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Title: Antimicrobial and Immunomodulatory Surface-Functionalized Electrospun Membranes for Bone Regeneration

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Guided Bone Regeneration (GBR) is a surgical procedure utilizing occlusive membranes for providing space maintenance and enabling selective repopulation of the damaged area. While this technique is effective in regenerating bone, bacterial infiltration occurs frequently and can compromise the regenerative outcome. In this study, we describe the development and characterization of a GBR membrane made of medical grade polycaprolactone (mPCL) electrospun fibers with antibacterial and immunomodulatory properties. This is achieved by the immobilization of an antibiotic, azithromycin into the membrane *via* a solvent evaporation technique leading to a sustained release of the drug over 14 days. *In vitro* testing showed that this controlled release of azithromycin is proficient at inhibiting the growth of *Staphylococcus aureus* for 14 days. Implantation of azithromycin loaded mPCL membrane in a rodent calvarial defect induces macrophage polarization towards the M2 phenotype after 1 week and resulted in significantly more bone regeneration eight weeks post-surgery. Our results suggest that this antibacterial membrane should be effective at preventing infection and also impacts on the macrophage polarization enhancing bone regeneration.

The drug loading technique developed in this study is simple, effective with a strong potential for clinical translation and can be applied to different types of scaffolds and implants for applications in craniofacial and orthopaedics applications.

1. Introduction

Guided Bone Regeneration (GBR) is a technique extensively utilized for restoring osseous defects. The GBR technique involves the surgical application of an occlusive membrane in order to prevent the infiltration of connective tissue and create space for the repopulation of the osseous defect with osteoprogenitor cells that facilitate bone regeneration.[1-3] However, in many cases, regeneration is hindered due to the early exposure of the membrane to the external micro-environment with consequential contamination of the healing tissues.[4] A local drug delivery approach is an inherently sound strategy for improving clinical outcomes whereby the GBR membrane is loaded with an antibiotic to prevent or inhibit bacterial contamination in the early stages of wound healing.

GBR membranes must fulfil several criteria such as biocompatibility, space maintaining capacities and the preservation of occlusive properties for not only the tissue regeneration time period yet the whole duration of the wound maturation and remodeling process.[1, 5, 6] In addition to these physical criteria, bioactivity can be considered a desirable feature of GBR membranes in order to initiate and enhance bone formation.[7, 8] Medical devices fabricated by solution electrospinning are potential candidate for application in GBR [6, 9] and our group has developed and characterized a medical grade polycaprolactone (mPCL) membrane.[10, 11] possessing many of these aforementioned properties

such as flexibility, space maintenance, micro to nano-fibers diameter, small pore size that imparts occlusive properties, and a highly interconnected porous network favorable for long term tissue infiltration and integration.[12] In addition, mPCL is a slow resorbing polymer, degrading *via* an erosion mechanism, [13] hence avoiding the rapid release of acidic by-products detrimental to tissue compatibility as seen in several other fast degrading aliphatic polyesters such as polyglycolic acid, poly(D, L lactic) acid and polydioxanone us. The degradation profile of PCL also enables several cycles of tissue remodeling before the membrane is losing its mechanical properties and then finally degraded, thus resulting in the formation of a mature tissue prior the loss of the occlusive and space maintenance properties [14]. In addition to the electrospun membrane long term occlusive properties, it is possible to incorporate a drug of interest for local delivery using various techniques such as direct blending, [15, 16] emulsion[17] or co-axial [18] electrospinning

Azithromycin is a macrolide antibiotic area (**Supplementary figure 1**) extensively used for the treatment of a wide range of infections and in addition to its antibacterial properties, it displays immunomodulatory and anti-inflammatory properties, which has attracted increased attention from the clinical and scientific community.[19-21] However, the clinical application of azithromycin is limited by its low bio-availability (37%) as a result of its poor water solubility, [22] as well as its systemic side effects such as diarrhea, abdominal pain, and other gastrointestinal symptoms.[23]

The objective of this study was to develop a calcium phosphate coated mPCL electrospun membrane loaded with azithromycin for local and controlled drug delivery, thus addressing the clinical challenge of infection during guided bone regeneration GBR and circumventing the limitations associated with the systemic administration of azithromycin. The method of incorporating the antibiotic in the electrospun membrane is described along with the evaluation of the physical and

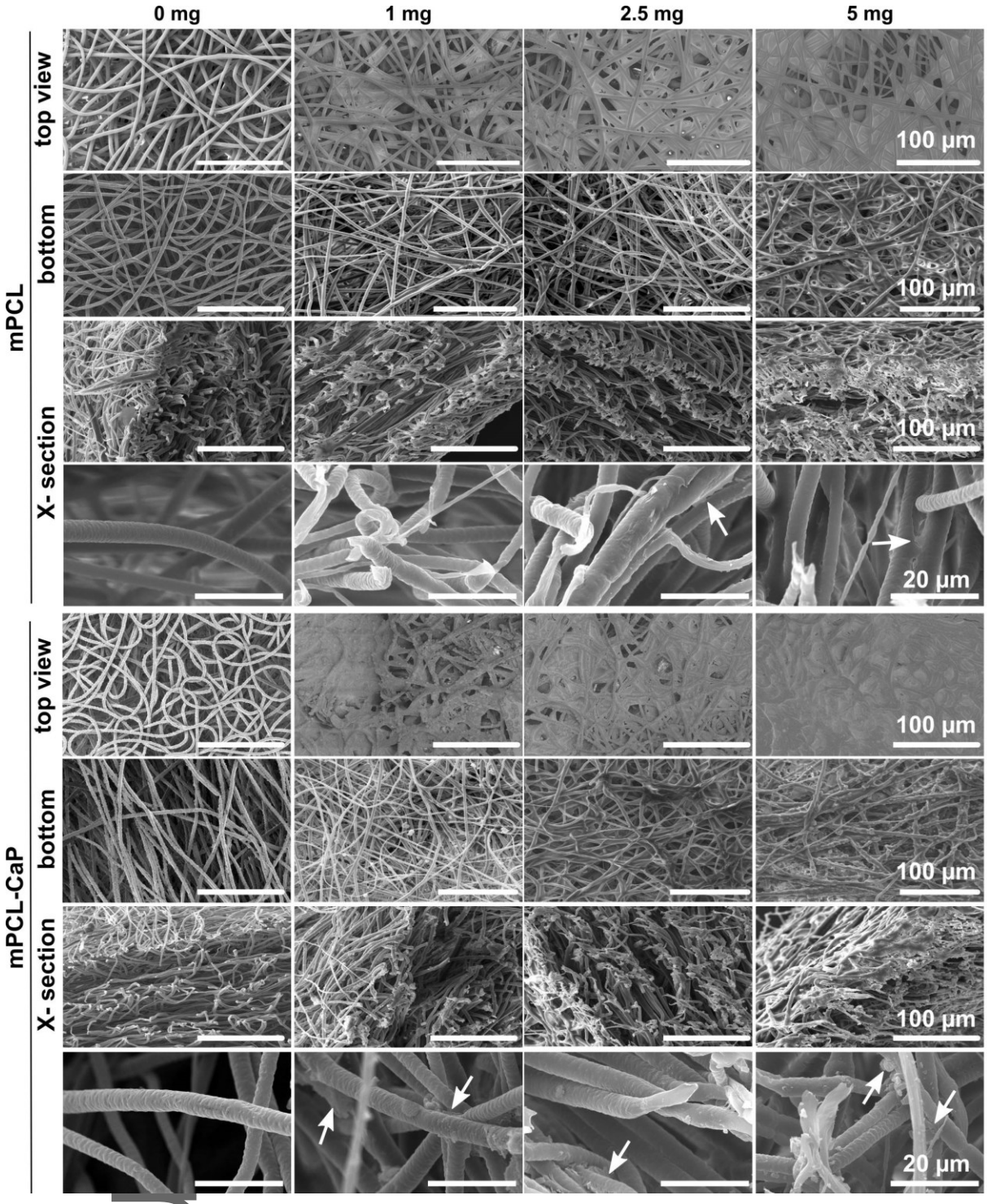
biological properties. The *in vitro* antibacterial properties and the *in vivo* performance in polarizing the macrophage population phenotype along with regenerating a critical size rodent calvarial defect model are also reported.

