



## 1 **Abstract**

2           Hollow hydroxyapatite (HA) microspheres showed the ability to facilitate bone regeneration  
3 in rats with non-healing calvarial defects. However, new bone formation in the rat calvarial defect  
4 implanted with the closed HA microspheres was limited. The objective of this work is to evaluate  
5 size-, time, and structure-dependent bone regeneration between open and closed HA microspheres in  
6 an osseous model. Open HA microspheres were obtained by sectioning closed HA microspheres.  
7 The open HA microsphere had dense convex surface and rough and porous concave surface. For  
8 both size ranges ( $\phi 106-150 \mu\text{m}$  vs.  $\phi 212-250 \mu\text{m}$ ), the open HA microsphere were more effective in  
9 facilitating bone regeneration than the closed HA microspheres in rat calvarial defects. Bone  
10 regeneration in the open HA microspheres ( $49 \pm 7\%$  for  $\phi 106-150 \mu\text{m}$ ;  $40 \pm 8\%$  for  $\phi 212-250 \mu\text{m}$ )  
11 were higher than the closed HA microsphere ( $26 \pm 8\%$  for  $\phi 106-150 \mu\text{m}$ ;  $30 \pm 9\%$  for  $\phi 212-250 \mu\text{m}$ )  
12 at 12 weeks. Furthermore, the open HA microspheres of smaller size showed a significant increase in  
13 bone regeneration than the open HA microspheres of larger size at both 6 weeks and 12 weeks. The  
14 difference in bone regeneration between these microspheres could be due to their differences in  
15 microstructures, namely curvature, concavity, porosity, surface roughness, and total surface area  
16 available for cells to attached to.

## 17 **Keywords**

18 Hollow hydroxyapatite microspheres; Osteogenesis; Bone regeneration; Rat calvarial defect model

# 1 **Introduction**

2           Effective regeneration of bone defects caused by trauma or chronic diseases is a significant  
3 clinical challenge. Over the past few decades, researchers have investigated the mechanism of bone  
4 regeneration to better inform the designs of healing strategies [1-3]. Bone healing involves three  
5 primary stages: the early inflammatory stage; the repair stage and the late remodeling stage [4].  
6 These three stages are distinct, but continuous. In the inflammatory stage, a hematoma forms and  
7 inflammatory cells infiltrate the bone, resulting in the formation of granulation tissue, vascular tissue  
8 and immature tissue. During the repair stage, new blood vessels are developed to facilitate tissue  
9 regeneration and a soft callus is formed around the repair site. Bone healing is completed during the  
10 remodeling stage in which the bone is restored to its original shape, structure and mechanical  
11 strength.

12           Clinically, bone deficiency is overcome using treatments that rely on bone regeneration and  
13 augmentation. While various treatments have been investigated with encouraging results [5],  
14 complete and predictable bone reconstruction is often difficult [6]. Autologous bone grafts are the  
15 gold standard for treatment because they contain osteoinductive growth factors, osteogenic cells and  
16 a structural scaffold. However, disadvantages of this treatment include limited tissue availability,  
17 increased surgery time, additional pain and cosmetic imperfection at the donor site [6-8]. Many of  
18 these issues can increase the health care cost for the patient [9]. An alternative to autogenous bone is  
19 allogenic bone, which can induce moderate healing results due to its preserved osteoinductivity.  
20 However, allografts are costly, can have unpredictable effects on growth due to donor variance,  
21 cause adverse immune reactions, and increase the risk of disease transference [10-12]. Synthetic bone

22 grafts have advantages such as consistent quality, safety, and good tissue tolerance, but they usually  
23 function as inert or merely osteoconductive implants. Encouraging results have been reported.

24 Hydroxyapatite (HA), the main component and essential ingredient of human bone, can be  
25 prepared by chemical reactions. Studies have demonstrated that HA supports bone regeneration and  
26 bonding to surrounding tissue because of its biocompatibility, bioactivity, and osteoconductivity  
27 [13]. Our studies with the closed HA microspheres showed the ability to regenerate bone in non-  
28 healing rat calvarial defects [14, 15]. Experiments with  $\phi 106-150 \mu\text{m}$  and  $\phi 150-250 \mu\text{m}$  closed HA  
29 microspheres showed differences in mechanical properties and biological tests [16]. The size  
30 variation of closed HA microspheres could affect the structure of HA microspheres. The changes in  
31 structure can influence on the biological tests in return. We sporadically observed that there tended to  
32 be better bone regeneration with broken closed microspheres with micro-concavity [15, 17]. This  
33 observation motivated us to design this study that focused on enhanced bone regeneration with open  
34 microspheres. We hypothesize that open HA microsphere with special geometric characters can yield  
35 better bone regeneration compared with the closed HA microspheres. Our goal is to investigate  
36 whether bone regeneration in an osseous model is microgeometry-, size-, and time-dependent. To  
37 achieve our goal, two size ranges ( $\phi 106-150 \mu\text{m}$  and  $\phi 212-250 \mu\text{m}$ ) of closed and open HA  
38 microspheres were created. Bone regeneration was conducted with a rat calvarial defect model. No  
39 osteoinductive agents were added in order to distinguish the intrinsic osteogenic properties of the  
40 open HA microspheres.

## 41 **Materials and Method**

## 42 **Preparation of closed and open hollow hydroxyapatite (HA)** 43 **microspheres**

44 The closed hollow HA microspheres were prepared by conversion solid glass microspheres in  
45 aqueous phosphate solution as described in a previous study. Briefly, calcium-lithium-borate glass  
46 with the composition of 15CaO, 11Li<sub>2</sub>O and 74B<sub>2</sub>O<sub>3</sub> (wt. %), designated as CaLB3-15, was prepared  
47 by melting CaCO<sub>3</sub>, Li<sub>2</sub>CO<sub>3</sub>, H<sub>3</sub>BO<sub>3</sub> (Alfa Aesar, Haverhill, MA, USA) in a platinum crucible at 1200  
48 °C for 45 min and then quenching the melt between stainless steel plates. Glass particles of were  
49 obtained by grinding the glass *via* a mortar and pestle, crashing in a shatter box and sieving through  
50 100 and 140 mesh sieves for  $\phi$ 106-150  $\mu$ m in size, or 60 and 70 mesh sieves for  $\phi$ 212-250  $\mu$ m in  
51 size. Glass microspheres were obtained by dropping the crushed particles down through a vertical  
52 furnace at 1200 °C. The closed hollow hydroxyapatite microspheres were obtained by reacting the  
53 glass microspheres in a 0.02 M K<sub>2</sub>HPO<sub>4</sub> solution at 37 °C and pH = 9 for 7 days. In the conversion  
54 process, 1 g glass was immersed in a 200 ml phosphate solution and the system was stirred gently  
55 and continuously. The converted microspheres were washed with distilled water and anhydrous  
56 ethanol, and then dried at room temperature for at least 12 h and at 90 °C for at least 12 h.

57 The open hollow HA microspheres were obtained by sectioning the closed hollow HA  
58 microspheres using a microtome. Briefly, the closed HA microspheres were fixed on a wax block  
59 using a water-soluble tape and were sectioned by microtome. The open HA microspheres were  
60 washed with distilled water and ethanol, and then dried at room temperature for at least 12 h and at  
61 90 °C for at least 12 h. The debris in open HA microspheres were removed using sieves.

## 62 **Characterization of closed and open hollow hydroxyapatite (HA)** 63 **microspheres**

64 The microstructures of the closed HA microspheres, cross-section of closed HA microspheres,  
65 and open HA microspheres were observed using a scanning electron microscope (SEM; S4700  
66 Hitachi, Tokyo, Japan) with an accelerating voltage of 15kV and working distance at 12 mm. The  
67 local composition of the surface layer, middle layer and inner layer of the mesoporous shell wall of  
68 the HA microspheres was investigated using energy dispersive X-ray (EDS) analysis in SEM with an  
69 electron beam spot size of 1  $\mu\text{m}$ .

70 The specific surface area (SSA) of the closed and open HA microspheres and pore size  
71 distribution of the shell wall were measured by using nitrogen absorption (Autosorb-1;  
72 Quantachrome, Boynton Beach, FL) as described in a previous study. Three hundred milligrams of  
73 closed or open HA microspheres were weighted and evacuated at 120 °C for 15 h to remove  
74 absorbed moisture. The volume of nitrogen absorbed and desorbed at different relative gas pressure  
75 was measured and used to construct adsorption-desorption isotherms. The first twelve points of the  
76 adsorption isotherm, which initially followed a linear trend implying monolayer formation of  
77 adsorbate, were fitted to the Brunauer-Emmett-Teller equation to determine the specific surface area.  
78 The pore size distribution of the shell wall of the hollow HA microspheres was calculated using the  
79 Barrett-Joiner-Halenda method applied to the deposition isotherms [18].

## 80 **Animals and surgical procedures**

81 All animal use and care procedures were approved by the Missouri S&T Institutional Animal  
82 Care and Use Committee in compliance with the NIH Guide for Care and Use of Laboratory  
83 Animals (1985). The rat calvarial defects were implanted with four groups of implants composed of  
84 closed or open hollow HA microspheres for 6 weeks and 12 weeks (Table 1). The implantation time  
85 was based upon considerable bone regeneration in rat calvarial defects implanted with hollow HA  
86 microspheres observed in previous studies. The closed or open HA microspheres of  $\phi$ 212-250  $\mu$ m  
87 were randomly implanted to defect areas. The closed or open HA microspheres of  $\phi$ 106-150  $\mu$ m  
88 microspheres were randomly implanted to defect areas, but mixing implants of closed and open  
89 microspheres in the same animal was avoided due to the possible migration of low-weight open HA  
90 microspheres.

91 **Table 1. Implants groups composed of closed or open hollow hydroxyapatite microspheres.**

Group	HA microspheres		Sample size (n)	
			6 weeks	12 weeks
1	106-150 $\mu$ m	Closed	5	5
2		Open	5	5
3	212-250 $\mu$ m	Closed	5	10
4		Open	5	10

92 The male Sprague-Dawley rats (3 months old, weight =  $350 \pm 30$  g, Envigo, USA) were  
93 acclimated for 2 weeks to diet, water, and housing under a 12 h/12 h light/dark cycle. The rats were  
94 anesthetized with a combination of ketamine and xylene (0.15  $\mu$ l per 100 g) and maintained under

95 anesthesia with isoflurane in oxygen. The surgery area was shaved, scrubbed with 70% ethanol and  
96 iodine, and draped. With sterile instruments and using an aseptic technique, a 1 cm cranial skin  
97 incision was made in an anterior to posterior direction along the midline. The subcutaneous tissue,  
98 musculature and periosteum were dissected and reflected to expose the calvaria. Bilateral full  
99 thickness defects (4.6 mm in diameter) were created in the central area of each parietal bone using a  
100 saline-cooled trephine drill. The sites were constantly irrigated with sterile PBS to prevent  
101 overheating of the bone margins and to remove the bone debris. Each defect was randomly implanted  
102 with HA microspheres of each group. After the implantation of the hollow HA microspheres, one  
103 drop of Ringer's solution was added to each defect. The periosteum and skin were repositioned and  
104 closed with wound clips. Each animal received an intramuscular injection of ~200 µl buprenorphine  
105 and ~200 µl penicillin post-surgery. All animals were monitored daily for the condition of the  
106 surgical wound, food intake, activity and clinical signs of infection. After 6 weeks, the animals were  
107 sacrificed by CO<sub>2</sub> inhalation, and the calvarial defect sites with surrounding bone and soft tissue  
108 were harvested for subsequent evaluations.

