Designing bioactive porous titanium interfaces to balance mechanical properties and in vitro cells behavior towards increased osseointegration

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ABSTRACT

Titanium implant failures are mainly related to stress shielding phenomenon and the poor cell interaction with host bone tissue. The development of bioactive and biomimetic Ti

scaffolds for bone regeneration remains a challenge which needs the design of Ti implants with enhanced osseointegration. In this context, 4 types of titanium samples were fabricated using conventional powder metallurgy, fully dense, dense etched, porous Ti, and porous etched Ti. Porous samples were manufactured by space holder technique, using ammonium bicarbonate particles as spacer in three different ranges of particle size (100-200 μm, 250-355 μm and 355-500 μm). Substrates were chemically etched by immersion in fluorhydric acid at different times (125 and 625 seconds) and subsequently, were characterized from a micro-structural, topographical and mechanical point of view. Etched surfaces showed an additional roughness preferentially located inside pores. In vitro tests showed that all substrates were biocompatible (80% of cell viability), confirming cell adhesion of premioblastic cells. Similarly, osteoblast showed similar cell proliferation rates at 4 days, however, higher cell metabolic activity was observed in fully dense and dense etched surfaces at 7 days. In contrast, a significant increase of Alkaline phosphatase enzyme expression was observed in porous and porous etched samples compared to control surfaces (dense and dense etched), noticing the suitable surface modification parameters (porosity and roughness) to improve cell differentiation. Furthermore, the presence of pores and rough surfaces of porous Ti substrates remarkably decreased macrophage activation reducing the M1 phenotype polarization as well M1 cell marker expression. Thus, a successful surface modification of porous Ti scaffolds has been performed towards a reduction on stress shielding phenomenon and enhancement of bone osseointegration, achieving a biomechanical and biofunctional equilibrium.

1. INTRODUCTION

Titanium (Ti) and some of its alloys are well recognized as the clinical grade metallic biomaterial with the best prognostic for bone replacement. However, the major issues associated with titanium implants failures are related to stress shielding phenomenon and lack of osseointegration. [1–3]. Many research studies have reported the biomechanical mismatch between Ti implants and the surrounding bone tissue, highlighting the higher elastic Young's modulus of Ti as the main cause associated with bone resorption and weakening of implants area. Under these circumstances, the implant may suffer micro-loosening and further combined with poor osseointegration will finally be rejected from the body [4].

Many efforts have been developed to create bioactive scaffolds with similar mechanical properties as bone tissue. Indeed, porous metallic biomaterials have become an interesting approach to reduce the stress shielding effect and to improve long-term fixation of Ti devices [4,5]. The porosity factor has been described before as a strategy to reduce the stress shielding effect due to reproducing similar stiffness and Young's modulus as native bone tissue. Several manufacturing techniques enable porous titanium scaffolds fabrication, such as freeze casting [6,7], rapid prototyping [8,9], laser processing [10,11], electric sintering [12–14], and powder metallurgy [15,16]. Unfortunately, many of those methodologies are complex processes and expensive, leaving toxic residues compromising their use in tissue reconstruction applications. Powder metallurgy technology has been used with space holder (SH) particles offering an easy and scalable method to fabricate highly random interconnected pores with suitable mechanical properties [17,18] older references; The

interconnected pores are key factor in the development of healthy bone tissue since pores allow the required irrigation of blood and nutrients to feed the bone tissue in-growth. This SH technique has shown the advantage to control the space volume fraction which allows tailoring similar pore density and structure as cortical bone [16,19]. Earlier work showed similar Young's modulus values on porous samples (20 -28 GPa) as those of cortical bone (25GPa) using a variation in type and percentage of space holder [20,21] during processing. Although for bone regeneration certain stiffness and mechanical properties are required, according to literature, a pore size between 100 to 500 μm enhances vascularization process promoting tissue bone in-growth [22]. In addition, other studies also demonstrated that the pores generated by this space holder technique yielded intrinsic surface roughness along pore walls, improving the cell adhesion process and inhibiting bacteria attachment [23,24]. However, the increment of porosity may reduce the mechanical properties and compromise the internal stability of the implant which in turn can negatively affect in-vivo tissue reconstruction. In this context, the control of parameters such as pore size, pore volume fraction and pore morphology, as well as surface roughness are key factors to guarantee biomechanical and biofunctional stability with an enhancement in the osseointegration response in living bone tissue [25,26].

On the other hand, the inert biological character of titanium surfaces may lead low levels of cell interactions at the bio interface. This poor integration may delay or even impair the proper reconstruction of the damaged bone tissue, driving to the failure of the implant [27]. For this reason, it is essential to develop bioactive and biomimetic titanium surfaces, to promote the adhesion and differentiation of bone marrow mesenchymal stem cells, osteoblast, macrophages, and osteoclast recovering the required bone homeostasis [28].