2. Results

2.1. Effect of drug loading on the membrane physical properties

Azithromycin was loaded onto solution electrospun mPCL membranes *via* a solvent evaporation technique. Three different doses (1mg, 2.5mg and 5mg) of azithromycin were dissolved in ethanol (100%) and pipetted (100µL) onto mPCL and calcium phosphate (CaP) coated mPCL membranes (6 mm diameter, 0.5mm thick). The membranes were soaked in the antibiotic solution for 8 hours and then the solvent was evaporated at room temperature, allowing azithromycin to precipitate onto the electrospun porous network which resulted in entrapment of the drug into the membrane as shown in **Figure 1.** With lower doses (1 and 2.5 mg), azithromycin entrapment was limited to the upper surface of the membranes while with higher dose (5mg), azithromycin deeply penetrated into the electrospun membrane and traces were still visible at the bottom side of the membrane (**Figure 1**) for both of the CaP coated and non-coated groups.

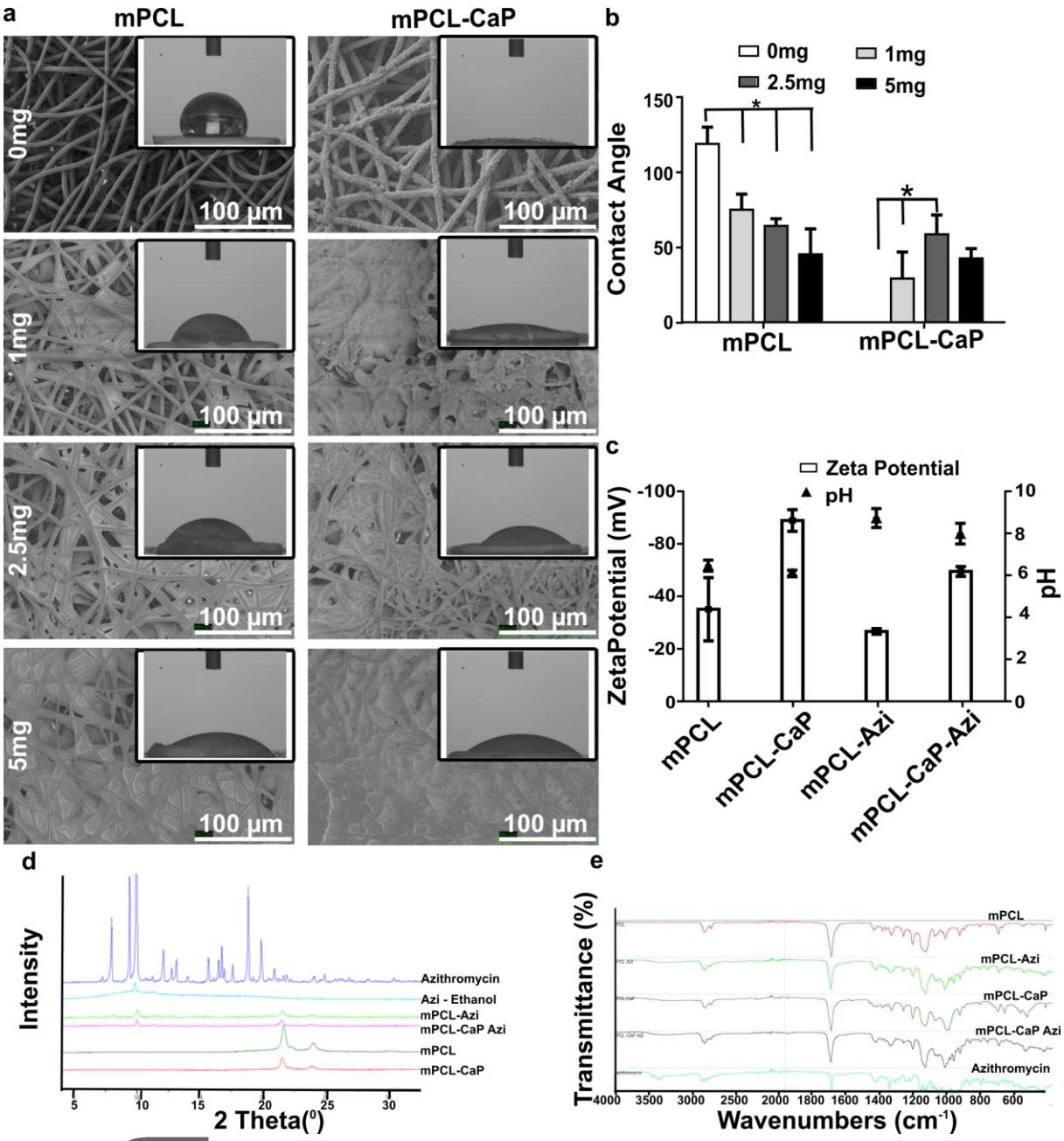
Figure 1. SEM micrograph of mPCL and mPCL-CaP loaded with 0, 1, 2.5 and 5mg of azithromycin. White arrows indicate the presence of azithromycin in the depth of the membrane.



Contact angles were measured on coated and non-coated membranes revealing that the non-coated mPCL membranes were highly hydrophobic with a contact angle of 118±11º, whereas the CaP coated mPCL membranes where highly hydrophilic (**Figure 2a ,b**) which is consistent with our previous report.[24] The loading of various doses of azithromycin onto mPCL and CaP coated mPCL membranes also had an effect on the overall hydrophilicity as the mPCL membranes became more hydrophilic with increasing azithromycin doses. Conversely, the azithromycin loading reduced the hydrophilicity of the CaP coated mPCL membranes. However, both mPCL and CaP coated mPCL membranes displayed similar hydrophilicity above 2.5mg of azithromycin loading suggesting that the drug had covered the entire surface of the membrane and hence was the determining factor for the hydrophilicity. Surface charge of the mPCL/mPCL-CaP membranes with or without azithromycin (5mg) was also measured indicating that mPCL and mPCL-CaP membranes were highly negatively charged and that the azithromycin loading reduced the overall negative charge of the membrane (**Figure 2c**). In addition, the azithromycin loading altered the local pH of the membranes and rendered the membranes slightly basic **(Figure 2c)**. The effect of the drug loading process on azithromycin crystallinity was also analysed; azithromycin is crystalline in nature and sharp diffraction peaks were present in the XRD pattern of the 'as received' azithromycin dihydrate (**Figure 2d**). However, most of these diffraction peaks (except 9.5º) disappeared for ethanol evaporated azithromycin and for both the loaded mPCL and mPCL-CaP membranes, suggesting that the drug became amorphous. FTIR spectrum confirmed the XRD results and indicated a decrease in crystallinity upon loading the azithromycin onto mPCL and mPCL-CaP membranes (**Figure 2e**).A sharp peak at 3463cm^{-1} corresponds to free O-H stretching indicative of the presence of tightly

bound water in the crystal lattice. The broad band at 3600-3300 cm^{-1} (less intense peak) in azithromycin loaded mPCL and mPCL-CaP membranes is due to the O-H stretching of the loosely bound water characteristic of the amorphous phase. Distribution and uniformity of H-bonding in the amorphous region was poor and hence H bond rupture took place resulting in a decrease in the intensity of the band in azithromycin coated mPCL and mPCL-CaP membranes.

Figure 2. SEM micrograph (a) and contact angle measurement (a-b) of mPCL and mPCL-CaP membranes loaded with different doses of azithromycin. (c) Zeta Potential and pH (d) XRD spectrum and (e) FTIR spectrum of mPCL and mPCL-CaP membranes with or without azithromycin (5mg) (* indicates statistical significance, $p<0.05$).