## 109 **Histological processing**

110 Harvested calvarial samples were fixed in a 10% formaldehyde solution for five days. The  
111 samples were cut into half after being washed with deionized water. Half of the sample was for  
112 paraffin embedding, and the other half was for poly (methyl methacrylate) (PMMA) embedding. The  
113 paraffin-embedded samples were decalcified in 14 wt. % ethylenediaminetetraacetic acid (EDTA,  
114 Sigma-Aldrich, USA) for 2 weeks, dehydrated in ethanol, and then embedded in paraffin using  
115 standard histological techniques. These samples were sectioned using microtome. The thickness of



116 the tissue section with paraffin was 5  $\mu\text{m}$ . These slices were then stained with hematoxylin and eosin  
117 (H&E) [19]. Without decalcification, the samples for PMMA embedding were dehydrated in ethanol  
118 and embedded in PMMA. These samples were sectioned, affixed to acrylic slices, and ground to a  
119 thickness down to 50  $\mu\text{m}$  using a micro-grinding system (EXAKT 400CS, Norderstedt, Germany).  
120 The von Kossa staining was used to observe mineralization [20].

## 121 **Histomorphometric analysis**

122 Histomorphometric analysis was carried out using optical images of stained sections and  
123 Image J software (National Health Institute, USA). The percentage of new bone formed in calvarial  
124 defect was evaluated from the H&E stained sections. The newly formed bone was identified by  
125 outlining the edge of the defect, with the presence of old and new bone being identified by lamellar  
126 and woven bone, respectively. The total defect area was measured from one edge of the old calvarial  
127 bone, including the entire implant and tissue within it, to the other edge of the old bone. The newly  
128 formed bone within this area was then outlined and measured; the amount of the new bone was  
129 expressed as a percentage of the total defect area. The amount of von Kossa positive area was shown  
130 as a percent of the total defect area.

## 131 **Statistical analysis**

132 Measurements of the percentage of new bone (relative to the entire defect area) were  
133 expressed as a mean  $\pm$  SD. Analysis for differences between groups was performed using one-way  
134 analysis of variance (ANOVA) followed by the Tukey's post hoc test; the differences were  
135 considered significant at  $P < 0.05$ .

## 136 **Results**

### 137 **Geometry of the closed and open hydroxyapatite microspheres**

138       The closed HA microspheres were prepared by converting glass microspheres in a phosphate  
139 solution. The diameters of the starting glass microspheres were  $\phi$ 106-150  $\mu\text{m}$  and  $\phi$ 212-250  $\mu\text{m}$ ,  
140 respectively. After conversion, changes in the diameter of the microspheres were negligible. The  
141 SEM images revealed a spherical shape of closed HA microspheres with two size ranges:  $\phi$ 106-150  
142  $\mu\text{m}$  (thereafter, small size; Fig. 1A1 and A2) and  $\phi$ 212-250  $\mu\text{m}$  (thereafter, large size; Fig. 1C1 and  
143 C2). Open HA microspheres were sectioned from closed HA microspheres using a microtome. The  
144 SEM images confirmed precise sectioning of open HA microspheres of both sizes (Fig. 1B1, B2, D1  
145 and D2). Compared to the complete spherical structure of closed HA microspheres, the open HA  
146 microspheres were near hemispherical. The hollow microsphere had a mesoporous shell and a  
147 hollow core (0.6 of the microsphere diameter). The shell wall consisted of two distinct layers: a  
148 denser external layer and a more porous internal layer. For both size ranges of HA microspheres, the  
149 thickness of the denser layer was  $\sim 5 \mu\text{m}$ . The open HA microspheres of both sizes showed the dense  
150 external part and rough and porous internal part of the shell wall (Fig. 2). Both size ranges of the  
151 closed and open HA microspheres showed similar microstructures of the shell wall. The HA  
152 microspheres were formed by needle-like hydroxyapatite nanoparticles. The external surface tended  
153 to be denser than the internal surface.

154 **Figure 1. SEM images of 106-150  $\mu\text{m}$  closed HA microspheres (A1, A2) and open HA**  
155 **microspheres (B1, B2) and 212-250  $\mu\text{m}$  closed HA microspheres (C1, C2) and open HA**  
156 **microspheres (D1, D2).**

157 **Figure 2. SEM images of external surface (A) and internal surface (B) of 106-150  $\mu\text{m}$  open HA**  
158 **microspheres and external surface (C) and internal surface (D) of 212-250  $\mu\text{m}$  open HA**  
159 **microspheres.**

160 The BET surface area and average pore size of closed HA microspheres in two size ranges are  
161 summarized in Table 2. The surface areas of small and large closed HA microspheres were 101  $\text{m}^2/\text{g}$   
162 and 168  $\text{m}^2/\text{g}$ , respectively. The average pore sizes of small and large closed HA microspheres were  
163 13 nm and 10 nm, respectively. The surface area was higher in the large HA microspheres, while the  
164 average pore size was higher in the small HA microspheres.

165 **Table 2. Surface area and average pore size of 106-150  $\mu\text{m}$  and 212-250  $\mu\text{m}$  HA microspheres.**

HA microspheres	Surface area ( $\text{m}^2/\text{g}$ )	Average pore size (nm)
106-150 $\mu\text{m}$	101	13
212-250 $\mu\text{m}$	168	10

## 166 **Composition of the closed and open hollow hydroxyapatite** 167 **microspheres**

168 A high-resolution cross-section of the hollow HA microspheres in both sizes is shown in Fig.  
169 3. The shell walls of the microspheres were divided into three regions: external layer, middle layer  
170 and inner layer. Compositions at the midpoint of each region were analyzed by EDS for the Ca/P

171 atomic ratio (Table 3). The Ca/P atomic ratios of the HA microspheres of small size from the surface  
 172 layer to the inner layer were  $1.63 \pm 0.11$ ,  $1.63 \pm 0.11$ , and  $1.60 \pm 0.14$ . The Ca/P atomic ratios of the  
 173 HA microsphere of large size from the surface layer to the inner layer were  $1.67 \pm 0.10$ ,  $1.63 \pm 0.08$   
 174 and  $1.63 \pm 0.06$ . There was no significant difference in Ca/P ratio within the three regions or between  
 175 the two size ranges of HA microspheres ( $n=10$ ,  $p>0.05$ ). The Ca/P atomic ratios of the three regions  
 176 were close to the theoretical Ca/P value of stoichiometric hydroxyapatite, 1.67.

177 **Figure 3. SEM images of cross section of 106-150  $\mu\text{m}$  open HA microspheres (A) and 212-**  
 178 **250 $\mu\text{m}$  open HA microspheres (B).**

179 **Table 3. Ca/P atomic ratio ( $n = 10$ ; mean  $\pm$  SD) for the three regions for 106–150  $\mu\text{m}$  and**  
 180 **212–250  $\mu\text{m}$ .**

HA microspheres	Cross-sectional zone	Ca/P atomic ratio (mean $\pm$ SD)
106-150 $\mu\text{m}$	Surface layer (A)	$1.63 \pm 0.11$
	Middle layer (B)	$1.63 \pm 0.11$
	Inner layer (C)	$1.60 \pm 0.14$
212-250 $\mu\text{m}$	Surface layer (D)	$1.67 \pm 0.10$
	Middle layer (E)	$1.63 \pm 0.08$
	Inner layer (F)	$1.63 \pm 0.06$

## 181 **Evaluation of bone regeneration in rat calvarial defects**

182 H&E and von Kossa stained sections of the implants with closed and open hollow HA  
 183 microspheres of the two size ranges after 6 weeks in rat calvarial defects are shown in Fig. 4 and Fig.

184 5. Bone regeneration was limited and confined mainly to the edge of the defects and some bone  
185 bridging along the bottom of implants. Fibrous tissues (light blue in H&E stained sections) filled the  
186 space between the microspheres. New bone formation in the implants with the smaller size of closed  
187 and open HA microspheres was  $12 \pm 3\%$  and  $17 \pm 6\%$ , respectively (Fig. 4 and Table 4). The von  
188 Kossa positive areas in in the implants with the smaller size of closed and open HA microspheres  
189 were  $41 \pm 3\%$  and  $49 \pm 5\%$ , respectively (Table 5). The percentages of new bone in the implants  
190 with the larger size of closed and open HA microspheres were  $6 \pm 2\%$  and  $12 \pm 3\%$ , respectively  
191 (Fig. 5). The von Kossa positive areas in the implants with the larger size of closed and open HA  
192 microspheres were  $30 \pm 3\%$  and  $35 \pm 3\%$ , respectively. Open HA microspheres showed significant  
193 improvement in bone regeneration compared with closed HA microspheres for both size ranges at 6  
194 weeks in rat calvarial defects ( $n = 5$ ,  $p$ 's  $< 0.05$  for both sizes, Fig. 8 and 9). Smaller closed HA  
195 microspheres showed a significant increase in bone regeneration than the larger closed HA  
196 microspheres ( $n = 5$ ,  $p < 0.05$ ). Based on the H&E results, there was a borderline difference in new  
197 bone formation between the two size ranges of open HA microspheres ( $n = 5$ ,  $p = 0.050$ ). However,  
198 based on von Kossa results, the smaller open HA microspheres showed a significant enhancement in  
199 bone growth compared to the larger open HA microspheres ( $n = 5$ ,  $p < 0.001$ ).

