferential directions were performed. In general, all membranes were extremely ductile. They were able to be stretched to more than three times their original length (Figures 5–7). Also, they did not fail instantaneously but instead sheared apart until they no longer bore load (Figures 2C, 5–7). While these general characteristics were observed of all samples, specific differences were measured between groups. Membrane mechanical properties were altered by spacer topography. Roughened spacers produced membranes capable of over 40% higher tensile strains at yield in both test directions ($p=0.01$) (Figures 5–6). Tensile failure point strain was also significantly affected by spacer finish ($p=0.037$). However, there was a significant interaction between spacer finish and testing direction for this outcome ($p=0.018$). Tensile failure strain was on average 25% higher in the axial direction for both roughened groups but was similar between groups in the circumferential direction (Figures 5–7). Smooth spacer induced membranes had, on average, a 58% higher tensile modulus of elasticity than membranes induced by roughened spacers in both test directions ($p=0.01$) (Figure 7). No differences in were found between groups or testing direction for stress at any point or overall tensile toughness (Figure 7).

Shrinkage, although modest in all groups (*i.e.* <10% of original area or length), was also impacted by spacer topography but not material (Figure 8). Membranes evoked by roughened spacers shrank more in the circumferential direction than did ones evoked by smooth spacers ($p=0.01$) (Figure 8B). No differences between groups were found in the axial direction (Figure 8A). Area significantly decreased over time ($p<0.001$) and trended to be greater in the roughened groups, but this did not reach significance ($p=0.057$).

Barrier Testing

To determine if barrier properties were affected, fluorescently labeled dextrans of differing sizes (10kDa, 70kDa, and 500kDa) were injected into the membrane compartment after spacer removal.

Control samples lacking dextrans showed very low levels of signal in all channels (*i.e.* less than 7 intensity units, Figure 9). For the 10kDa dextrans, fluorescence intensity was

significantly higher in PMMA induced membranes compared to TI ($p=0.0009$). No differences in fluorescence intensity were seen between smooth and rough spacers. Fluorescence intensity was similar in the non-birefringent to birefringent layers. Fluorescent intensity dropped moving from the birefringent layer to the second fibrous tissue ($p=0.0245$). Finally, no difference was seen between the second fibrous and muscle tissue layers. However, signal for this dextran was relatively low (max: 35) for all groups and regions.

For the 70kDa and 500kDa dextrans fluorescence intensity levels were again significantly higher in the PMMA induced membranes versus TI ($p<0.0001$ for both sizes). Spacer finish had no effect. In comparing fluorescence intensity location, the signal was significantly higher in the non-birefringent layer than in the birefringent layer ($p=0.0005$ & 0.0066 , 70kDa and 500kDa, respectively). For both sizes, there was a drop in signal moving from the non-birefringent to secondary fibrous tissue area. However, it failed to reach significance for either dextran ($p=0.0529$ & $p=0.0994$, 70kDa and 500kDa, respectively). Similar to the 10kDa dextran, there was no difference in fluorescence intensity between the second fibrous and muscle tissue layer for either larger size. Although, there was a significant drop between the non-birefringent layer and muscle ($p=0.047$ both dextrans).

Discussion

Few differences were seen in the matrix composition, tensile or shrinkage properties of membranes induced by spacers of different materials. This was contrary to the original hypothesis that predicted the membranes induced by TI would have different material properties than those induced around PMMA spacers. These membranes did exhibit different barrier properties. Roughening had more of an effect on mechanical properties creating membranes that were more compliant.

Research regarding the mechanical and shrinkage properties of induced membranes has not been previously performed. Therefore, it is difficult to put our findings into context. Although, the induced membrane is often compared to periosteum due to similarities in structure and theorized function. This work suggests that the induced membrane is, in fact, distinct from the periosteum. The periosteum is an anisotropic, non-linear material. (Bertram et al., 1998; McBride et al., 2011; Popowics et al., 2002; Uchiyama et al., 1998) When stressed in the axial direction, initially the periosteum is relatively compliant. At some transition strain, it becomes much stiffer. (Bertram et al., 1998; McBride et al., 2011; Popowics et al., 2002; Uchiyama et al., 1998) When stressed in the circumferential direction, the compliance remains linear at a value somewhere between compliance's of the two axial regions. (McBride et al., 2011) This was in contrast to our results showing the induced membrane to be isotropic under tensile stress.

Shrinkage of the periosteum also differed from the induced membrane. The periosteum shrinks over 40% in total area and axial length and 10–20% in circumferential length when removed from the bone surface. (McBride et al., 2011) The induced membrane did not shrink by more than 10% in area or either direction. This implies that the periosteum may be imparting different mechanical signals to residing cells than the induced membrane.