Particularly, the implantation of any biomaterial implies an immune host response leading by the action of macrophages which are the first cell line in the immune response process [29]. As any biomaterial, Ti scaffolds triggers an inflammatory response, which usually is resolved in several days once the implant is integrated by the organism. In some scenarios, this initial inflammation may endure several weeks, evolving to a chronic inflammation response by encapsulating the Ti implant [30,31]. This fibrotic capsule isolates the metallic device within a thin layer of fibrous tissue, which reduces cellular interactions with native bone tissue and promotes bacterial infections. This complex and well-orchestrated reaction also referred to as foreign body reaction (FBR) is driven by macrophages, one of the first immune cells which arrive at the implantation area [32–34]. These immune cells are exposed to multiple signals (biophysical and chemical cues from both implant surface and the surrounding tissues). Depending on the integration of these signals, macrophages become activated exhibiting a wide spectrum of polarization states. [35] At either end of such a spectrum, M1 is commonly referred to pro-inflammatory phenotype and M2 to anti-inflammatory. Each M1 and M2 phenotypes show specific features, such as different cell surface markers; expression of particular genes; and secretion of specific cytokines, chemokines, and enzymes, as well as different cell shape [34].

Some reports have shown the importance of surface modification to successfully control macrophages response and enhance osteoblast cell differentiation [33,36,37]. Hence, surface treatments of porous Ti implants have been developed with the aim of controlling the inflammatory reaction and promoting cell differentiation by enhancing the biological interactions at the bio-interface. Chemical surface modification among other treatments such anodic reaction, hydrogen peroxide, sol-gel, chemical vapor deposition, has contributed to

the microtopography by increasing the roughness and the surface free energy, being both essential factors that affect cellular processes such as protein adsorption, cellular adhesion, migration, and differentiation [38]. However, it should be pointed out the great value of nanoscale order in the surface modification role to control cells behavior and develop biomimetic surfaces is still under research by some authors of this group (Civantos and Allain) which are working on the novel nanopatterning of porous titanium scaffolds.

As pointed out above, there is some evidence showing the benefits of porous and rough Ti surfaces in implant fixation and long-term stability [1,36]. Nevertheless, their combined effect has been poorly understood and their interactions with different cellular lineages have not been evaluated before with the aim to reproduce a bone in vitro environment. Under this premise, the hypothesis in this work, based on the controlled chemical etching modification of designed porous Ti disks, will be a biomimetic and bioactive strategy to reestablish bone homeostasis and support faster osseointegration. The mechanical properties and surfaces topography, such as porosity (pore size, shape, and distribution) and roughness profile, were characterized to evaluate their influence on premioblast, osteoblast and macrophage behavior. This study reveals a porous surface treatment as a successful approach to activate the porous Ti scaffolds which will enhance the osseointegration and fixation in bone tissue.

4. CONCLUSIONS

In this work, it has been developed a chemical treatment of porous c.p.Ti surfaces to modify the roughness in a controlled manner by using an organic inhibitor to generate the observed additional micro-roughness. The etching treatment modified substrates surfaces, as well as porosity in terms of volume fraction and morphology. All this micro-structural and topographical variations involved a decrease of substrates mechanical properties, getting values closed to those of cortical bone, confirming more biomimetic porous ti scaffolds. This study correlates cell viability, adhesion, proliferation, cell morphology and immune response of premioblastic cells, pre-osteoblastic cells, and macrophages. Cell differentiation was improved, and the immune response was controlled to reduce M1 phenotype. Samples with the time of the attack of 0s and 125s favored the cell biocompatibility, whereas the samples with the chemical attack of 625s enhanced the processes of osteoblast differentiation. In vitro evaluation of porous Ti samples is not sufficient to determine the degree of osseointegration of the samples; making necessary in vivo studies with animal models that allow a better understanding of osseointegration processes in the complex environment.

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7. FIGURE CAPTIONS

Figure 1. SEM microphotographs of all c.p.Ti surfaces. Images showed the different surfaces, with and without HF treatment as well the three different pore sizes of space holder (BA). Insets correspond to macroscopic images of the samples revealing the main differences (color and pore size and morphology) of the samples.

Figure 2. Pore's detail of cp. Ti samples by SEM. These microphotographs highlight the pores structure and the HF influence as surface modification treatment. Noticed am increased roughness at the microscale order as well as the presence of geometrical features (upper insets) due to HF treatment and PM process respectively.

Figure 3. Roughness quantification, Sa (µm) of c.p.Ti substrates by the analysis of confocal images using Sensomap software. A, B, C, and D correspond to the Sa values comparing flat and pores surfaces of A, B C and D surfaces at the three times of etching modification (0, 125, and 625). Upper and left insets showed clearly a bigger scale of flat surfaces. Noticed the higher affinity of chemical attack for pore surfaces which reached higher Sa values.