200 **Table 4. Comparative new bone formation in all implants after 6 or 12 weeks based on H&E**  
201 **staining. The amount of new bone is expressed as a percent of the total defect area (mean  $\pm$**   
202 **SD).**

203

Hollow HA microspheres		New bone (%)	
		6 weeks	12 weeks
φ106-150 μm	Closed	12 ± 3	26 ± 8
	Open	17 ± 6	49 ± 7
φ212-250 μm	Closed	6 ± 2	30 ± 9
	Open	12 ± 3	40 ± 8

204 **Figure 4. H&E stained and von Kossa sections of implants composed of closed (A1, B1) and**  
 205 **open (A2, B2) hollow HA microspheres (φ106-150 μm) after 6 weeks in rat calvarial defects;**  
 206 **(C, D) higher-magnification images of boxed area in (A1, A2). HB: host bone; NB: new bone.**  
 207 **Blue arrow: new bone growth in micro-concavity.**

208 **Figure 5. H&E and von Kossa stained sections of implants composed of closed (A1, B1) and**  
 209 **open (A2, B2) HA microspheres (φ212-250 μm) after 6 weeks in rat calvarial defects; (C, D)**  
 210 **higher-magnification images of boxed area in (A1, A2). HB: host bone; NB: new bone. Blue**  
 211 **arrow: new bone growth in micro-concavity.**

212 **Figure 8. Comparative new bone formation in implants with closed and open hollow HA**  
 213 **microspheres with diameter of 106-150 μm or 212-250 μm after 6 weeks (6 W) and 12 weeks**  
 214 **(12 W) in rat calvarial defects (Mean ± SD; n = 5~10, \* significant difference between groups; *p***  
 215 **< 0.05).**

216 **Figure 9. Comparative von Kossa positive area for implants of closed and open hollow HA**  
 217 **microspheres with diameter of 106-150 μm or 212-250 μm after 6 weeks (6 W) and 12 weeks (12**

218 **W) in rat calvarial defects (Mean  $\pm$  SD; n = 5~10, \* significant difference between groups;  $p <$**   
219 **0.05).**

220 Higher magnification images of the closed and open HA microspheres of both sizes are shown  
221 in Fig. 4C and D (from the boxed areas of Fig. 4A1 and A2) and Fig. 5C and D (from the boxed  
222 areas in Fig. 5A1 and A2). For the closed HA microspheres in both size ranges, bone formation was  
223 scanty, while the fibrous tissues filled the pore space between the closed HA microspheres and  
224 infiltrated into the hollow core of some broken closed HA microspheres. In comparison, more bone  
225 regeneration was observed in the micro-concavity of open HA microspheres (indicated by blue  
226 arrows) in both sizes ( $\phi$ 106-150  $\mu$ m and  $\phi$ 212-250  $\mu$ m).

227 The outcomes from the implants with the closed and open HA microspheres of the two size  
228 ranges after 12 weeks in rat calvarial defects are shown in Figs. 6 and 7. New bones were formed  
229 from the edge of the defects and on the bottom of the implants. For the open HA microspheres of  
230 both size ranges, more new bone growth in the micro-concavity can be found; the remaining open  
231 HA microspheres can be observed in the new bone bridging the ends of defects. For the closed and  
232 open HA microspheres of small size (Fig. 6), the percentages of new bone formation were  $26 \pm 8\%$   
233 and  $49 \pm 7\%$ , respectively; the von Kossa positive areas were  $55 \pm 5\%$  and  $76 \pm 4\%$ , respectively.  
234 For the closed and open HA microspheres of large size (Fig. 7), the percentages of new bone were  $30$   
235  $\pm 9\%$  and  $40 \pm 8\%$ , respectively; the von Kossa positive areas were  $56 \pm 5\%$  and  $65 \pm 5\%$ ,  
236 respectively. The open HA microspheres showed significant improvement in bone regeneration when  
237 compared to the closed HA microspheres in both size ranges during a period of 12 weeks in rat  
238 calvarial defects (n = 5,  $p$ 's < 0.001 for small size; n = 5,  $p$ 's < 0.05 for large size). There was no

239 significant difference in new bone formation between the two size ranges of closed HA microspheres  
240 ( $n = 5\sim 10, p > 0.05$ ). However, smaller open HA microspheres showed a more significant increase in  
241 bone regeneration than larger closed HA microspheres ( $n = 5, p < 0.05$ ). Bone regeneration was  
242 time-dependent for both size ranges; new bone formation increased significantly from 6 weeks to 12  
243 weeks in rat calvarial defects ( $n = 5, p's < 0.001$  for closed HA microspheres;  $n = 5\sim 10, p's < 0.001$   
244 for open HA microspheres).

245 **Figure 6. H&E and von Kossa stained sections of implants composed of closed (A1, B1) and**  
246 **open (A2, B2) HA microspheres ( $\phi 106\text{-}150\ \mu\text{m}$ ) after 12 weeks in rat calvarial defects; (C, D)**  
247 **higher-magnification images of boxed area in (A1, A2). HB: host bone; NB: new bone. Blue**  
248 **arrow: new bone growth in micro-concavity.**

249 **Figure 7. H&E and von Kossa stained sections of implants composed of closed (A1, B1) and**  
250 **open (A2, B2) HA microspheres ( $\phi 212\text{-}250\ \mu\text{m}$ ) after 12 weeks in rat calvarial defects; (C, D)**  
251 **higher-magnification images of boxed area in (A1, A2). HB: host bone; NB: new bone. Blue**  
252 **arrow: new bone growth in micro-concavity.**

253 A comparison of closed and open HA microspheres in both sizes at 12 weeks is shown in  
254 higher magnified images in Fig. 6C and D (from the boxed areas of Fig. 6A1 and A2) and Fig. 7C  
255 and D (from the boxed areas of Fig. 7A1 and A2). Bone regeneration in the cores of some broken  
256 closed HA microspheres was identified. A higher degree of new bone formation in the micro-  
257 concavity of open HA microspheres was observed.

## 258 **Discussion**



259 The capability of HA microspheres to regenerate bone can presumably be affected by the  
260 differences between closed and open HA microspheres in microstructure. In this study, the  
261 microstructure of closed and open HA microspheres in two size ranges ( $\phi$ 106-150  $\mu\text{m}$  vs.  $\phi$ 212-250  
262  $\mu\text{m}$ ) were analyzed. To test HA microspheres in facilitating bone regeneration, rat calvarial defects  
263 were created and HA microspheres were implanted. Bone regeneration was evaluated in weeks 6 &  
264 12.

265 For both size ranges, the thickness of the denser (outer) layer was  $\sim 5 \mu\text{m}$ , while the ratio of  
266 the hollow core diameter to the microsphere diameter is  $\sim 0.6$ . The factors leading to these two  
267 distinct layers are still unclear. In the glass conversion process [16, 21-23], ions are dissolved from  
268 glass (i.e.,  $\text{Ca}^{2+}$ ,  $\text{Li}^+$ ,  $\text{B}^{3+}$ ) to the aqueous solution. The  $\text{Ca}^{2+}$  from glass reacts immediately with  
269 phosphate anions from solution to form calcium phosphate. The calcium phosphate precipitates onto  
270 the glass surface due to its insolubility in the system. As the glass dissolves, the calcium phosphate  
271 layer continues to thicken until the glass is completely converted to calcium phosphate. The kinetics  
272 and mechanism of the formation of the HA layer in borate glass is investigated in several studies [22,  
273 24-26]. The conversion rate is initially described by a reaction-controlled model (linear kinetics);  
274 however, at the later stage, a three-dimensional diffusion model (parabolic kinetics) better explains  
275 the conversion rate. Presumably, the denser layer and porous layer results from these two kinetic  
276 models. Additional experiments can be set-up to further investigate the dynamic changes of SSA and  
277 pore size.

278 Our *in vivo* experiment showed the effectiveness of open HA microspheres in bone  
279 regeneration. For both size ranges of the open HA microspheres, new bone formation was observed

280 in both 6 weeks and 12 weeks post-implantation. The amount of new bone growth increased from 6  
281 weeks to 12 weeks. In the study of 12-week implantation with small microspheres, new bone  
282 formation with the implants of open microsphere was about twice that of the closed microspheres;  
283 for large microspheres, new bone formation in the implants of open microspheres was about 30%  
284 higher than that of the closed microspheres. Thus, the open microspheres were more effective in  
285 facilitating bone regeneration than the closed microspheres. Compared to the closed microsphere, the  
286 open microsphere had a micro-concave region with a more porous and rougher surface (see Fig  
287 1&2). These characters (i.e., micro-concavity, porosity, roughness) could contribute to the difference  
288 in bone regeneration between the closed and open microspheres.

289         The effectiveness of micro-concavity in bone regeneration has been investigated by others  
290 [27-32]. Substantial mineralization of simulated body fluid on the discs made of calcium phosphate  
291 ceramic were observed inside concavities but not at the planar surface [31]. Smaller concavity (0.4  
292 mm in diameter) can induce much more mineralization than larger concavities (0.8 mm or 1.8 mm in  
293 diameter) [31]. An *in vivo* study demonstrated that concavity appeared to stimulate formation of  
294 blood vessels, a critical process for bone formation [32]. Stem cells showed better outcomes on a  
295 concave surface than a flat surface in terms of cell maturation, osteodifferentiation, and specific  
296 protein production [28]. Bone formation by intramembranous ossification preferred to occur on a  
297 concave surface as well [30]. Concavity is also conducive to accumulation of growth factors such as  
298 BMPs [27]. Differences in microstructure may also be a contributing factor to the outcome of bone  
299 regeneration. The internal concave surface was more porous and rougher compared to external  
300 convex surface.

301 The differences in porosity and roughness could influence dissolution/degradation of  
302 biomaterials, adsorption of growth factors, and mineral deposition from body fluid [33-40]. For  
303 instance, the degradation of a porous surface could lead to faster  $\text{Ca}^{2+}$  release which is a key factor in  
304 facilitating angiogenesis [41]. Further, a more porous and rougher surface could be a more suitable  
305 substrate for adsorption of biologically active molecules, such as BMPs and growth factors.  
306 Together, these lead to enhanced cell attachment, proliferation and differentiation.

307 Dissolution/degradation of HA have been shown to be affected by the ratio of Ca/P of the  
308 microspheres [42-44]. The dissolution of HA in water increased as the Ca/P ratio decreased [42].  
309 Higher dissolution/degradation of HA could release more  $\text{Ca}^{2+}$  and phosphate ions, which could  
310 facilitate bone regeneration. In this study, there was no significant difference in Ca/P ratio within the  
311 three regions or between the two size ranges of HA microspheres. It is possible that the Ca/P ratio of  
312 our HA microspheres can be manipulated to achieve varying degree of dissolution.

313 The current study demonstrated that the small open microspheres induced a more significant  
314 increase in bone regeneration than the large open microspheres at both 6 weeks and 12 weeks. One  
315 reason for this difference may be attributed to total surface area on microspheres where cells can be  
316 attached to. A simulation of the difference in available surface area for cell attachment was made.  
317 Given the same mass, the same size distribution pattern of the open and closed microspheres of the  
318 same size, and the same density of the shell of the microspheres of different sizes, the open  
319 microspheres of the same size have larger surface area than that of the closed microspheres for cells  
320 to attached to. For instance, the closed and open microspheres of  $\phi 106\text{-}150\ \mu\text{m}$  have surface areas of  
321  $584\ \text{cm}^2$  (assuming the total volume of the microsphere shell is  $1\ \text{cm}^3$ ) and  $981\ \text{cm}^2$ , respectively.

322 The closed and open microspheres of  $\phi$ 212-250  $\mu\text{m}$  surface areas of have 328  $\text{cm}^2$  and 552  $\text{cm}^2$ ,  
323 respectively. Another reason for the difference in bone regeneration could be due to the curvature.  
324 The small microspheres have higher curvature than the large microspheres. It remains to be  
325 investigated how the curvature of the microspheres affect cellular physiology leading to the  
326 differential outcome of bone regeneration.

327 An apparent observation is that new bone formation with implants of the small open  
328 microspheres was able to completely bridge the defects at the bottoms of all the implants. In  
329 comparison, not all animals with closed microspheres were able to bridge the entire defects. During  
330 the regeneration process, new bone formation started from the edge of the host bone and from the  
331 bottom of the defect (dura matter), where osteogenic cells and blood supply were abundant. The  
332 open microspheres might absorb the osteogenic factors by diffusion or fluid transport and trigger  
333 bone growth in the micro-concavity. The open microspheres at the bottom of the implants had the  
334 best chance of contact with the osteogenic factors not only from dura matter but also from the edges  
335 induced by the open microsphere in periphery. We observed that a large number of smaller pieces of  
336 open microspheres was found in the bottom of the implants. This might be caused by the rats'  
337 physical activity of daily living.