This study's findings are significant because they have implications for optimization of the MDBF Technique in bone healing and regeneration. Specifically, roughened spacers produced membranes with significantly higher yield strains, which could indicate they are more likely to be deformed in situations such as graft overfilling or normal gait. This in turn could modulate the behavior of the cells residing in the membrane and affect bone regeneration. Even without differential exogenous mechanical signals, the cells may behave differently based on their sensing of the matrix environment (Discher et al., 2005; Engler et al., 2006). Further, the increased strain at yield and failure suggests they are more likely to maintain their integrity under high strains and better protect the graft environment.

Another important finding is that the membrane does act as a barrier to particles close in size to physiologically relevant proteins and cells. It is widely thought that graft failure is mediated by cellular immunity and activation of the complement cascade (Burchardt, 1983; Hausmann et al., 1973). This is why importance has been placed on the possible barrier function of the membrane. Reduced mobility of cells and proteins across the membrane could enhance graft incorporation and healing by excluding negative mediators originating from surrounding tissues and retaining positive regulators. On the other hand, healing could be hindered if influx of positive regulators and efflux of negative regulators is slowed. Alternatively, factors that are typically positive to the healing process could be concentrated to hyper-physiological levels that are actually detrimental. For example, Dr. Masquelet conducted a small prospective human trial where BMP7, a well-known osteogenic factor, was added to the bone grafts with the intent of accelerating graft incorporation. Unfortunately it was found that adding BMP-7 did not increase bone healing rates and caused unexpected complications. (Masquelet and Begue, 2010) If the membrane's barrier function is critical, then TI-evoked membranes may be superior to PMMA-evoked ones. TI-induced membranes significantly inhibited dextran transport. Many of the signaling molecules important to the bone repair cascade are within the sizes investigated. For example, BMP-2 functions as a dimer of 30 kDa (Wozney et al., 1988) and VEGF is 19 kDa (Leung et al., 1989); cells are typically orders of magnitude larger.

It should be noted that the surface topographies investigated here may or may not reflect what is achieved clinically. It is well known that preparation methods like vacuum vs hand-mixing greatly affect PMMA's material and surface properties as well as antibiotic elution (Bistolfi et al., 2011; Lee, n.d.; Macaulay et al., 2002; Saha and Pal, 1984; Vaishya et al., 2013; Wang ' et al., 1993; Wixson et al., 1987). In the Masquelet literature, PMMA/antibiotic mixing techniques are variably reported. Many do not report which method was used (Accadbled et al., 2013; Aurégan and Bégué, 2014; Azi et al., 2016). Of those that do, vacuum mixing is the primary technique specified (Chadayammuri et al., 2015; Stafford et al., 2010). Additionally, spacer implantation methods vary. For example, some papers report packing malleable PMMA by hand into the defect, (Aurégan and Bégué, 2014; Biau et al., 2009; Wang et al., 2016) while others used plastic tubing or other molds to prefabricate the spacer before definitive placement (Chadayammuri et al., 2015; Gouron, 2016; Micev et al., 2015; Stafford et al., 2010). Further, even when identical preparation methods are used, bone cement surface roughness can vary significantly between manufacturers or product lines. One study that used different brands of bone cement that was hand-mixed formed in identical, smooth molds resulted in surface roughness ranging from 0.16 to 0.49 μm (van de

Belt et al., 2000). The authors felt this was due to the particle sizes of the pre-reacted PMMA and other additives. The differing techniques for PMMA mixing and canal placement used clinically may result in a very wide range of surface topographies. However, we feel our selected microroughness levels are within this range. More importantly, the roughnesses chosen for this study (1 and 8 μm) were specifically chosen to be within what was known to affect cellular behavior in orthopaedic scenarios (Goriainov et al., 2014) and what is likely to be detectable by important immune and osteogenic cells. Macrophages and osteoblasts are approximately 10–20 μm in diameter (Krombach et al., 1997). So surface topographies larger than this range will be perceived as ‘flat’ by the cells. Although, it may be worth defining the full surface roughness range used clinically and investigating multiple topography levels in future studies.