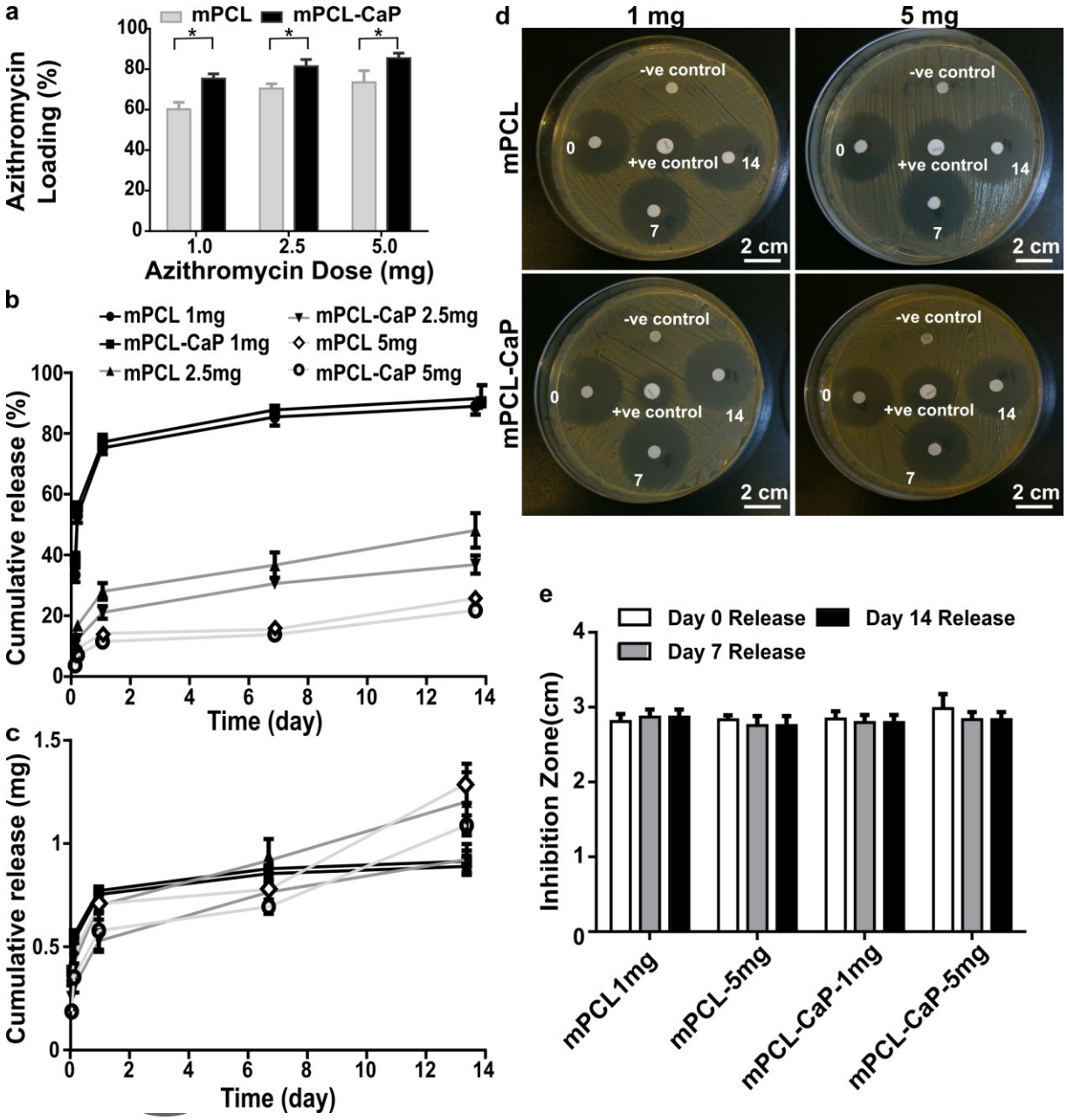


2.2. Drug loading efficacy and release profile

Azithromycin entrapment using a solvent evaporation technique also enabled the reproducible loading of azithromycin onto the electrospun membranes with an unprecedented high loading efficacy for such a poorly water soluble compound. It was also demonstrated that the loading efficiency was dependent on the surface topography of the mPCL membranes as calcium phosphate deposition significantly enhanced azithromycin loading efficiency. When loaded with 1mg, 2.5mg and 5mg of azithromycin, the mPCL membranes displayed a loading efficiency of 60.2±3.3%, 70.4±2.3% and 73.5±5.7%, while CaP coated mPCL membranes showed a significantly higher loading efficiency of 75.3±2.3%, 81.4±3.3% and 85.4±2.5% respectively (**Figure 3a**). It was demonstrated that the deposition of a calcium phosphate layer onto the mPCL fibers significantly modified the topography and pore size distribution of the mPCL fibers and hence contributed to an increase in surface area (**Supplementary figure 2**). Indeed, Brunauer Emmet Teller (BET) surface area analysis (calculated in relative to pressure range P/P0 =0.01-0.9) of the mPCL and CaP coated mPCL membranes was found to be 1.2 ±0.007m^2/g and 3.2±0.041 m^2/g respectively. This demonstrated that the larger surface area *per* volume (2.6-fold increase) available for the deposition of the antibiotic on the CaP coated membranes resulted in a higher loading efficacy. The release kinetics of various doses (1mg, 2.5mg and 5mg) of azithromycin loaded mPCL and mPCL-CaP membranes was investigated *in vitro* over 14 days. The release followed a biphasic profile with an initial burst release of azithromycin in the first 24 hours (**Figure 3b) and c)**) followed by a more sustained release over the next 14 days. Bacterial colonization generally occurs within a few hours post-implantation and it is generally considered that the first 6 hours are critical.[25] Hence, a burst release of antibiotics for the first few hours of implantation is an important requirement for preventing bacterial colonization

of the implanted material. The initial dose of azithromycin significantly affected the early and late phases of the release as shown in **Figure 3b.**

Figure 3. Loading efficiency (a) and release profile of azithromycin loaded on mPCL or mPCL-CaP electrospun membranes. The graph represents the percentage of azithromycin encapsulation (a) release (b) and total amount azithromycin released (c) at various time points from 1hr to 14 days. (d) *Staphylococcus aureus* agar plate growth inhibition assay showing that the antimicrobial properties of the loaded membranes were maintained over 14 days of *in vitro* release in PBS (0, 7 and 14 correspond to the time immersed in PBS prior to performing the inhibition assay), the negative control consists of the electrospun membrane without azithromycin demonstrating no antimicrobial activity and the positive control contains 15µg of the antibiotic. (d) and (e) quantification of the growth inhibition for the 1mg and 5mg loaded azithromycin over 0 to 14 days of release in PBS at 37ºC, (* indicates statistical significance, $p<0.05$).



Indeed, 35%, 12% and 5% release after 3 hours of incubation at 37ºC in PBS was obtained for initial doses of 1mg, 2.5mg and 5mg respectively. After 14 days of release, the 1mg group displayed ~ 90% release, while the 2.5mg group showed ~ 45% release and the 5mg group released only 25% of the

drug. Although CaP coating had an impact on loading efficiency, no significant differences in the release profile were observed as shown in **Figure 3b and c**.

SEM imaging also demonstrated the release of azithromycin over the experimental time frame, as the porous network formed by the electrospun fibers became exposed as the drug was release in PBS for 7 and 14 days (**Supplementary Figure 3**). Traces of azithromycin, as observed by SEM, were still found on both mPCL and mPCL-CaP membranes after 7 and 14 days release in PBS at 37ºC indicating that total release was not yet achieved after 14 days.

2.3. Antibacterial properties

The antibacterial efficacy of azithromycin loaded mPCL/mPCL-CaP membranes against *Staphylococcus aureus* (*S. aureus*) was longitudinally assessed at various release time points (7 and 14 days), and compared to a negative control (membrane without drug) and a positive control containing 15µg of the antibiotic. A distinct zone of inhibition was visible around all drug-loaded membranes (**Figure 3d**) regardless of the CaP coating or azithromycin doses, but not around the negative control. The membranes previously immersed in PBS at 37ºC for 7 and 14 days prior to performing the assay displayed a similar antibacterial efficacy. It is important for a GBR membrane to preserve the bioactivity of the antibiotics over the time frame of the drug release and the present study demonstrated that there was sufficient remaining drug loading, even after 14 days of release, to impart excellent antibacterial properties and that azithromycin bioactivity was maintained for more than 14 days.

Interestingly, the drug bioactivity was also well preserved despite the amorphous transformation that occurred during the encapsulation procedure. Hence, this indicated that the change in crystallinity of azithromycin occurring during the loading process did not affect its bioactivity.