Figure 4. Cell viability of C2C12GFP cells at 1 day. Results were expressed as a percentage of D0 levels. All substrates showed similar metabolic activities with no statistical differences ($P \geq 0.05$).

Figure 5. Fluorescent microphotographs of cell adhesion and proliferation of C2C12-GFP premioblastic cell line. A) Cell attachment of D, DE, P and PE at1 day of cell incubation. All samples allowed cell attachment, noticing a slight increase in cell density in D0 which has not rough surface and no pores. Etched Ti scaffolds showed similar cell density as controls revealing the cell biocompatibility of the treatment. B) Cell proliferation of C2C12-GFP

growing on D, DE, P and PE samples at 4 days of cell incubation. Noticed the increase of cell density showing the cytocompatibility character of the treatment.

Figure 6. Cell proliferation of MC3T3E1 preosteoblastic cells expressing cell metabolic activity results as relative fluorescence units at 4 and 7 days of cell culture (A and B respectively). These fluorescence levels showed similar cell proliferation as control and TCP. Noticed a slight reduction trend in porous samples of cell metabolic levels.

Figure 7. Microphotographs of cell morphology of osteoblast MC3T3E1 growing on all c.p. Ti samples at day 7. Actin fibers appeared in red and cell nuclei in blue due to Texas Red Phalloidin and Dapi staining. From these images, Ti samples achieved similar cell density covered the whole surface by preosteoblast showing the cytocompatibility character of the samples.

Figure 8. Evaluation of cell differentiation at 7 days. Detection of Alkaline phosphatase enzyme (ALP), an earlier bone marker, on osteoblast growing on cp. Ti samples. The higher ALP levels were observed in 625 HF treatment and in the pore of bigger size B and C.

Figure 9. Evaluation of cell mineralization at 14 days. Microphotographs of calcium deposits in red color after alizarin test. The higher calcium deposits were increased on B and C samples with 125 HF treatment.

Figure 10. RAW 264.7 seeded on titanium implants with different porosity and roughness. A. Samples of titanium implants (D without porosity and A with porosity; 0s of chemical attack without roughness and 625s of chemical attack with roughness). B. Actin fibers, cell nuclei by Hoechst staining, and M1 cell surface marker (CCR7) staining at 48h. Cells growing on D0 (without porosity and roughness) presented an increased polarization of M1 that was corroborated with the positive signal of CCR7 staining. Moreover, some

multinucleated cells, that could differentiate into FBGC (see yellow arrows), were observed on these samples. In contrast, D625 and A625s, as well as A0, showed less M1 polarization compared to D0 revealing the positive effects of both parameters roughness and porosity on the control of immune response.