As with any experimental study, there are limitations in the ability to mimic the *in vivo* process. The first of these limitations is the animal age. Ten-week old rats were used, which are approximately equivalent to 18 year old humans.(Flurkey et al., 2007) While younger individuals have a greater healing capacity than older, (Boskey and Coleman, 2010; Brodt and Silva, 2010; Leucht et al., 2013; Silva et al., 2012) the MDBF Technique is not only used in adult populations; it is now the gold standard for treatment in pediatric patients with primary bone malignancy.(Gouron, 2016) In these situations, our studies may serve as an adequate model. A second limitation was that some muscle radially outward from the membrane was left on the sample and was subsequently included in mechanical testing. This was allowed in order to ensure minimal tissue disruption. However, inclusion may have better approximated the *in vivo* situation, as the membrane and surrounding muscle do not exist as separate entities but as a continuous tissue. A third limitation is that the membrane strains measured here are probably well beyond what is experienced *in vivo*. Bone strains that typically cause an anabolic response are on the order of 1200 to 2000 microstrain (0.0012 to 0.0020 strain)(McBride and Silva, 2012), which is a very small part of the range measured here (0 to 1.7500+ strain). During fracture repair, interfragmentary strain greatly dictates callus volume and repair mechanism (intramembranous vs endochondral) (Bartnikowski et al., 2017; Gardner et al., 2011; Glatt et al., 2016; Willie et al., 2011). It’s been reported that strains less than 5% (0.0500) result in intramembranous repair, up to 15% (0.1500) in endochondral, and greater than 15% in failure.(Claes and Heigele, 1999; Glatt et al., 2016) More work is needed to adequately define the *in vivo* loading environment for the Masquelet technique. The cortical gap is much larger than in a fracture, and there is more opportunity for interfragmentary movement between the individual pieces of the graft material.

In summary, our studies have shown that altering spacer topography can significantly impact membrane tensile and shrinkage mechanical properties and that altering spacer material can impact solute transport through the membrane. The next step in this research would be to understand if cellular populations, factor expression or bone regeneration/healing are impacted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Overview of Spacer Fabrication & Animal Model

(A) Examples of PMMA (left) and TI (right) spacers. (B) SEM images demonstrating the surface topography of each spacer group. (C) Phase I of the MDBF Technique was performed on 10-week-old, male Sprague Dawley rats. An external fixator was applied and a 6mm osteotomy was made. One of four spacer types was inserted into the defect and the incision site closed for healing. Four weeks were allowed for foreign-body membrane development.

Figure 2. Tissue harvesting and mechanical testing

(A) Soft tissue/spacer constructs were removed from the bone, leaving the spacer in place so as not to disturb the membrane. Most overlying muscle was removed before the cylindrical membrane was incised (dotted line) and flattened to expose the membranous sheet on the inner surface. (B) Samples for mechanical testing were marked on two corners to maintain orientation and the membrane was cut in half for testing in the axial and circumferential directions. (C) The two halves were stretched until failure. (D) Samples for shrinkage tests were placed immediately in 100 centistoke silicon oil and subsequently imaged (5X).

Figure 3. Barrier testing through dextran diffusion

(A) Spacers were removed and (B–C) dextran solution was inserted via silicon tube sealed in place with cyanoacrylate (blue line). Diffusion continued for 5 minutes at which the femurs were harvested and processed for histology. (D) Cryosections were mounted in aqueous media and imaged under UV, blue, and green fluorescent light. Each channel's average intensity above a negative control area (i.e. spacer void) was determined for three to four regions: non-birefringent layer, birefringent layer, muscle, and, when present, secondary fibrous layer.

Figure 4. Histology Results

Collagen type I and elastin levels showed no difference based on material or finish. Elastin levels were significantly higher in the non-birefringent layer ($p=0.0001$).

Figure 5. Axial Tensile Testing Results

Yield strain and failure strain were higher for the membranes created by roughened spacers. There were no other differences between groups nor were there results significantly different from the circumferential direction.

Figure 6. Circumferential Tensile Testing Results

Similar to axial tensile testing results, yield strain was higher for the membranes created by roughened spacers. There were no other differences between groups nor were there results significantly different from the axial direction.

Figure 7. Tensile Testing Results – Characteristic Curves, Elastic Modulus, and Toughness
 Membranes evoked by roughened spacers were more elastically compliant and failed at significantly higher strains. However, all membranes were equally as tough. Also, there were no differences between axial and circumferential testing directions.

Figure 8. Shrinkage Study Results

There was no difference in shrinkage between groups in the axial direction. Membranes produced by roughened spacers showed significantly higher levels of shrinkage in the circumferential direction.

Figure 9. Barrier Results

For all dextrans, the intensity in TI evoked membranes was less than that of PMMA evoked membranes. Fluorescence intensity of 10kDa dextrans dropped significantly moving from the birefringent layer to the secondary fibrous tissue with no further significant signal decay. Fluorescence intensity of 70kDa and 500kDa dextrans dropped significantly moving from the inner NB layer to the BR layer. For both sizes, there was a drop in signal moving from the non-birefringent to secondary fibrous tissue area. However, it failed to reach significance for either dextran ($p=0.0529$ & $p=0.0994$, 70kDa and 500kDa, respectively). Although, there was a significant drop between the non-birefringent layer and muscle ($p=0.047$ both dextrans). Spacer finish had no significant effects.