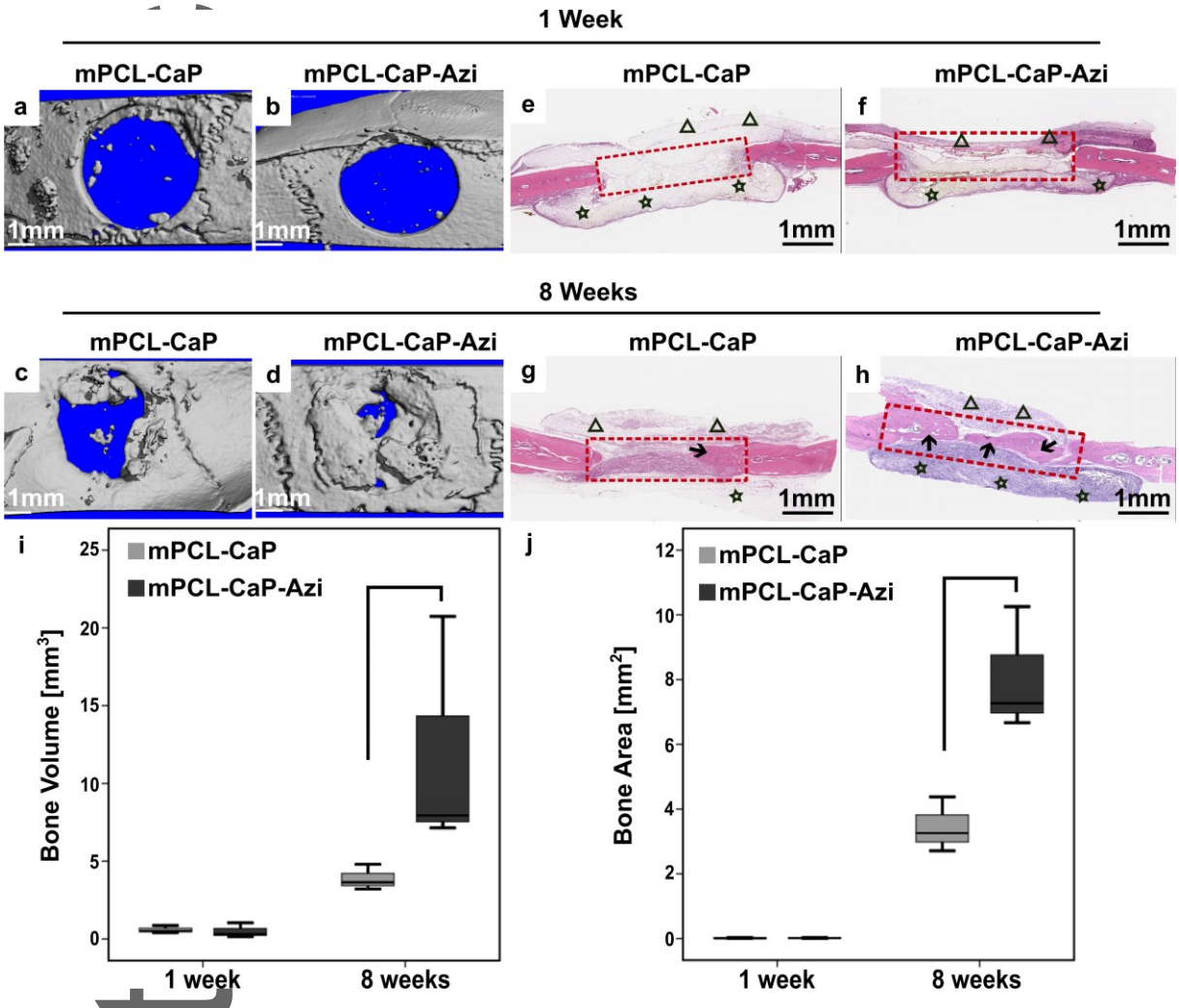
2.4. *In vivo* immunomodulatory effect of the loaded membranes

To determine the bone regeneration capacity of azithromycin, mPCL-CaP membranes with or without azithromycin were implanted contra laterally in a rodent calvarial defect and bone regeneration was assessed at one and eight week post-implantation. micro CT reconstruction and histomorphometry were used to evaluate bone healing in the defect at the various time points. This revealed that there was little newly formed bone at week 1, which was mostly localized to the marginal regions of the surgically created defect in both the mPCL-CaP and azithromycin loaded mPCL-CaP (5mg) groups (**Figure 4 a, b, e and f**).

However, new bone formation occurred for both the mPCL-CaP and azithromycin loaded mPCL-CaP (5mg) groups at 8 weeks post implantation, albeit to a different extent (**Figure 4 c, d, g and h**). The antibiotic loaded membranes enhanced bone regeneration within the defect (**Figure 4 i**) which was significantly higher compared to the control (unloaded mPCL-CaP), as determined by microcomputed tomography and confirmed by histomorphometry (**Figure 4 j**).

Figure 4. Bone regeneration outcome in a rodent calvarial defect as assessed by micro-computed tomography(μCT) (a-d and i) and histology (e-h and j)) after 1 week and 8 weeks implantation with or without 5mg azithromycin. (i) Quantification of bone formation as measured by μCT (j) new bone

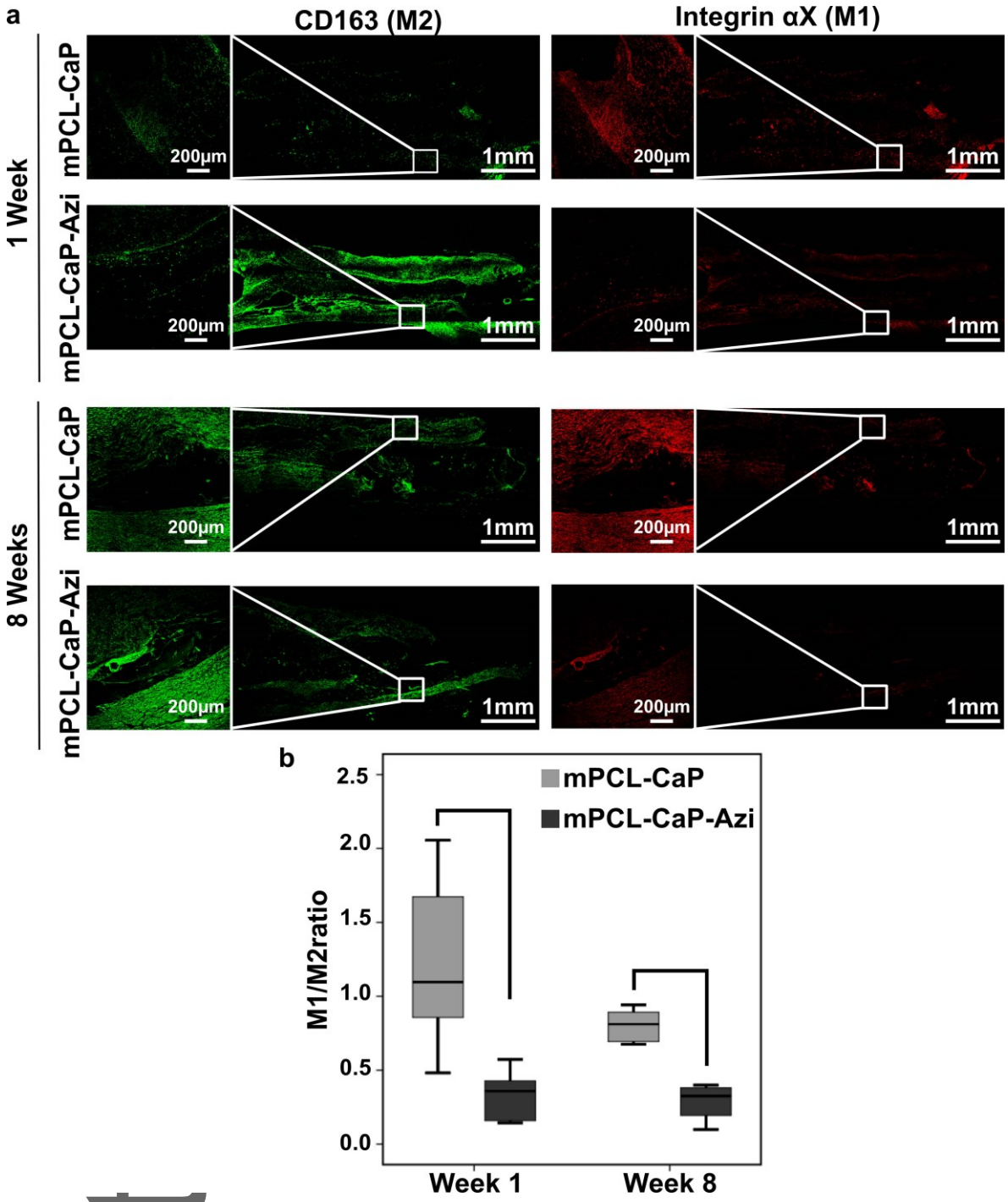
formation as measured by histomorphometry. The red boxes outline the defect boundaries, the stars indicate the thick and cell-impermeable mPCL membrane that was placed on top of the exposed dura mater, the black arrows show new bone formation and the triangles indicate the thin electrospun mPCL membrane placed above the drug loaded membrane to stabilize the defects. Bar indicates statistical significance, $p<0.05$.



In order to analyze the macrophage response in early and late wound healing stages, immunohisto-fluorescence was performed targeting the M1 and M2 phenotype markers Integrin αX and CD163

respectively. As depicted in **Figure 5**, Integrin αX (M1 in red) staining appeared qualitatively lower for the 5 mg azithromycin loaded mPCL-CaP group when compared to mPCL CaP control group at both 1 and 8 weeks post-implantation. This was in contrast to the M2 staining which was higher in the azithromycin loaded mPCL-CaP group at 1 week. The M1/M2 ratio, as calculated by the fluorescence intensity, was significantly higher at 1 week for the mPCL-CaP control group (1.2±0.49) than for the azithromycin loaded mPCL-CaP group (0.36±0.15). This indicated an early shift in the macrophage polarization towards the pro-regenerative M2 phenotype in the presence of the azithromycin loaded implant. For both groups the M1/M2 ratio decreased at 8 week post-implantation, suggesting a switch from a pro-inflammatory to a pro-regenerative process. Here again the control group still had a significantly higher ratio at 8 week (0.75±0.08 and 0.28±0.12 for mPCL-CaP control group and azithromycin loaded mPCL-CaP group respectively) indicating that the immunomodulatory effect of the azithromycin was still maintained at an advanced healing phase.