338 In this work, the closed HA microspheres of  $\phi$ 106-150  $\mu\text{m}$  significantly enhance bone  
339 regeneration than those of  $\phi$  212-250  $\mu\text{m}$  at 6 weeks; no significant difference in bone regeneration  
340 between two size ranges at 12 weeks. Compared to the work by Fu [14], new bone formation with  
341 the closed HA microspheres of  $\phi$ 150-250  $\mu\text{m}$  was significantly greater than that with the closed HA  
342 microspheres of  $\phi$ 106-150  $\mu\text{m}$  at 12 weeks. It should be noted that there is a significant difference in

343 the size range of the large microspheres between these two studies; thus, they should not be viewed  
344 as conflicting results.

## 345 **Conclusion**

346 The open HA microspheres significantly enhance the bone regeneration as compared to the  
347 closed HA microspheres at both 6 weeks and 12 weeks. Compared with the larger size of open HA  
348 microspheres (smaller curvature), the smaller size of open HA microspheres (larger curvature)  
349 resulted in a more significant increase in bone regeneration. The differences in microstructures of the  
350 HA microspheres (i.e., curvature, concavity, porosity, surface roughness, total surface area available  
351 for cells to attached to) may deserve future attention of investigation.

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## 356 **Conflicts of Interest**

357 The authors declare that there are no conflicts of interest.

## 358 **Abbreviations**

359 BSA, bovine serum albumin; HA, hydroxyapatite; PBS, phosphate Buffer Saline; BMP-2, bone  
360 morphogenetic protein-2; H&E, hematoxylin and eosin; FBS, fetal bovine serum; EDTA,  
361 ethylenediaminetetraacetic acid

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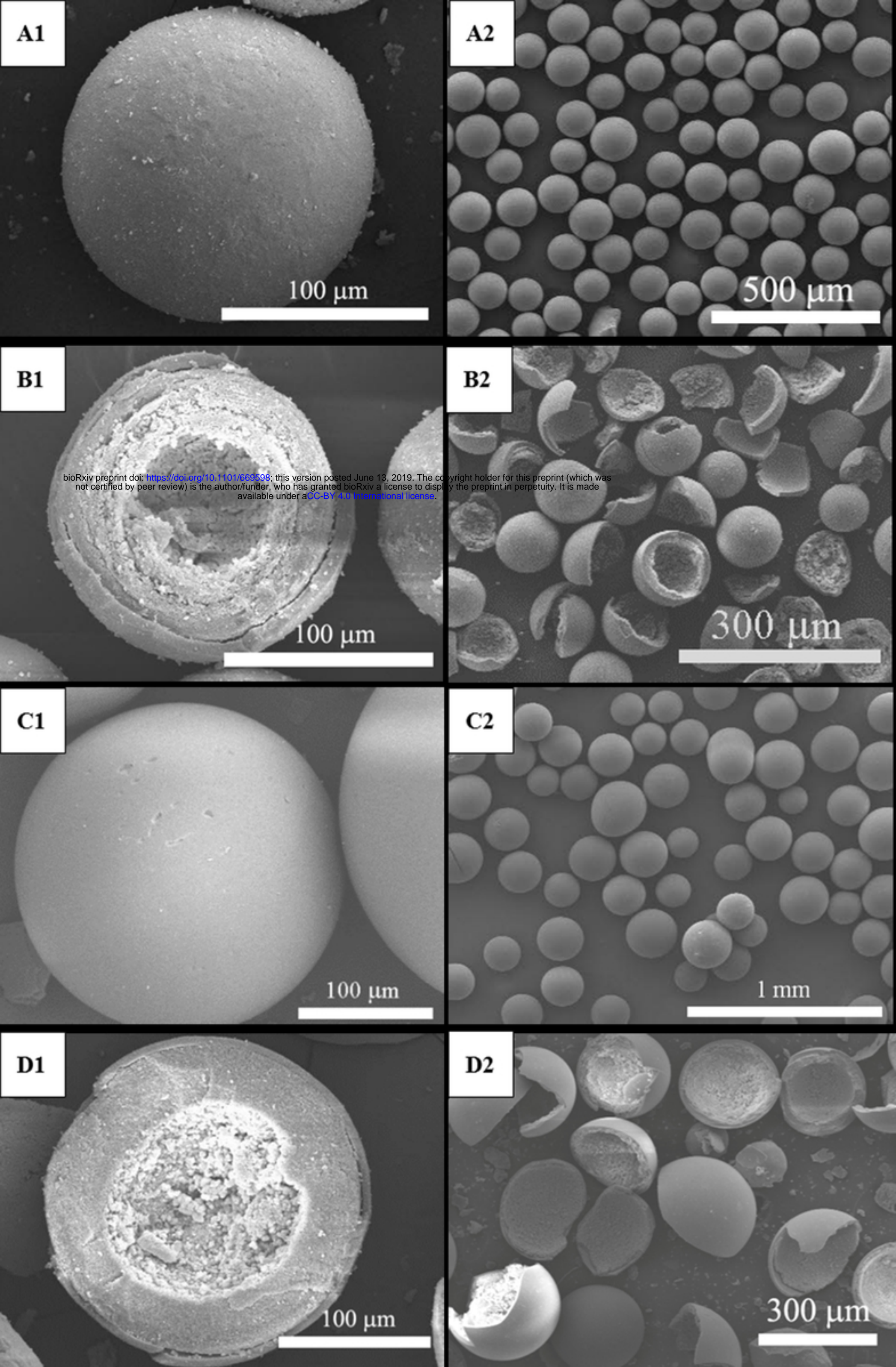


Figure 1



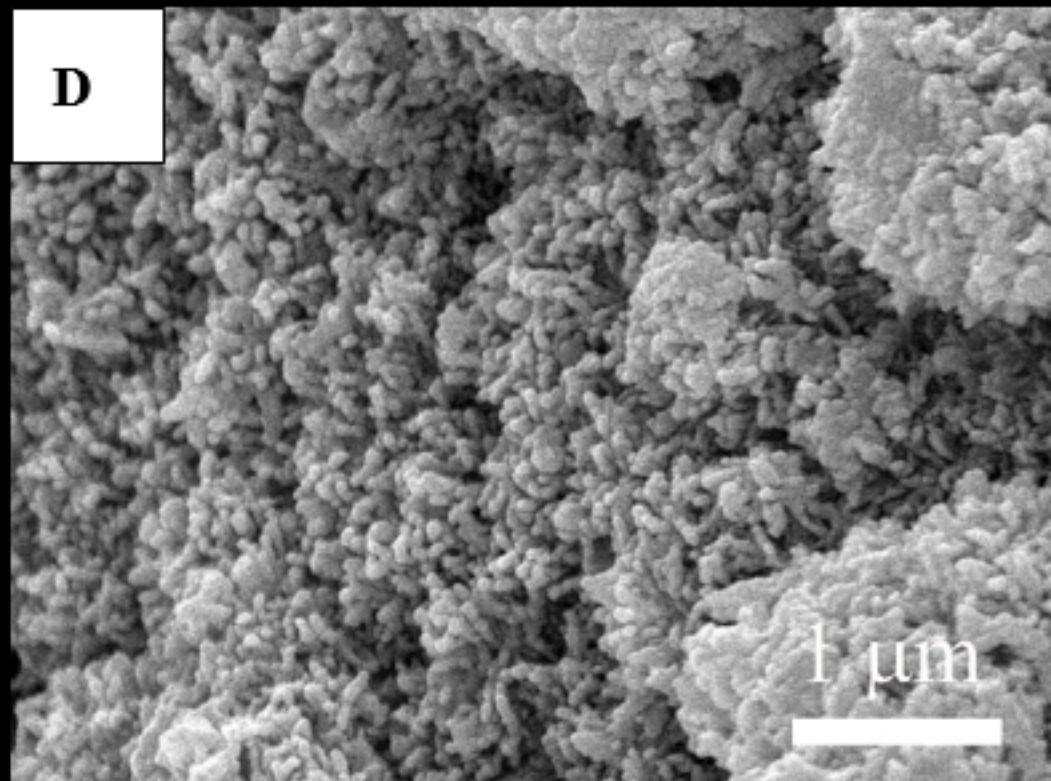
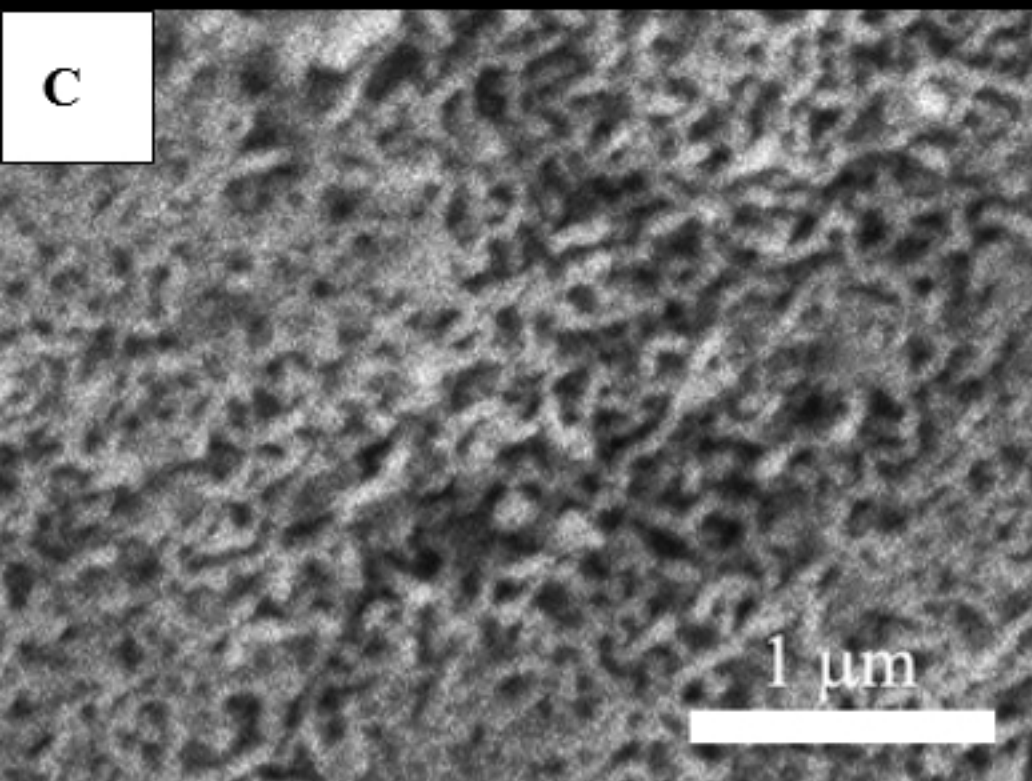
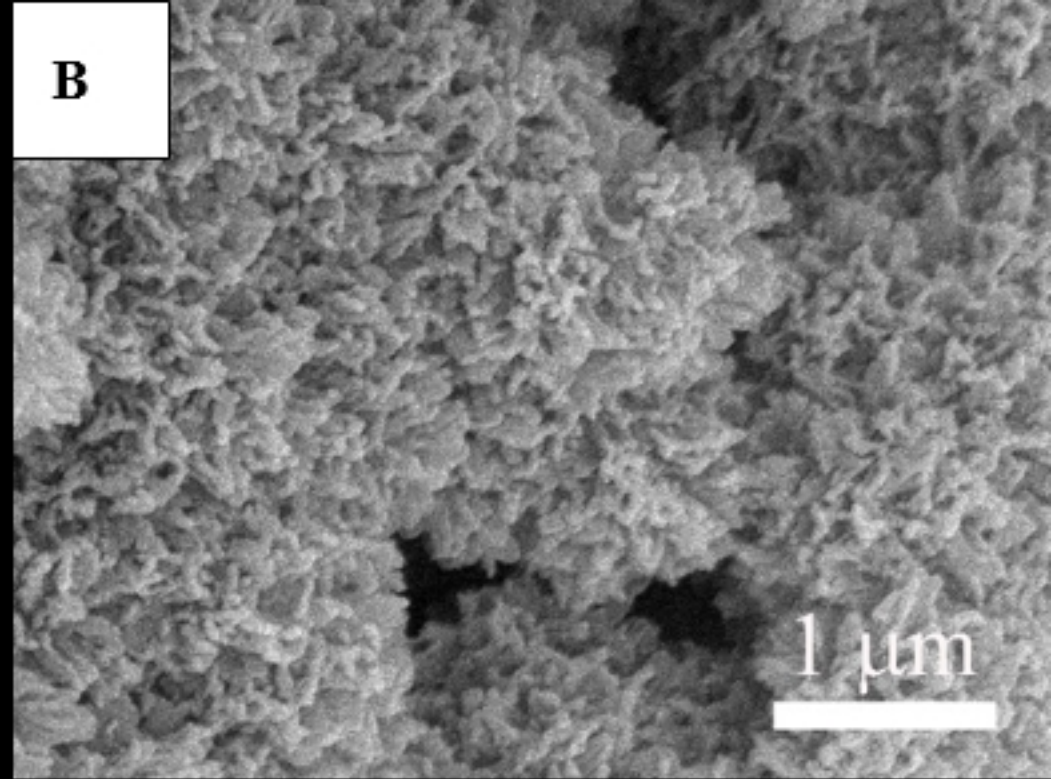
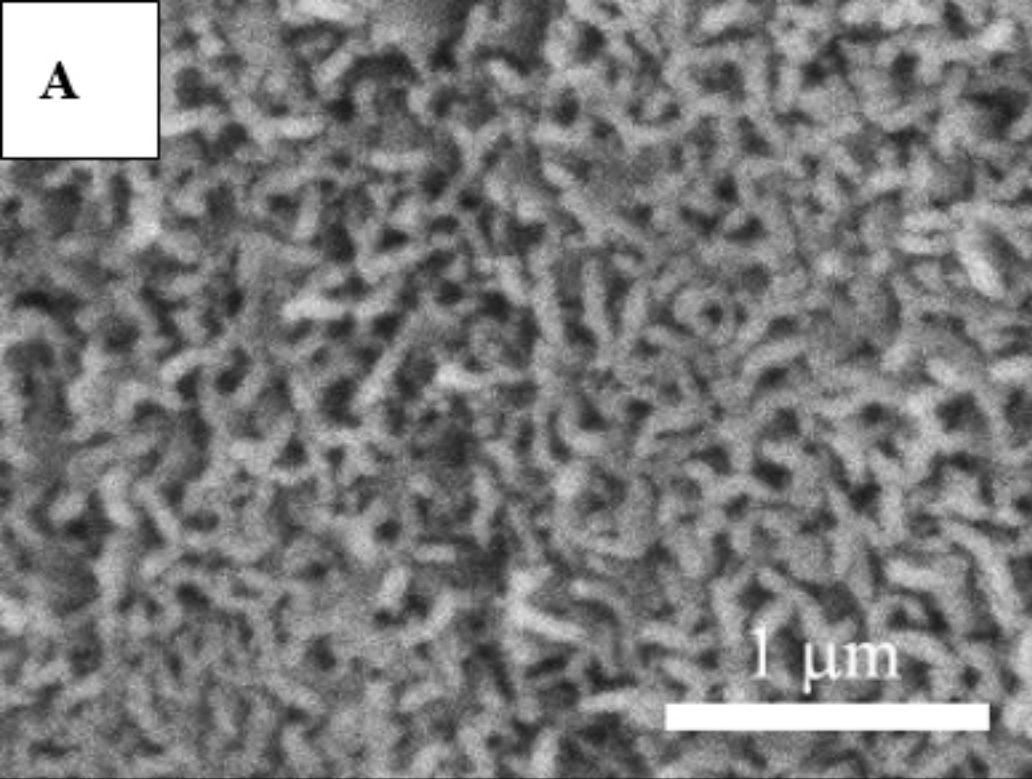