Figure 1

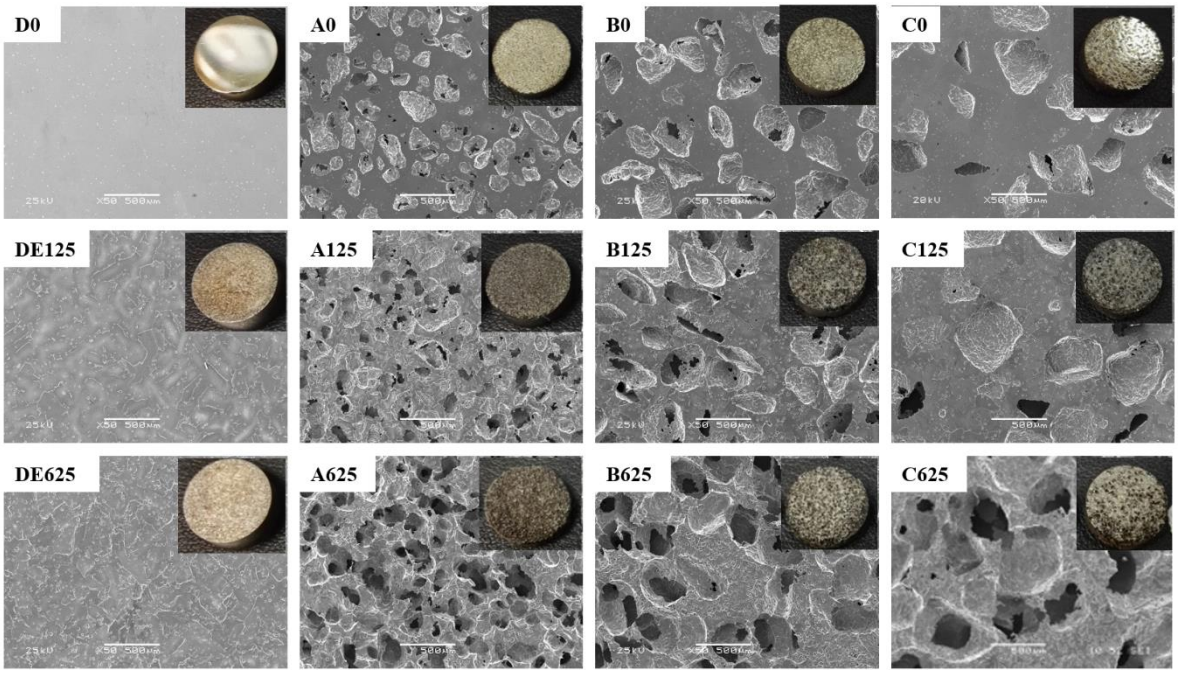

Figure 2

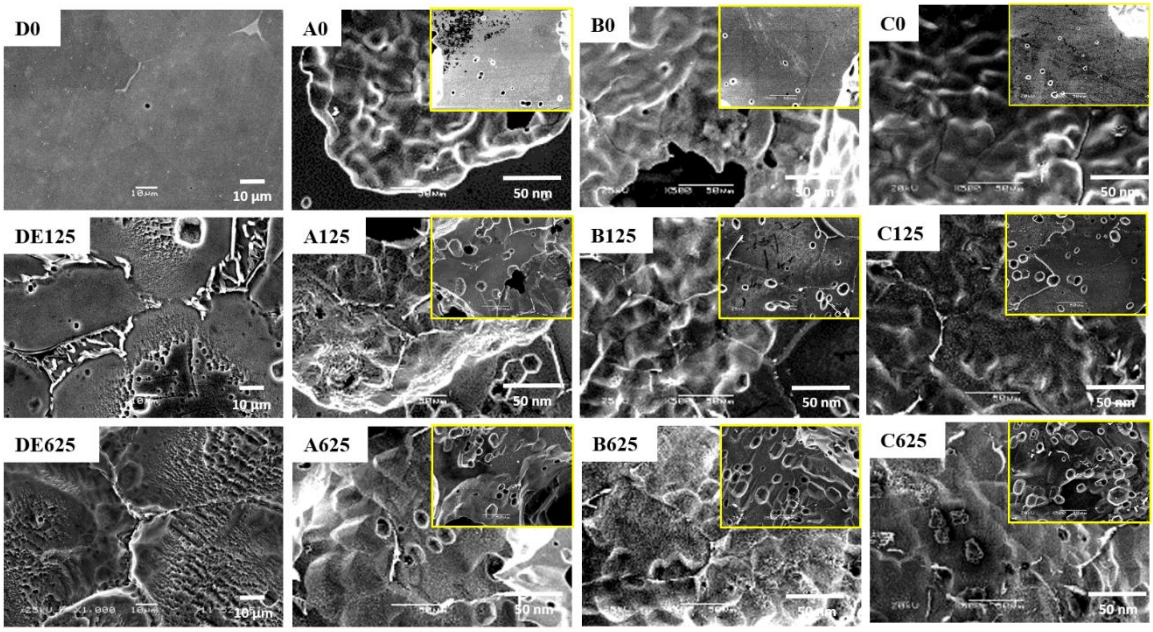

Figure 3

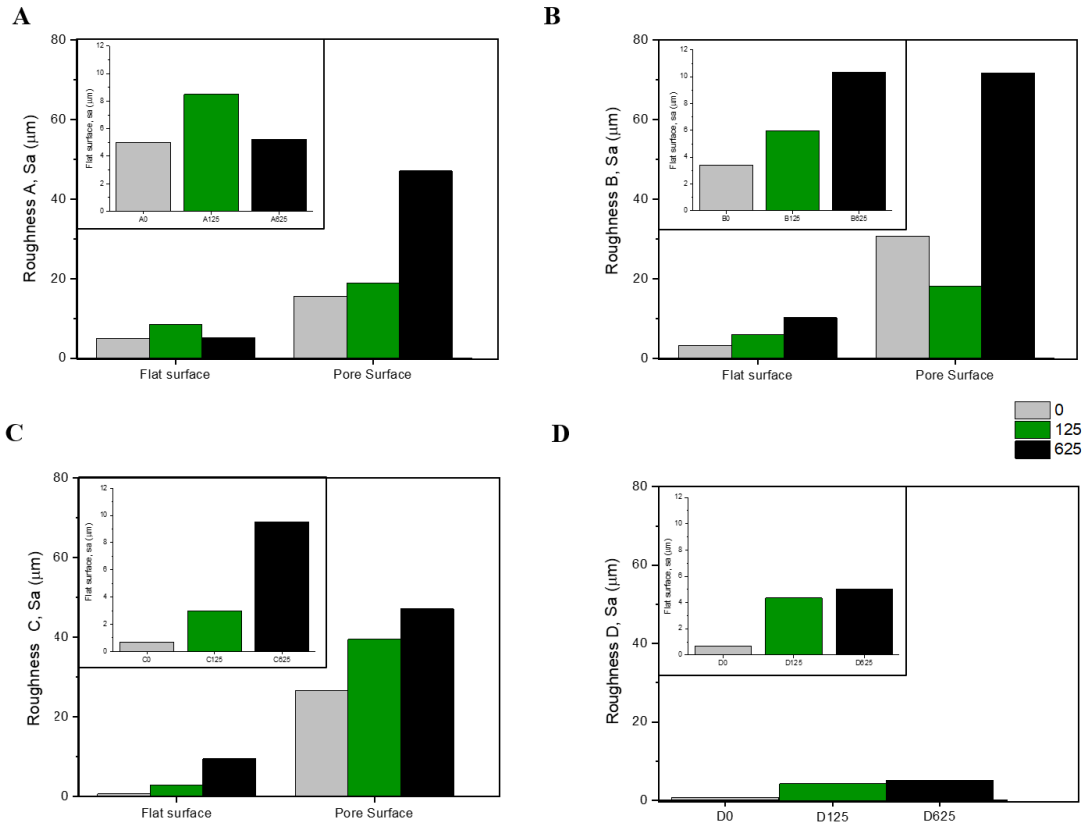
A
B
C
D
Roughness A, Sa (µm)
Roughness B, Sa (µm)
Roughness C, Sa (µm)
Roughness D, Sa (µm)
Flat surface
Pore Surface
0
125
625
D0
D125
D625