Figure 5. (a) Immunofluorescent staining of M1 (integrin, red) and M2 (CD163, green) macrophages of mPCL-CaP and azithromycin loaded (5mg) mPCL-CaP one week and eight weeks post implantation and (b) ratio of M1 and M2 macrophages one and eight weeks post implantation (Bars indicates statistical significance, $p<0.05$).



3. Discussion

This study demonstrated that the loading of azithromycin imparted anti-microbial properties onto mPCL-CaP membranes, as well as enhanced bone regeneration *via* immunomodulation of the inflammatory response through macrophage polarization favoring the M2 regenerative phenotype. The delivery of hydrophobic antibiotics is a challenge when compared to hydrophilic antibiotics, as high systemic doses cannot be prepared in aqueous solutions and also due to their low bioavailability. To overcome these drawbacks, various preparations of azithromycin have been proposed for local delivery, including microcapsules prepared *via* layer–by-layer self-assembly,[26] or polymeric nano/microspheres made using modified quasi emulsion solvent diffusion technique[27] or reduced pressure solvent evaporation technique.[28]

Azithromycin micro capsules prepared through layer-by-layer self-assembly onto the surface of silica microspheres and azithromycin loaded poly(lactide-co-glycolide) nanoparticles prepared by nano-precipitation e according to the modified quasi emulsion solvent diffusion technique exhibited a higher encapsulation efficiency ~ 78%, but resulted in a complete release within 24 hours.[26, 27] On the other hand, azithromycin microspheres prepared *via* a reduced pressure solvent evaporation technique exhibited a controlled release over 60 days, but the initial encapsulation efficiency was only around 60%. The solvent evaporation technique developed in the present study is a relatively simple technique, where the hydrophobic compounds are dissolved in an appropriate volatile solvent and the carrier system is added to the solution under agitation prior to evaporating the solvent under atmospheric pressure.[29] Although this method is generally employed to prepare

micro/nano spheres or capsules, this present study demonstrated that it can be utilized in combination with a electrospun membrane, resulting in high loading efficacy of up to 85% while maintaining homogenous drug distribution and release over 14 days.

This method can also be adapted to encapsulate other drugs and antibiotics onto polymeric membranes, films or 3D printed scaffolds (**Supplementary figure 4**) utilized for a range of applications and not necessarily confined to Guided Bone Regeneration.

The present study also demonstrated the favorable impact of the CaP-coating onto azithromycin loading efficiency. Previous studies[24] have shown that CaP-coated mPCL can significantly enhance bone regeneration and here we demonstrate that, synergistically with the azithromycin, the CaP-coated mPCL membrane can accelerate bone healing in the context of GBR.

The processing parameters utilized for loading the antibiotic can cause significant changes in its crystalline structure and bioactivity. XRD and FTIR results revealed that the crystalline structure of azithromycin was altered after being dissolved in ethanol, making it more amorphous in nature. Despite the change in internal crystal lattice, azithromycin still preserved its bioactivity, as evidenced by its antibacterial properties against *S. aureus* at all different concentrations. Moreover, azithromycin loading onto mPCL/mPCL-CaP membranes also resulted in the formation of a broad band at 3600-3300 cm^{-1} while, azithromycin dihydrate displayed a sharp peak at 3463cm^{-1}. previous studies has shown that azithromycin dihydrate have a sharp peak at 3463cm^{-1}, which corresponds to free O-H stretching indicative of the presence of tightly bound water, while azithromycin

monohydrates displayed a broad band at 3600-3300 cm^{-1} due to the O-H stretching of the loosely bound water.[30] This change of azithromycin from a dihydrate to a monohydrate state after loading on to mPCL/mPCL-CaP membranes is favorable for an application as medical device and implant. The accelerated dissolution rate resulted in an enhanced burst release in the initial 24 hours as previous study also suggests that azithromycin monohydrates have a faster dissolution rate than dehydrates.[30]

Membrane contamination and subsequent exposure to the external environment is a key risk factor for the failure of GBR therapy.[3] The possibility of such exposure is exacerbated by the fact that GBR is widely utilized in dentistry whereby the oral cavity presents a challenging microbial environment. Therefore, an initial burst release of an antibiotic from a GBR membrane is condition *sine qua non*, as the control over infection and inflammation is more prominent during the early stages of the wound healing process.[31] The present study has demonstrated that the loading of azithromycin onto mPCL/mPCL-CaP membranes *via* a solvent evaporation technique resulted in an initial burst release followed by a controlled and sustained release profile. The release profile and antibacterial performance suggest that the loaded membranes will be efficient at preventing bacterial infection following GBR surgery.

The risk of bacterial resistance can be exacerbated by the long term presence of antibiotics in tissues at sub-antibacterial concentration. The membranes with the higher doses of 2.5 and 5 mg displayed incomplete release of azithromycin after an *in vitro* incubation of 2 weeks and hence can potentially

increase this risk. Therefore the use of the 1 mg dose whereby the antibiotics is exhausted within 14 days would be preferable in reducing the risk of developing bacterial resistance. Furthermore it is worth noting that previous studies have demonstrated the retention of azithromycin in periodontal tissues for up to 6.5 days[32] and for up to 28 days in neutrophils after the last dose when taken systemically.[33] It has also been suggested that periodontal pathogens are generally less prone to developing resistance to azithromycin[34, 35] as it did not result in increased resistance when compared to other antibiotics.[36]

The action of azithromycin can be divided in three generally overlapping phases;[35] antibacterial, anti-inflammatory and pro-healing. It firstly exhibits an antibacterial activity phase during the initial 14 days of administration, which is partially overlapped with an anti-inflammatory phase extending for several months, leading ultimately to an enhanced healing phase.

The *in vivo* component of the present study was a proof of concept to demonstrate the safety of the utilization of the membrane with a relatively high loading dose (5 mg). This showed that the tissue integration of the membrane was complete and more importantly that no signs of tissue necrosis originating from a potentially cytotoxic local concentration of azithromycin were observed. It was also demonstrated that the azithromycin loaded membrane enhanced bone formation and that the temporal healing pattern was in agreement with the three phase model previously outlined.[35] Indeed, notable differences in bone formation were only observed at the late time-point (8 weeks post-implantation). Previous studies have demonstrated the immunomodulatory effect of azithromycin *in vitro*, resulting in decreased pro-inflammatory cytokine and macrophage polarization

towards a pro-healing phenotype.[37] The long term presence of azithromycin in the vicinity of the calvarial defect appears to have exerted a gradual immunomodulatory response which in turn resulted in enhanced bone formation. This is in agreement with several clinical studies which reported enhanced bone healing several months after the administration of the drug.[38, 39]

4. Conclusion

In conclusion, we have developed an efficient and effective technique to load hydrophobic azithromycin onto biodegradable membranes, resulting in a controlled drug release with favourable antimicrobial release profile. Enhanced bone regeneration was achieved by promoting an accelerated transition from a pro-inflammatory M1 macrophage response to a regenerative M2 phenotype. The straightforward and cost effective manufacturing process combined with a lack of obvious regulatory barriers suggests that the Azi-mPCL-CaP membranes have the potential for timely clinical translation in a variety of health disciplines, including dentistry, craniofacial surgery and orthopaedics.

5. Experimental section

5.1. Materials:

All reagents were obtained from Sigma–Aldrich (Australia), unless otherwise stated. All solvents were of analytical grade and used without purification.

5.2. mPCL membrane fabrication

Medical grade polycaprolactone (mPCL, 80kDa, PURAC), was utilized to fabricate mPCL membranes. mPCL was electrospun using an in-house solution spinning device as previously described.[11] Briefly, the polymer was first dissolved in a mixture of chloroform and dimethylformamide (9/1 vol/vol) at a concentration of 25% wt/vol. The polymer solution was loaded into a 5 ml syringe and electrospun at a feed rate of 2 ml/h, at 9 kV, using 21 G needle and at a 20 cm tip to collector distance for 30 min on a 10x10 cm^2 aluminium foil collector.