Figure 2

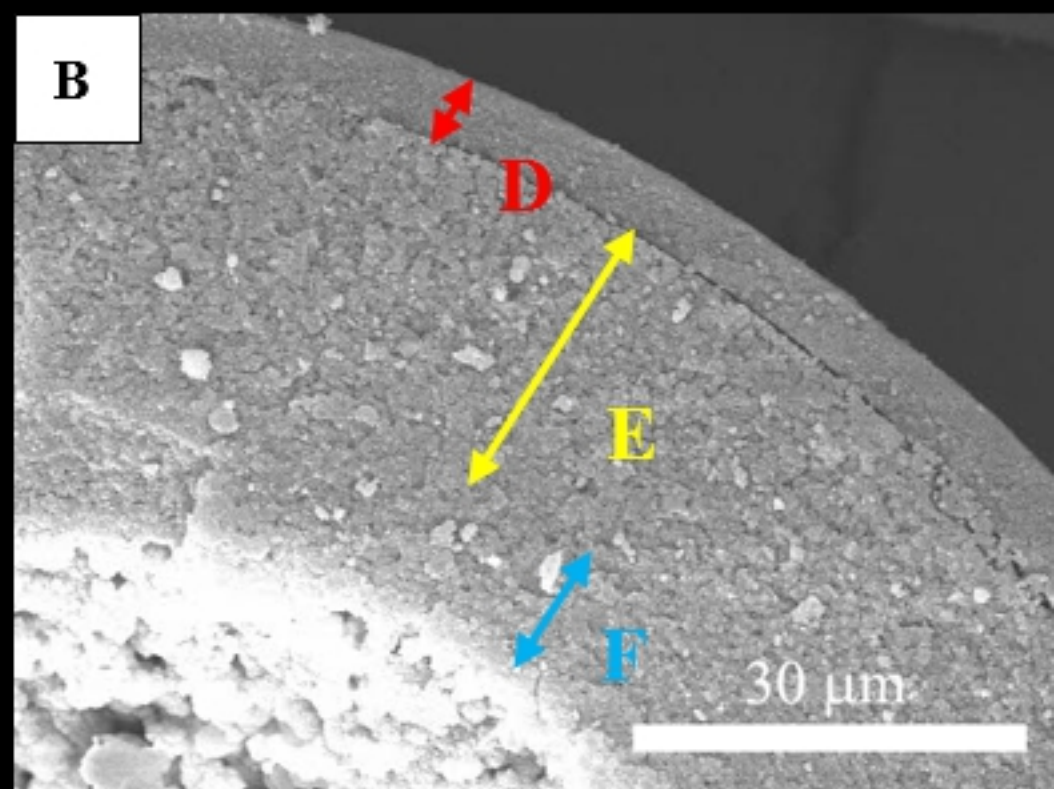
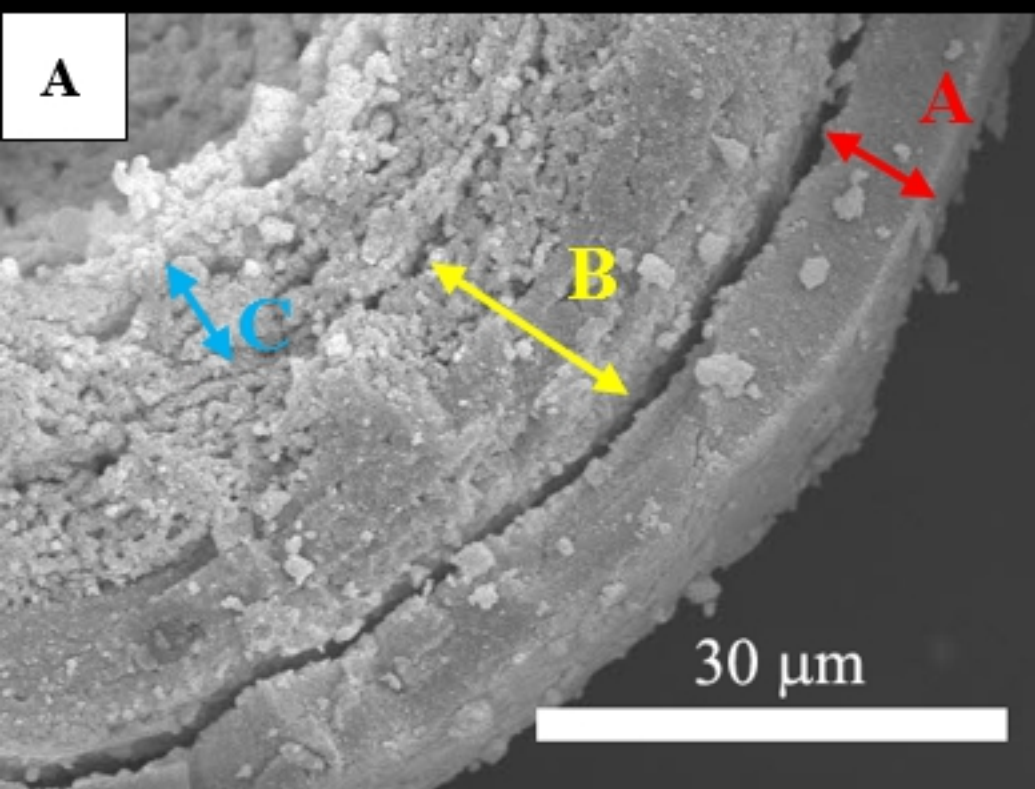