Figure 4

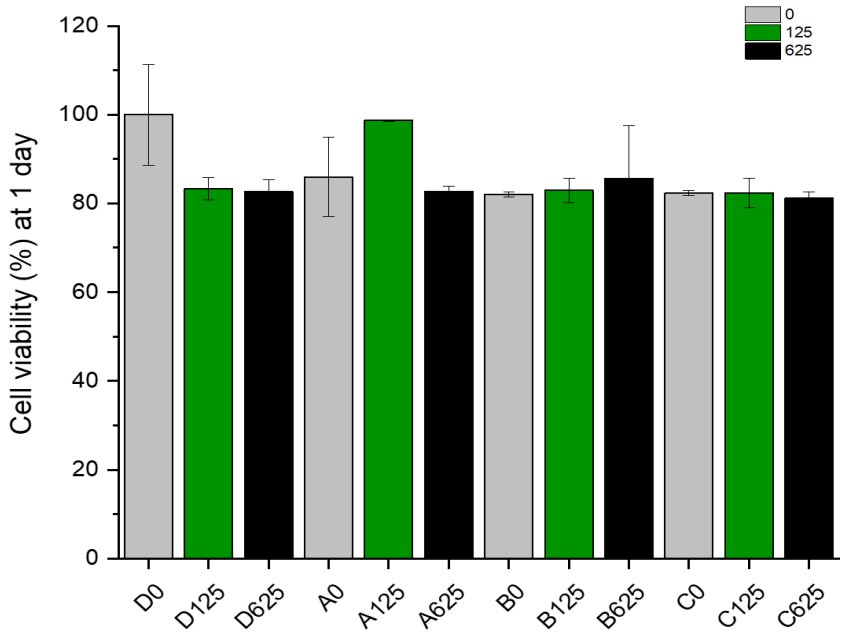
0
125
625
Cell viability (%) at 1 day
120
100
80
60
40
20
0
D0
D125
D625
A0
A125
A625
B0
B125
B625
C0
C125
C625