5.3.Calcium phosphate (CaP) coating on mPCL membranes

The mPCL membranes were submitted to a calcium phosphate coating process by successive immersion into specific reagents and solutions in three steps (surface activation, biomimetic mineralization and post-treatment) according to published protocol.[40] Briefly, during surface activation process, the scaffolds were immersed in 100% ethanol and placed under a vacuum for 15 min in order to remove entrapped air bubbles that could negatively impact on the coating homogeneity. The samples were then removed from ethanol and placed in a freshly prepared pre-warmed (37ºC) 2M NaOH for five min vacuum treatment. The samples were then placed at 37ºC for 30 min. The electrospun scaffolds were rinsed five times with Milli Q water to remove any residual NaOH. The pH of the last rinsing solution was checked to confirm it was approximately 7, thus preventing early precipitation of the simulated body fluid (SBF) solution in the subsequent step. During biomimetic mineralization process, the SBF solution was adjusted to pH 6 with $NaHCO_3$ under gentle stirring. This solution was then filtered using a 0.2 µm-filter. Surface activated mPCL membranes were placed on this filtered solution and vacuumed for five minutes. The pressure was gently released to avoid early precipitation at the surface of the SBF solution. Thereafter, the samples were placed at 37ºC for 30 min and the samples were gently shaken every 10 min. This last

step (except the vacuum treatment) was repeated one more time. The samples were then twice rinsed in Milli Q water. For the post-treatment step, the CaP coated samples were immersed in 0.5 M NaOH at 37ºC for 30 min. This step was performed in order to homogenize the CaP phase obtained after the SBF treatment. After post-treatment the mPCL membranes were rinsed with distilled water five times and the pH of the last solution was confirmed to be approximately 7.

5.4. Azithromycin loading on mPCL and CaP coated mPCL membranes

Azithromycin dihydrate was loaded onto mPCL/mPCL-CaP membranes by a solvent evaporation technique. Briefly, mPCL and mPCL-CaP membranes (6mm diameter, 0.5mm thick) were placed on a 2ml eppendorf tube (flat bottom). The following was performed as follows: 1, 2.5 and 5mg of azithromycin dissolved in 100µL of ethanol was added and incubated at room temperature for 8 hours under mild agitation. The membranes were left under fume hood for ethanol to evaporate. After complete evaporation of ethanol samples were then gently washed three times with distilled water to remove any unbound azithromycin and later dried and stored at -20 ºC until use.

5.5. Physical characterization of the electrospun loaded and unloaded mPCL/mPCL-CaP

5.5.1. Membrane Morphology

The morphology of the electrospun mPCL/mPCL-CaP membranes with or without azithromycin coating were gold coated and characterized with a scanning electron microscope (Zeiss Sigma VP Field Emission SEM) operating at 10 kV.

5.5.2. Surface Area

Surface area of the mPCL/mPCL-CaP membranes (n=3) were measured by performing argon adsorption and desorption at 77Kusing a surface area and porosity analyzer (Micromeritics Tristar II 3020 Surface Area Analyzer). All samples were freeze dried and degassed for 12 hours prior to the experiment Surface area was calculated using Braunauer Emmet Teller (BET) equation.

5.5.3. Contact Angle

The contact angle of non-coated and CaP-coated mPCL membranes (n =4) was measured using a FTA200 Contact Angle and Surface Tension Instrument (Poly-Instruments Pty. Ltd., Australia. 5µL droplets of Milli-Q water was used as the probe liquid. The contact angle was measured immediately after the droplet was deposited onto the surface.

5.5.4. Surface Charge Analysis

A SurPASS electrokinetic analyzer (Anton Paar) was used for surface zeta potential measurements of mPCL and mPCL-CaP membranes with or without azithromycin (5 mg) using three replicates. In each zeta potential recording, two identical samples were attached to the adjustable gap cell to face each other with a 100 µm gap between them. The streaming current was measured between two Ag/AgCl electrodes placed at both sides of the samples. The measurements were performed using 1 mM KCL solution as the electrolyte. The VisioLab-interface calculated the zeta potential from the streaming current measurements according to the Fairbrother-Mastin approach (1)

$$\text{Zeta Potential} = (dU/dp) \times [\eta/(\varepsilon \times \varepsilon_0)] \times K_B) \quad (1)$$

Where dU/dp is the slope of the streaming current versus pressure, η is the viscosity of the electrolyte, ε is the dielectric constant of the electrolyte, εo is the vacuum permittivity and K_B is the specific electrical conductivity of the electrolyte solution outside the capillary system.

5.5.5. X-ray diffraction and Fourier transform infrared spectroscopy

X-ray diffraction (XRD) studies were conducted on a PAN analytical X'Pert PRO MPD Powder X-ray Diffractometer equipped with Cu-K α source (40 kV, 40 mA) in a 2θ range of 4-50° at a scan rate of 5°/min with three replicates for each sample. Fourier transform infrared spectroscopy (FT-IR) was performed on a Nicolet 5700 FT-IR with a Smart Endurance Diamond ATR accessory on each sample (n=3). The spectra were collected by co-adding 64 scans at 4 cm^{-1} resolution, range 4000-600 cm-1. The spectra were ATR collected and displayed as absorbance spectra.

5.6. Drug loading efficacy

Azithromycin concentration was measured by colorimetry after adding the azithromycin to sulfuric acid solution (75 ml sulfuric acid was added to 100 ml distilled water (43%)) for coloration. At first, azithromycin solution standard curve was plotted. For this, a series of azithromycin solutions at different concentrations: 1, 5, 10, 15, 20, 25 and 30mg/mL, were prepared in PBS. 250µL of azithromycin solution was mixed uniformly with 250µL of sulfuric acid solution (43%). The mixture was incubated at room temperature for 30 min, and then was scanned using a microplate spectrophotometer (BIO-RAD). The absorbance at these solutions at 482nm was measured to establish the azithromycin solution standard curve. Based on the relationship between the azithromycin standard solution concentration and the corresponding absorbance at 482 nm, a linear equation for azithromycin standard curve was obtained. To determine the encapsulation efficiency

of azithromycin on to mPCL/mPCL-CaP membranes (n=6), each azithromycin coated membrane was placed on 2ml eppendorf tube. To this 500µL of ethanol was added to dissolve azithromycin from the membrane. The membranes were then removed after overnight incubation. Ethanol was evaporated and azithromycin was redissolved in 250µL sulfuric acid solution (43%) for coloration. The absorbance at 482nm was measured after 30mins incubation at room temperature. Azithromycin encapsulation was determined using the standard curve.

5.7. Azithromycin release from mPCL/mPCL-CaP membranes

The azithromycin release profile was determined using a colorimetric assay similar to that described in the previous section. Briefly, azithromycin solution standard curve was plotted. For this, a series of azithromycin solutions at different concentrations: 1, 5, 10, 15, 20, 25 and 30mg/mL, were prepared in PBS. 250µL of azithromycin solution was mixed uniformly with 250µL of sulfuric acid solution (43%). The mixture was incubated at room temperature for 30 min, and then was scanned using a microplate spectrophotometer (BIO-RAD). The absorbance at these solutions at 482nm was measured to establish the azithromycin solution standard curve. Based on the relationship between the azithromycin standard solution concentration and the corresponding absorbance at 482 nm, a linear equation for azithromycin standard curve was obtained.

Each mPCL/mPCL-CaP membranes (n=6) with the various doses of 1, 2.5 and 5 mg were placed in an eppendorf tube with 250µL PBS at 37ºC. At each time point (1 hour, 3hour, 1, 7 and 14 days) the membranes were removed and placed in new eppendorf tubes with 250µL PBS. To determine azithromycin concentration, 250µL of sulfuric acid solution (43 %) was added to 250µL PBS (obtained after each release time point) for coloration. The absorbance at 482 nm was measured after a 30

min incubation at room temperature. Azithromycin release was calculated using the linear equation obtained from the standard curve.