Figure 3



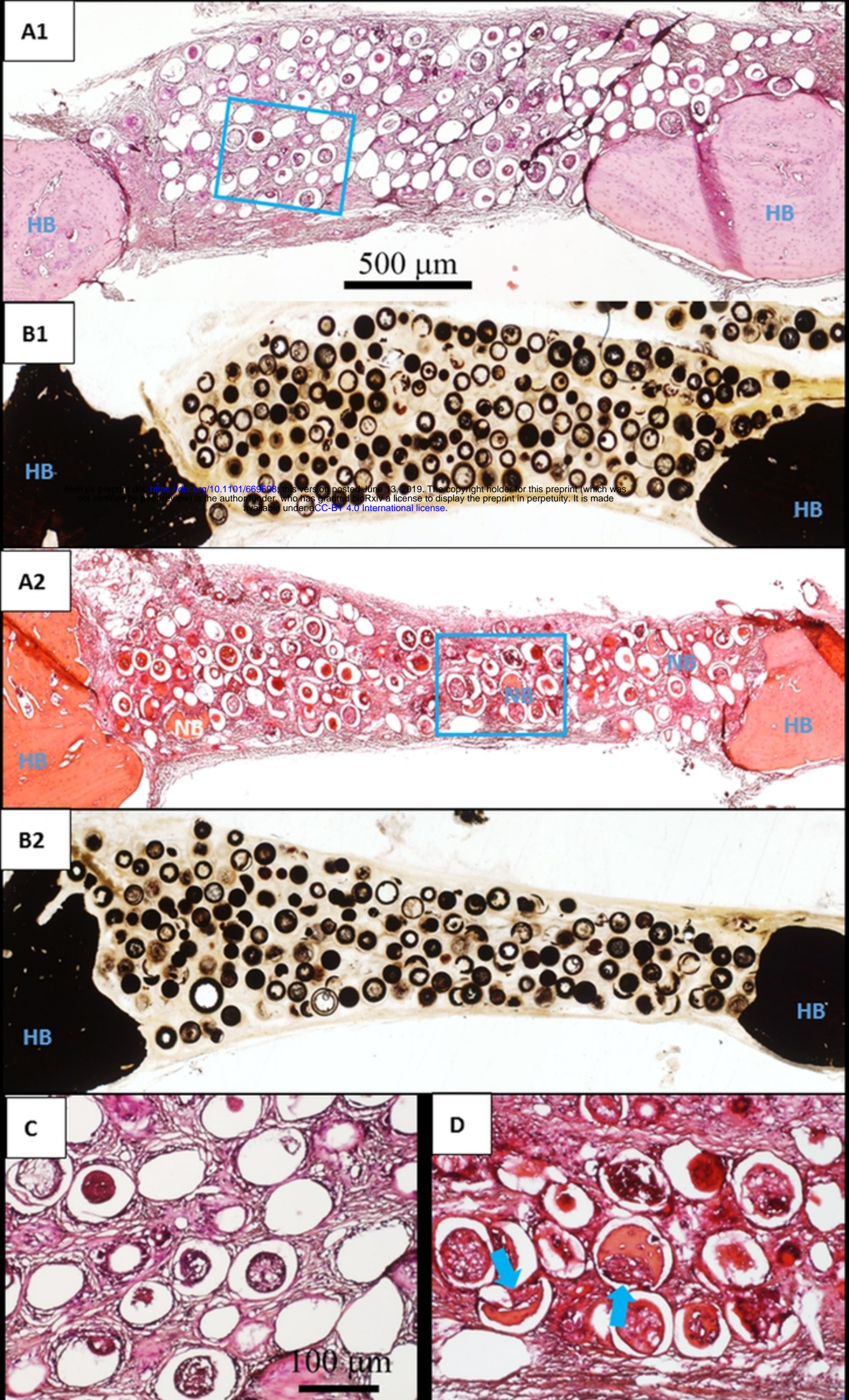


Figure 4



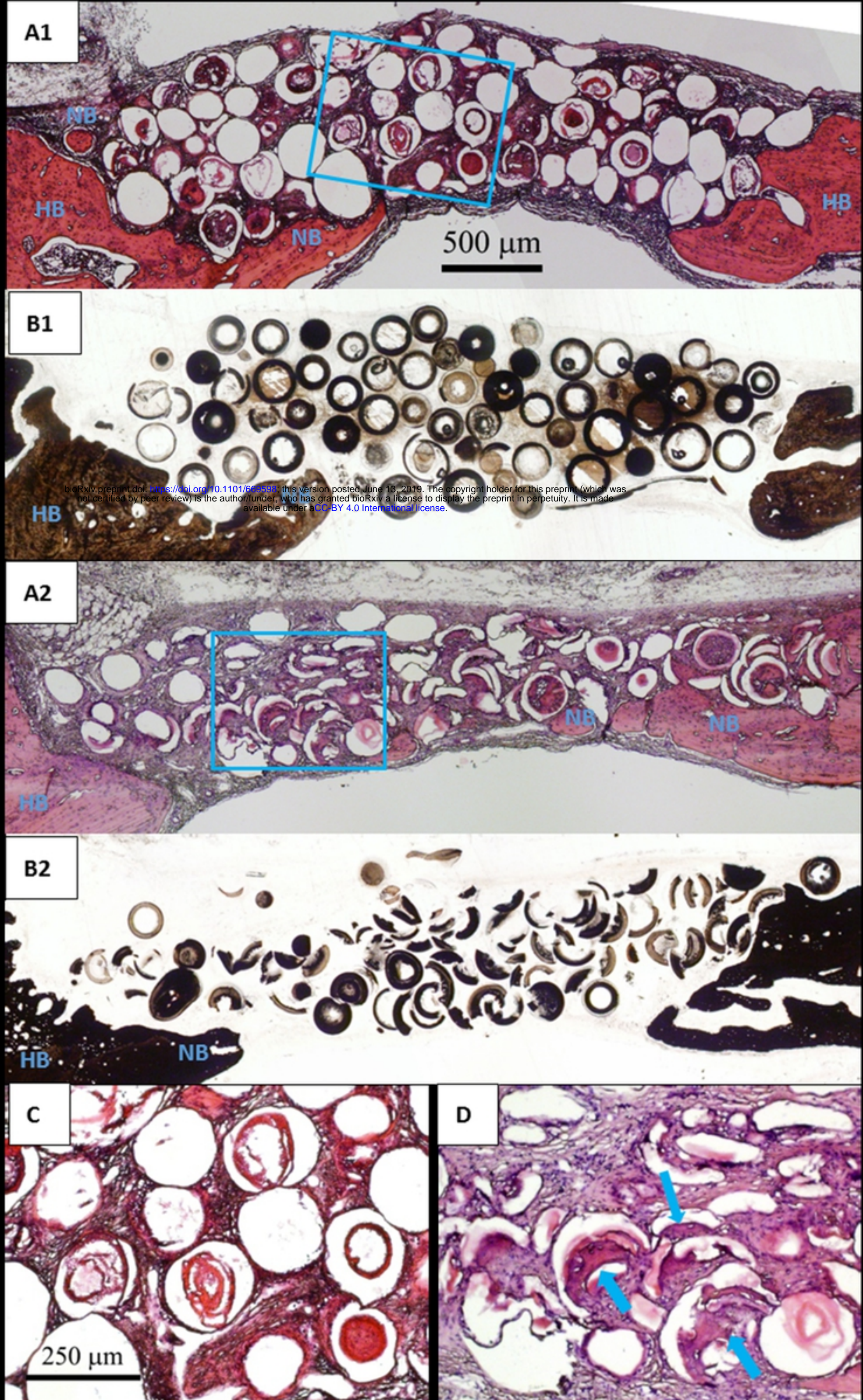
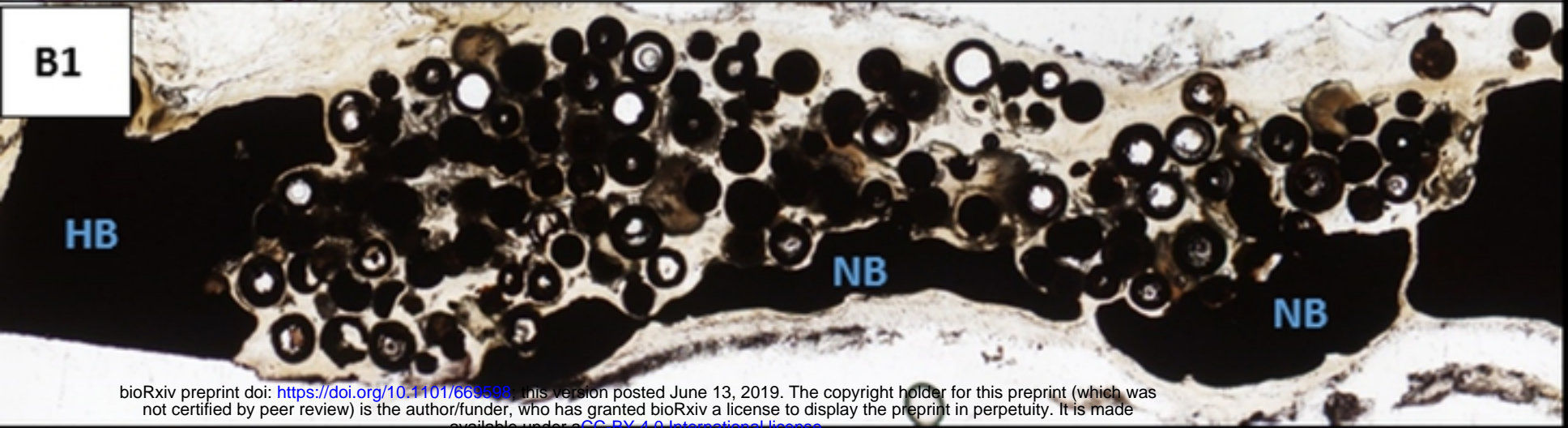
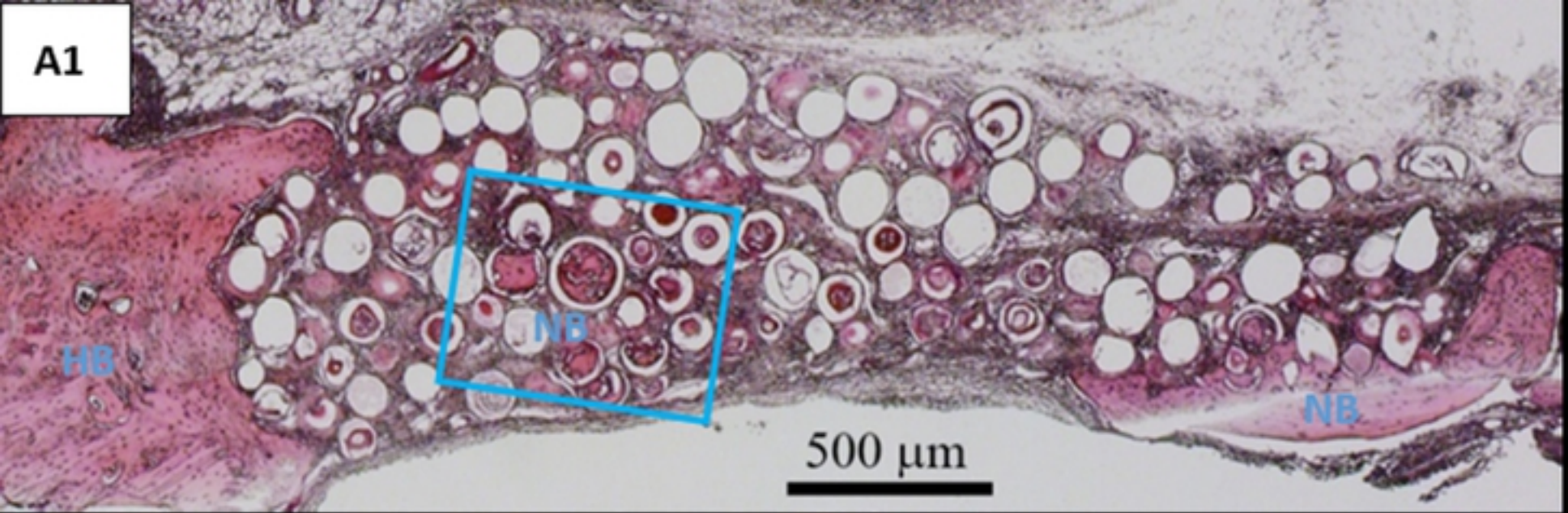