Figure 5

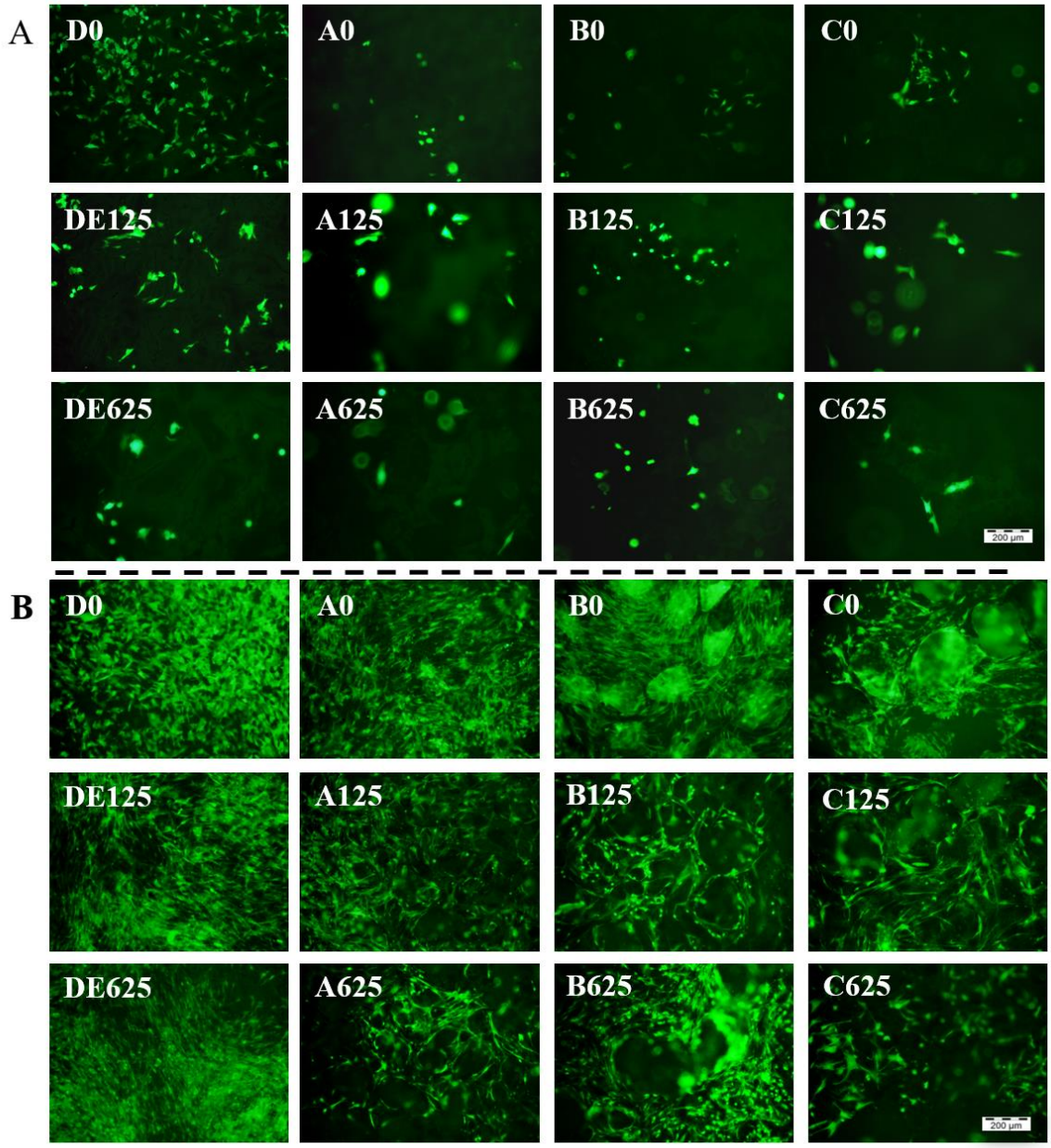
A
D0
A0
B0
C0
DE125
A125
B125
C125
DE625
A625
B625
C625
200 μm
B
D0
A0
B0
C0
DE125
A125
B125
C125
DE625
A625
B625
C625
200 μm