5.8. *In vitro* antibacterial activity

The antibacterial activity of the membranes against *S. aureus* ATCC 25923 (source), was determined by disc diffusion method.[41] Briefly, the bacterial strain was inoculated onto a Brain Heart Infusion Agar (Oxoid) and incubated at 37ºC for 24 hours. After incubation, isolated bacterial colonies of the stock culture were suspended in sterile saline until the turbidity was compatible with 0.5 Mac Farland. A 100 µL *S. aureus* suspension was spread onto a Mueller-Hinton agar (Oxoid) plate. The mPCL/mPCL-CaP membranes (6mm) with various doses of azithromycin (1, 2.5 and 5 mg, n=3 for each dose) after 0, 7 and 14 days of release in PBS at 37 ºC were pasted onto the agar plate and incubated for 18 hours at 37ºC. All the samples were sterilized for 30 mins under UV before they were pasted. Membranes without azithromycin were used as the negative control and azithromycin antimicrobial susceptibility disks (15µg, Oxoid) were used as the positive control. The bacterial growth on the plate was visualized directly, after incubation of the plates at 37ºC for 18 hours and the diameter of the inhibition zone was measured according to Clinical and Laboratory Standards Institute (CLSI M02-A10) recommendations.[42]

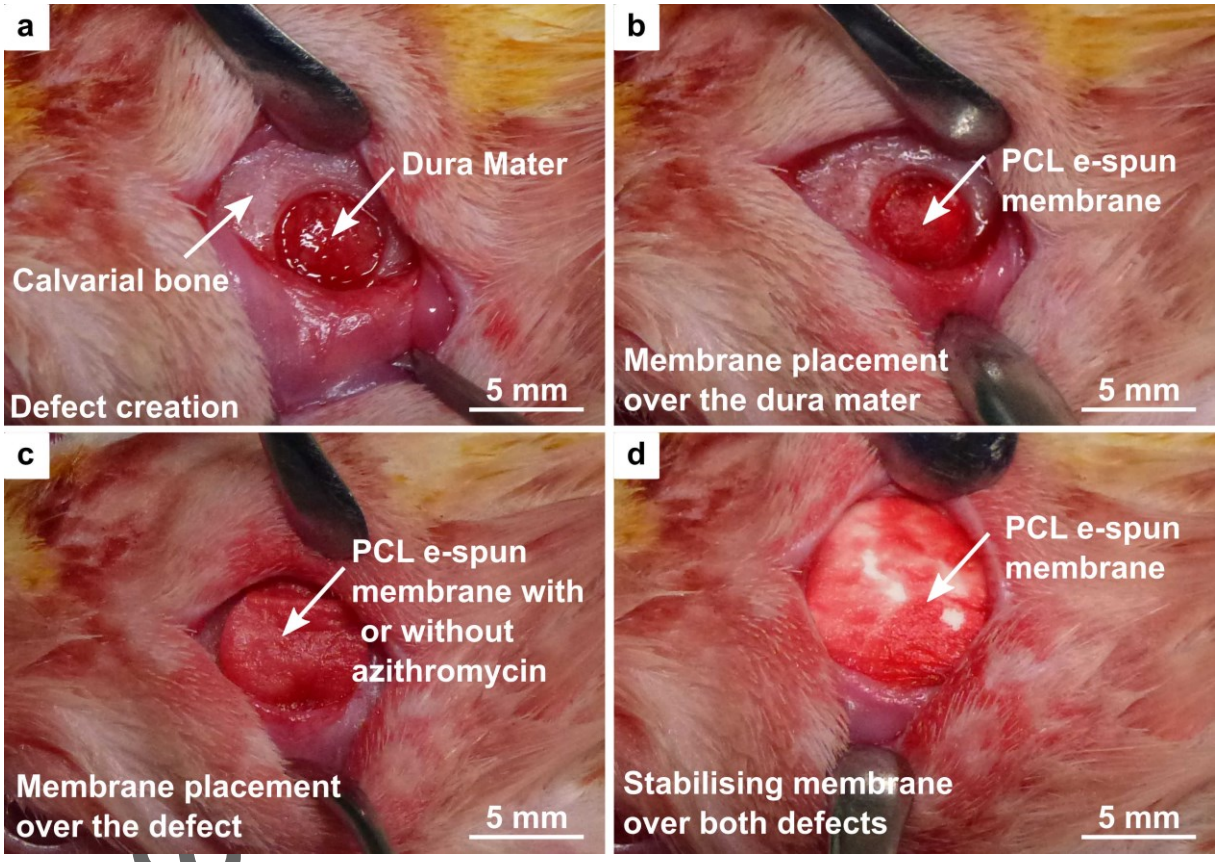
5.9. *In vivo* study

5.9.1. Surgical procedure

Animal ethics approval for the use of Sprague Dawley rats in this experiment was granted by the Animal Ethics Committee of Griffith University under the approval number DOH/02/15/AEC "Characterization and evaluation of antibacterial and anti-inflammatory polycaprolactone

membranes for bone regeneration applications: a rodent calvarial study". As a proof of concept for assessing tissue integration of the loaded membranes and the azithromycin impact on bone regeneration, the high dose 5mg was selected for the *in vivo* experiment. Prior to implantation, 6 mm electrospun CaP coated mPCL discs were sterilized in a biosafety cabinet by ethanol immersion for 30 min followed by a 30 min UV-irradiation. Following this, the membranes were loaded with 5 mg azithromycin according to the protocol described previously (5.4. Azithromycin loading on mPCL and CaP coated mPCL membranes). After solvent evaporation in the biosafety cabinet, the membranes were rinsed in sterile water, transferred to a new sterile dish and stored in the laminar hood until implantation. To this end, six male rats (Animal Resources Centre, Canning Vale, WA, Australia) were used. The animals were anaesthetized with isoflurane. The dorsal part of the cranium was shaved and disinfected with 50mg/mL Povidone-Iodine (Betadine, Mundipharma BV, Netherland). A sagittal incision was performed through the skin and the periosteum of the calvarium and the cranial vertex was exposed. After raising the full thickness periosteal flap, two circular osseous defects will be created on the calvaria by means of a trephine bur (internal diameter of 5.0 mm) with copious isotonic solution (0.9% Saline) irrigation as shown in **Figure 6a**.

Figure 6: Surgically created calvarial defect in a rodent model, a) 5mm diameter defect exposing the Dura Mater, b) placement of a 8 mm diameter polycaprolactone electrospun disc on top of the Dura Mater for preventing soft tissue infiltration in the bony defect, c) placement of a 6 mm electrospun membrane with or without azithromycin (5mg) d) stabilization of the defect using a rectangular PCL electrospun membrane positioned over the bilateral defects.



A 8 mm mPCL membrane was placed on top of the exposed dura mater in order to prevent soft tissue infiltration in the defect (**Figure 6b**). The mPCL-CaP membranes (6 mm in diameter) with or without azithromycin (5mg) was placed over each defect (**Figure 6c**). A thin electrospun mPCL membrane covering both defect (**Figure 6d**) was utilized in order to stabilize the mPCL-CaP membranes (with or without azithromycin) and to provide soft tissue occlusion. The wound was closed in layers using resorbable sutures (Vicryl 5.0 and 4.0, Ethicon, Germany).The animals were sacrificed after one and eight weeks and the implants (n=3 for each group at each timepoint) were

retrieved and fixed in 4% paraformaldehyde for 24 hours at 4 ºC and then placed in PBS at pH 7.4 until further analysis.

5.9.2. Micro-CT analysis

Bone formation within the defect was assessed using microcomputed tomography (micro-CT). The samples were scanned using a mCT40 (SCANCO Medical AG, Brüttisellen, Switzerland) with the following conditions: 55kVp, 145µA, 8 W, a voxel size of 30µm, at a greyscale threshold of 220 and 3D images was constructed by the micro-CT system software package. Quantification of the volume of bone formation was performed by selecting the region of interest from the scans.

5.9.3. Histology and Histomorphometry

After performing the micro-CT analysis, the samples were decalcified in 5% formic acid at room temperature for a month with bi-weekly change of solution and subsequently embedded in paraffin. Histological sections were prepared in the horizontal plane and included intact bone margins surrounding the reconstructed defects. Consecutive serial sections (5 µm) were deparaffinized and stained with hematoxylin–eosin. The stained slides were scanned using the AT2 Scanscope (Leica Biosystems, USA). Histomorphometrical analyses were performed using the Osteomeasure histomorphometry suite (Osteometrics Inc., Decatur, GA, USA). To determine the area of new bone formation, three sections of each specimen (n=3 for each group at each timepoint) were utilized and the area occupied by newly formed bone was manually drawn using a stylus provided with the Osteomeasure suite.

5.9.4. Immunofluorescence Microscopy

Paraffin sections were deparaffinized and antigens retrieved by heat in Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) at 95 ˚C for 20 minutes according standard protocols. The sections were blocked with 1% BSA in PBST (PBS with 0.1% Tween-20) for 1 hour at room temperature. Subsequently, the sections were probed for M1 with rabbit anti-human Integrin αX (R-113) polyclonal (Santa Cruz, SC-30137) and M2 with mouse anti-human CD163 (ED2) monoclonal (Santa Cruz, SC-58965) antibodies at 4^0C overnight at concentrations of 1:100. After washing 3 times with PBST for 5 minutes, the sections were incubated with secondary goat anti-rabbit IgG-PE (Santa Cruz, SC-3739) (for M1) and goat anti-mouse IgG-FITC (Santa Cruz, SC-2010) (for M2) antibodies at room temperature for 1 hour at a concentration of 1:200. The sections were washed 3 times with PBS, counterstained with DAPI and mounted on microscope slides using antifade gold (cell signaling technologies). Confocal fluorescence microscopy (Nikon A1R+) was used to image the whole sections and subsequently analyzed with the ImageJ software. The intensity of each channel was extracted from the imaged sections (red channel-M1, green channel-M2). Data is presented as the ratio of M1 intensity over M2.