Figure 5





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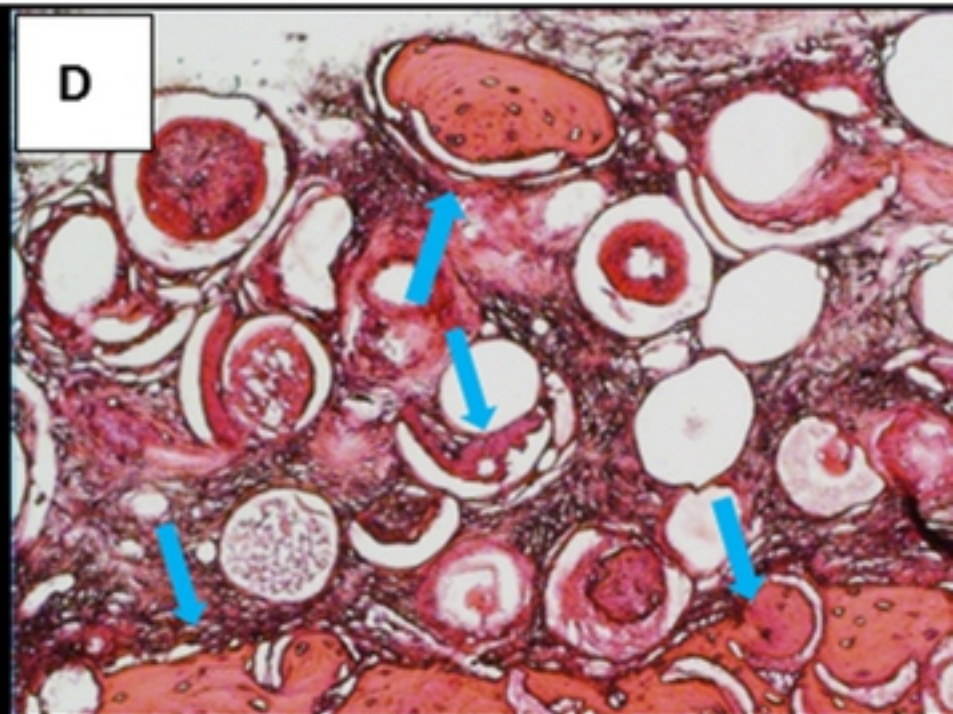
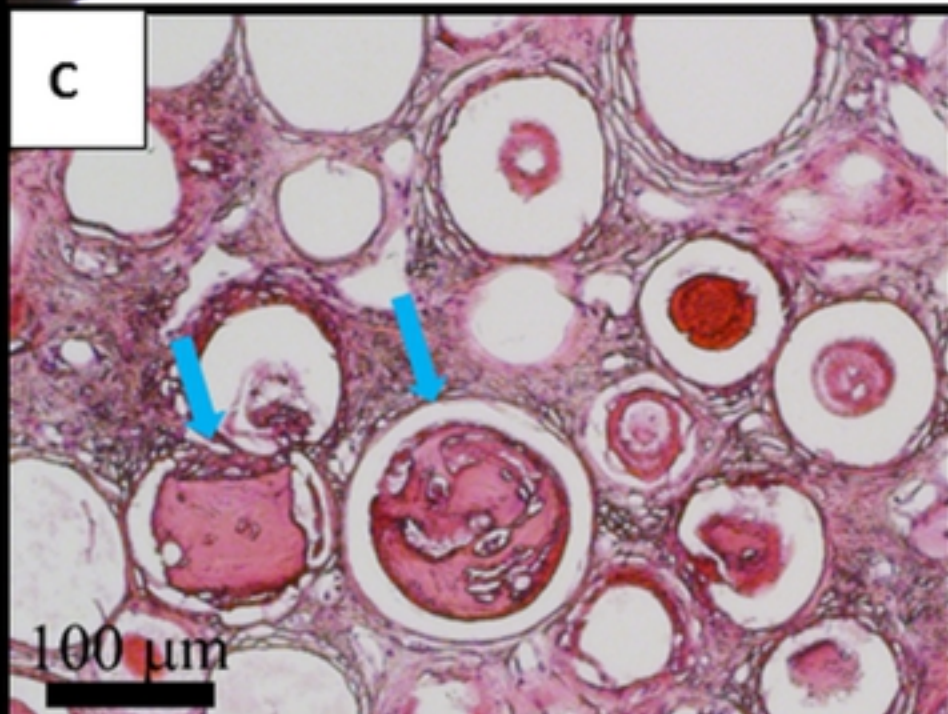
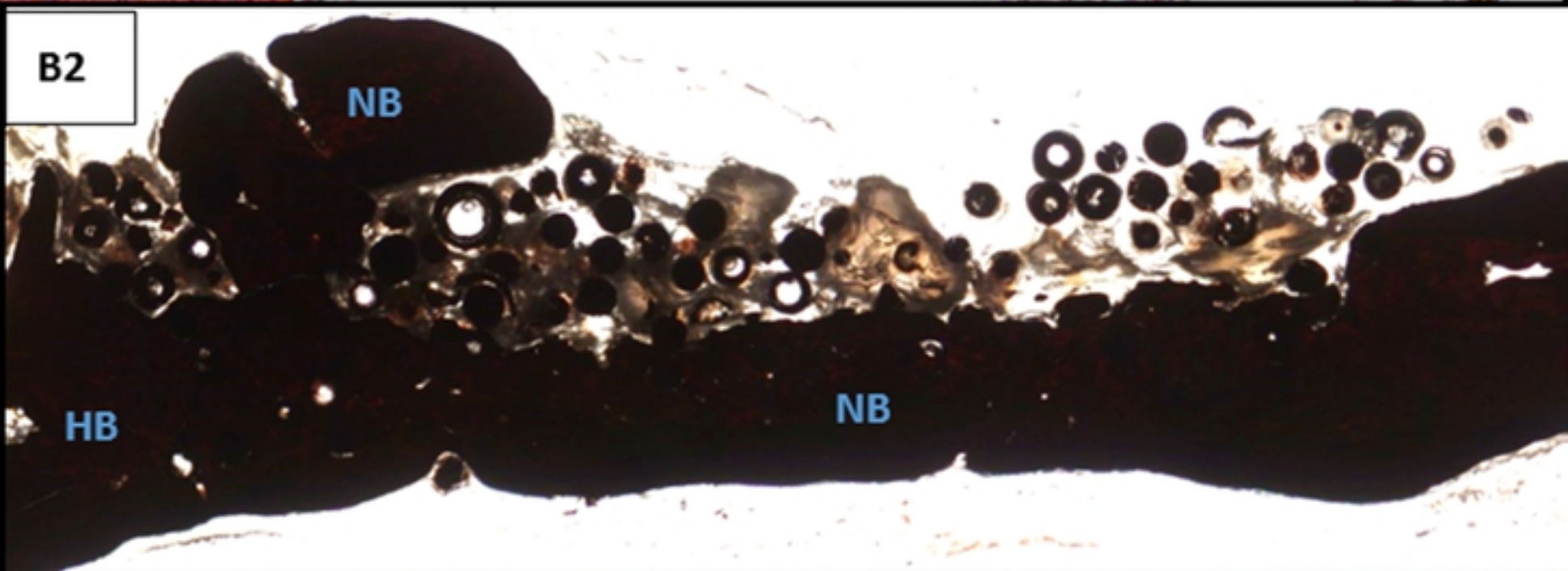
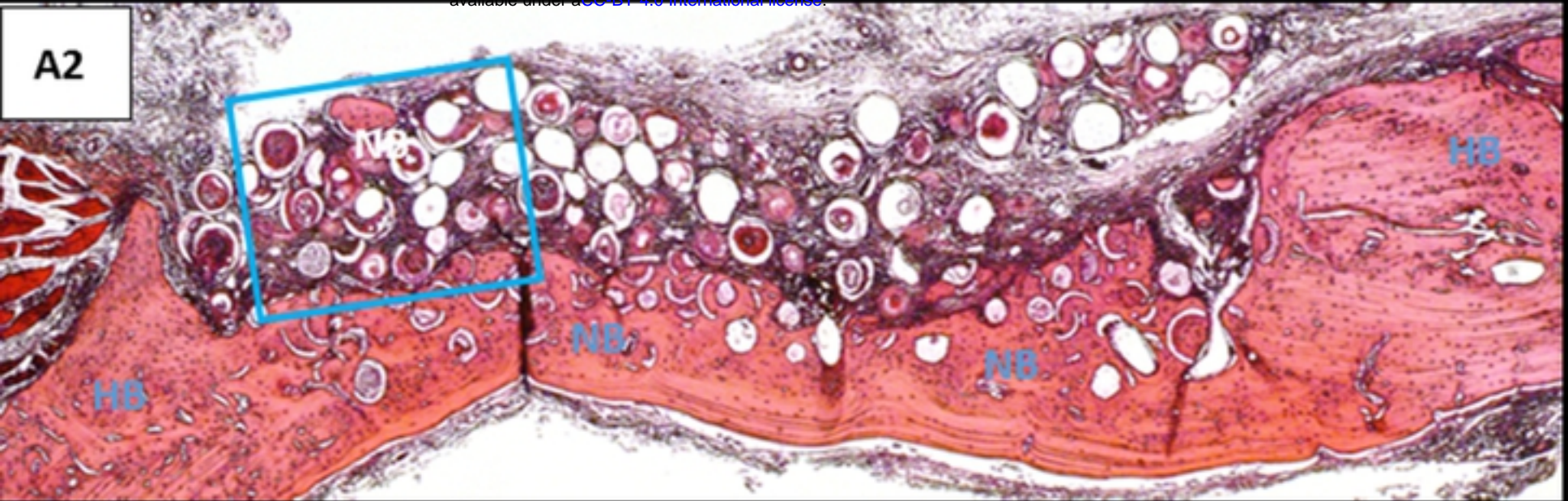