Figure 6

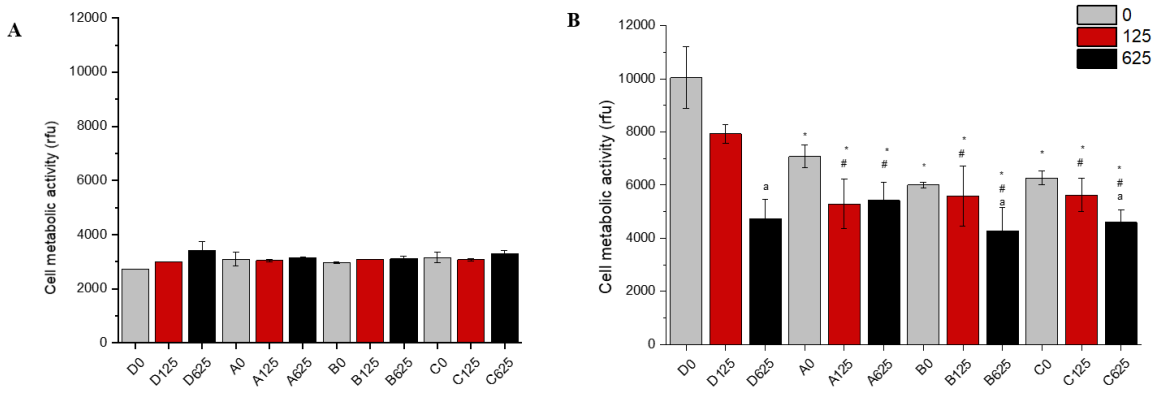

Figure 7

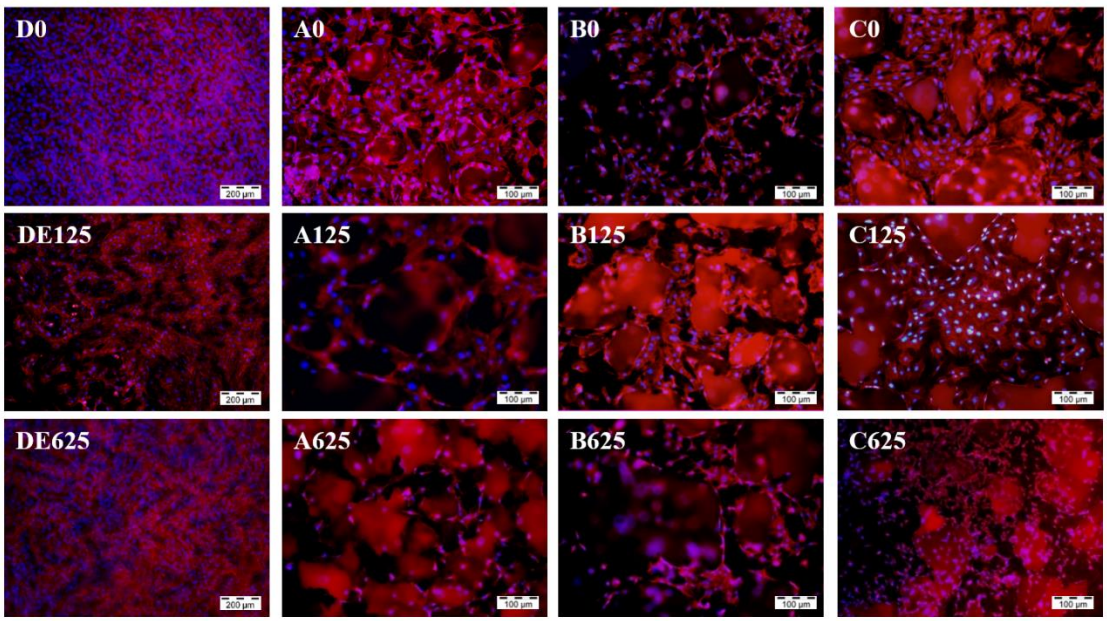

Figure 8

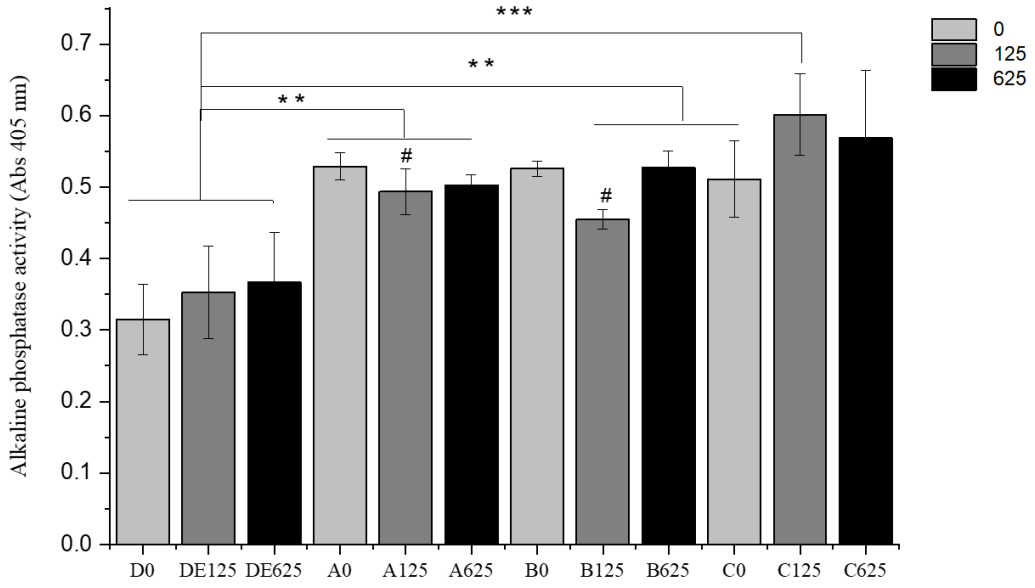

Figure 9