5.10. Statistical analysis

For statistical significance, samples were evaluated using a Student t-test where only two groups were compared. For multiple group and time points, a Generalized Estimating Equation model was utilized with a pairwise interaction comparison using a LSD Post-Hoc. Differences were considered significant when equal or less than p=0.05.

Supporting Information*:*

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

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Supporting Information

Title: Antimicrobial and Immunomodulatory Surface-Functionalized Electrospun Membranes for Bone Regeneration

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Figure S1. Chemical structure of Azithromycin.

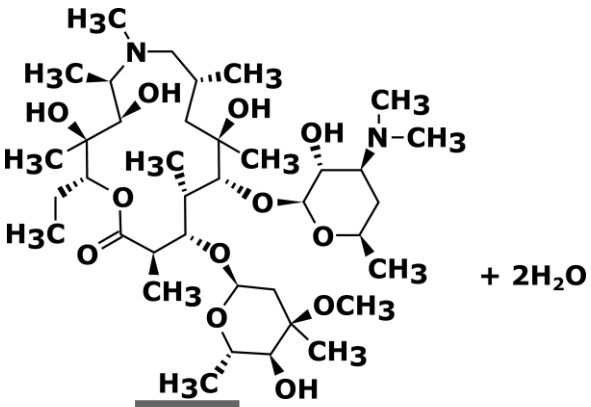

Figure S2. Argon adsorption –desorption isotherms (a, b) with corresponding pore size distribution (c, d) of mPCL (a,c) and mPCL-CaP (b,d) membranes.

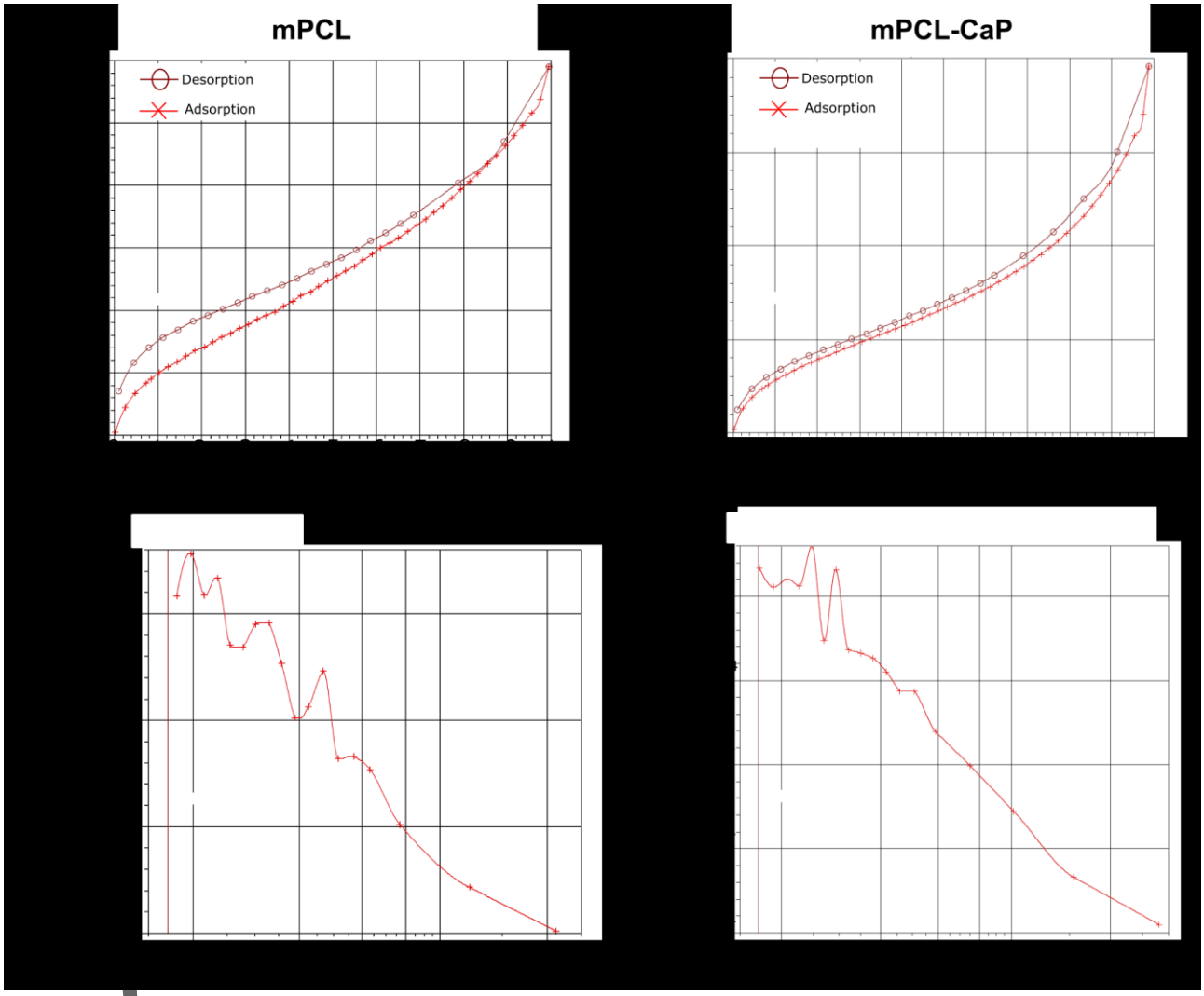

Figure S2. SEM images of azithromycin loaded on mPCL or mPCL-CaP electrospun membranes after 7 and 14 days of release in PBS at 37ºC.

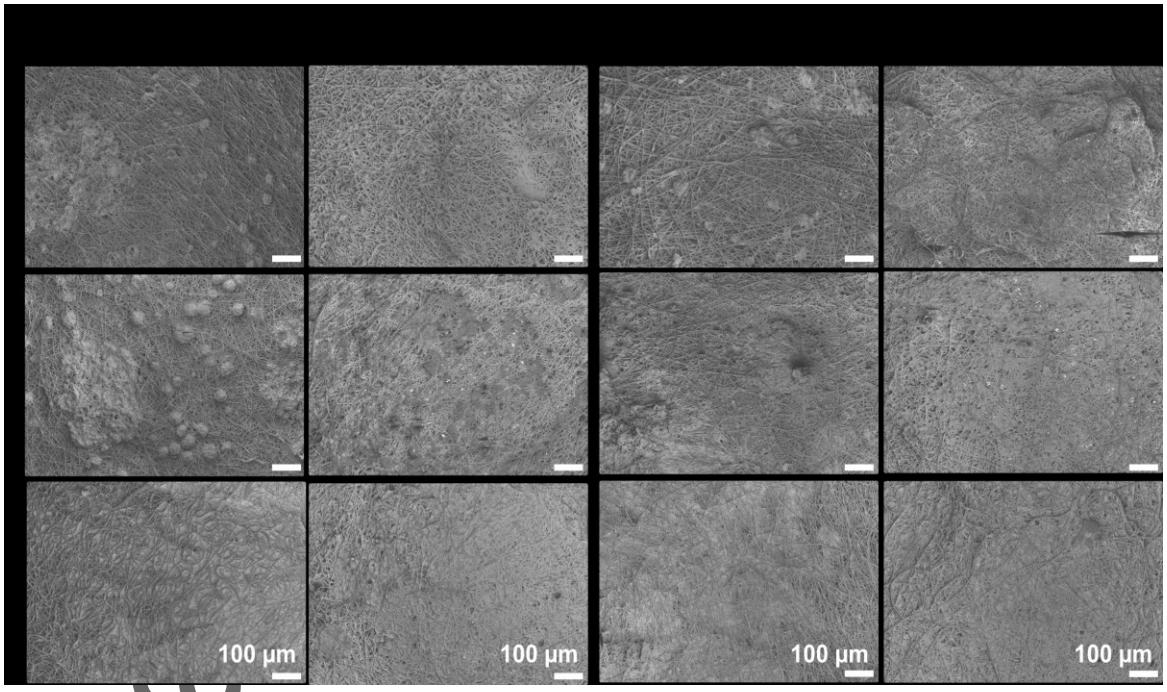

Figure S4. SEM Images of highly porous additively manufactured constructs via melt electrospinning writing. The CaP coated scaffolds were loaded using the technique described in the present manuscript with various doses ranging from 1 to 5mg. The square head arrows indicate the melt electrospun fibers and the arrows show the deposited azithromycin.