Figure 6



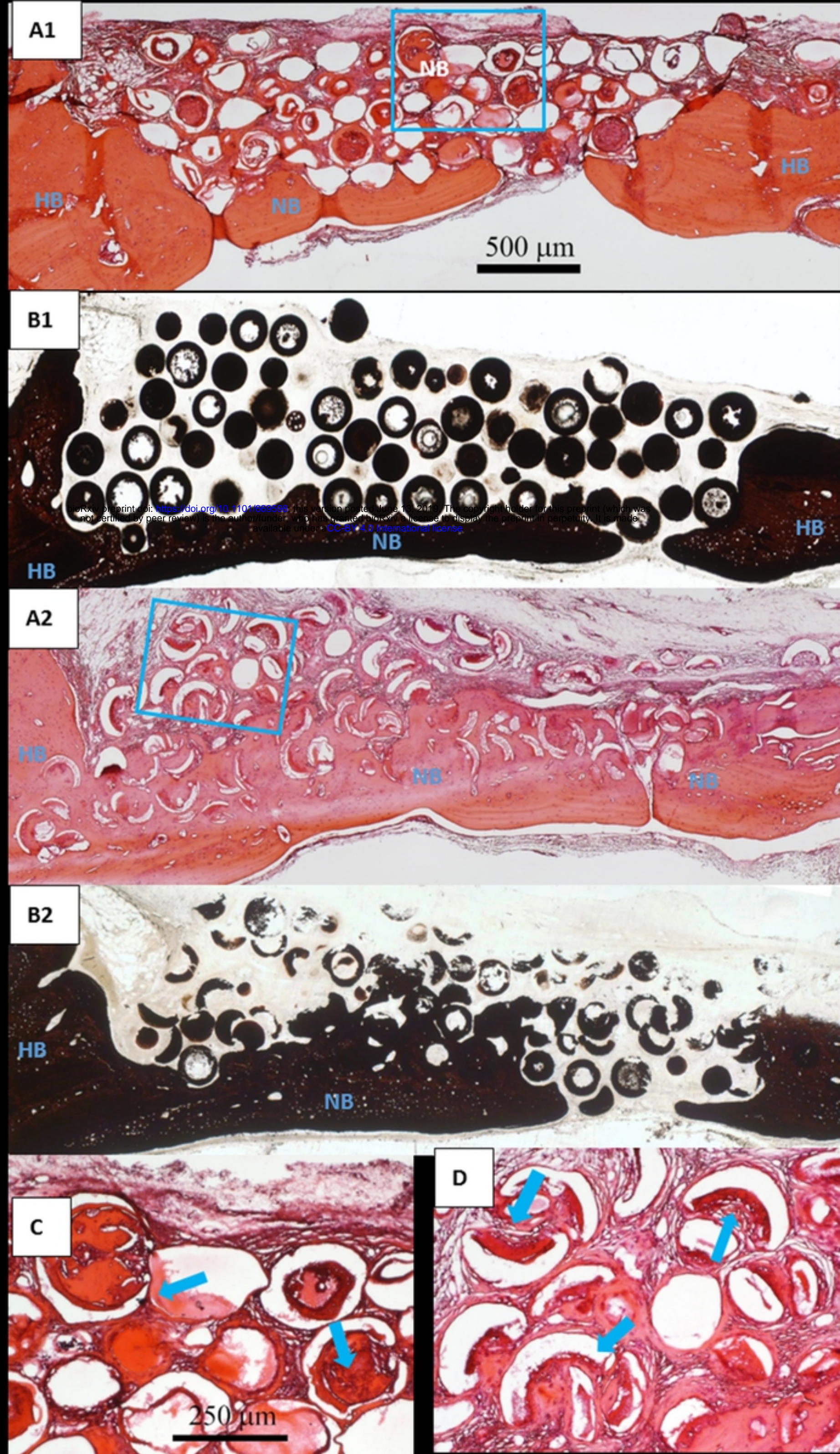


Figure 7



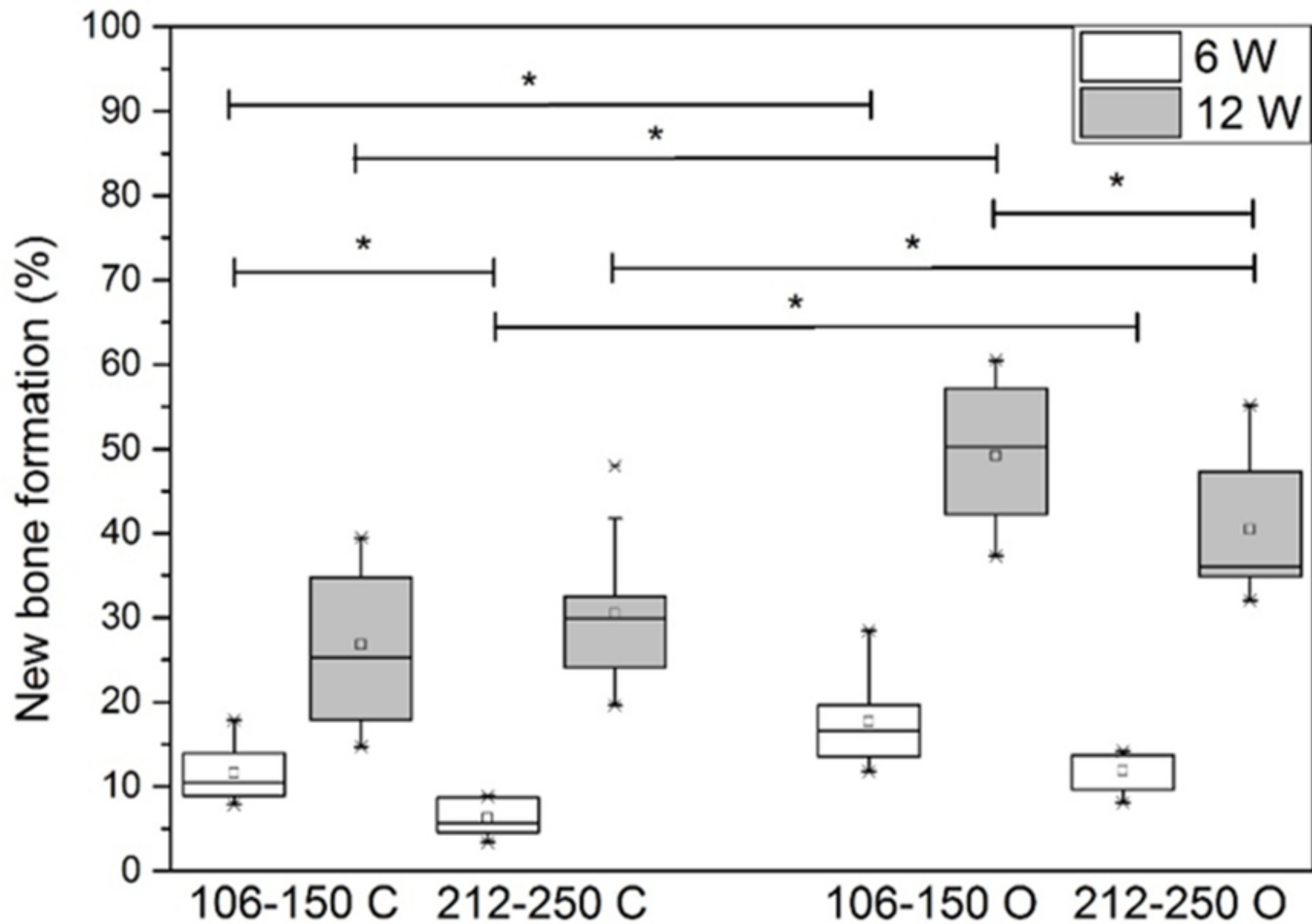


Figure 8

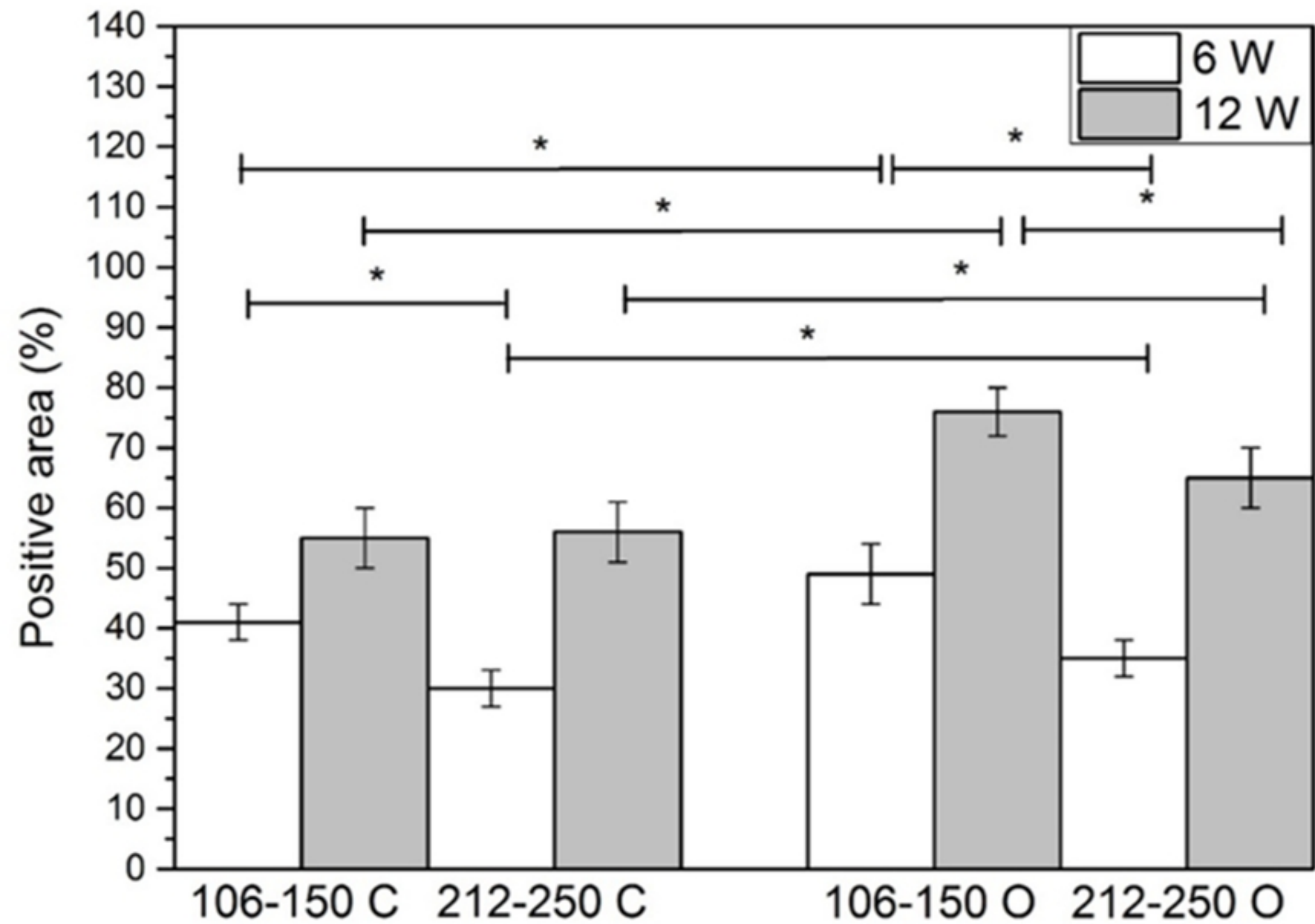


Figure 9