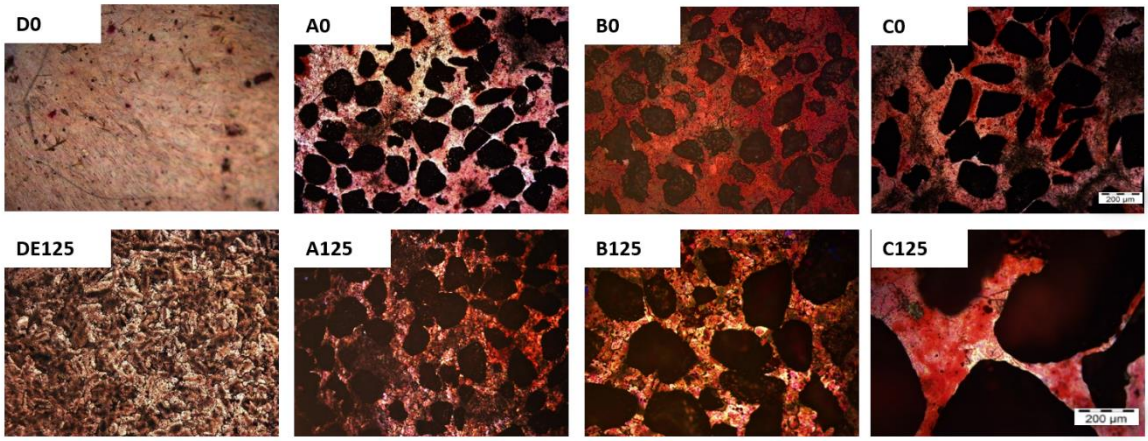

Figure 10

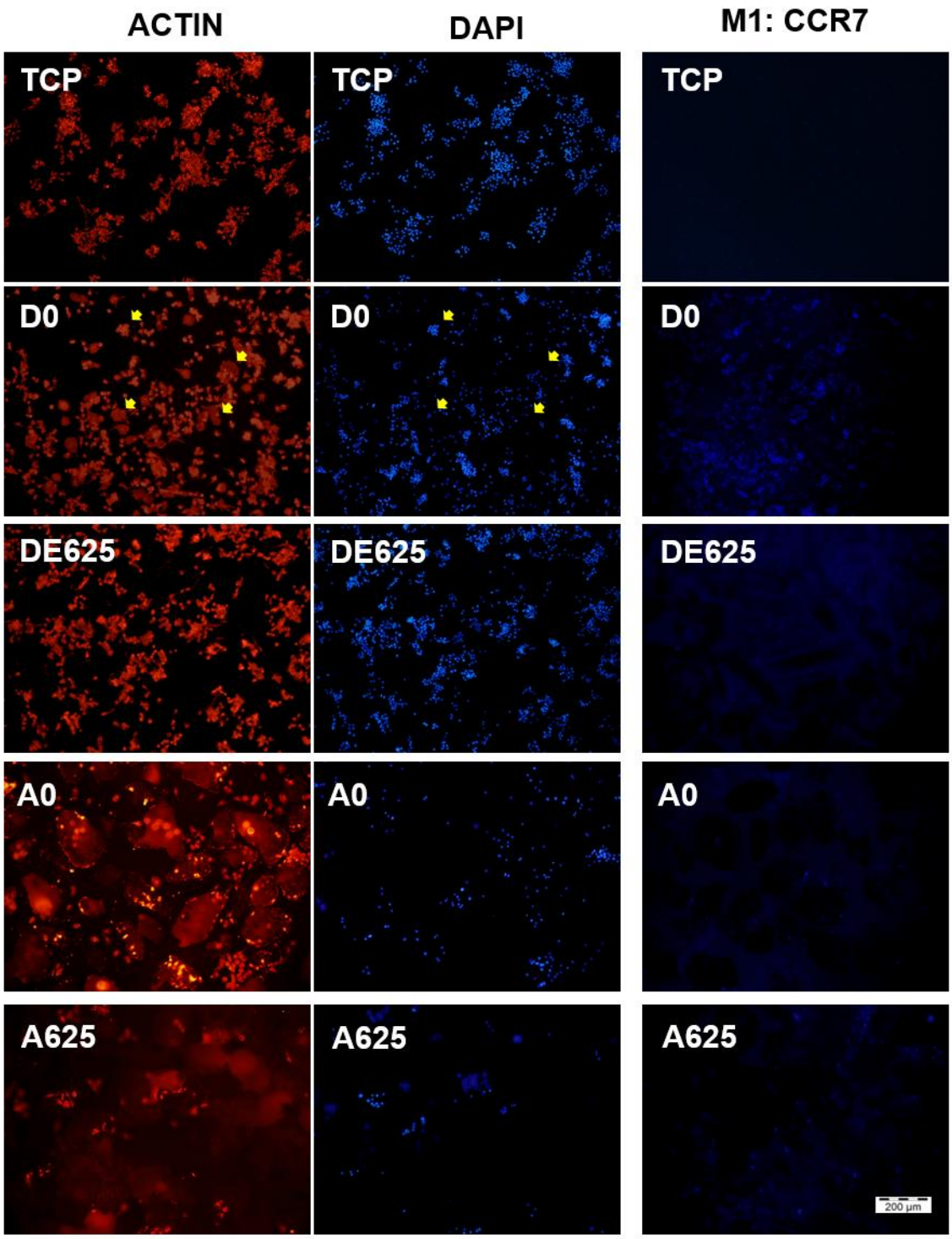
ACTIN
DAPI
M1: CCR7
TCP
TCP
TCP
D0
D0
D0
DE625
DE625
DE625
A0
A0
A0
A625
A625
A625
200 µm

Figure S1