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Abstract: Ti6Al4V is a popular biomaterial for load-bearing implants for bone contact, which can be fabricated by additive manufacturing technologies. Their long-term success depends on their stable anchoring in surrounding bone, which in turn depends on formation of new bone tissue on the implant surface, for which adhesion and proliferation of bone-forming cells is a pre-requisite.. Hence, surface coatings which promote cell adhesion and proliferation are desirable.
Here, Ti6Al4V discs prepared by additive manufacturing (EBM) were coated with layers of pectins, calcium-binding polysaccharides derived from citrus (C) and apple (A), which also contained alkaline phosphatase (ALP), the enzyme responsible for mineralization of bone tissue. Adhesion and proliferation of human bone marrow stromal cells (hBMSC) were assessed. Proliferation after 7 days was increased by A-ALP coatings and, in particular, by C-ALP coatings. Cell morphology was similar on coated and uncoated samples. In conclusion, ALP-loaded pectin coatings promote hBMSC adhesion and proliferation.

Cover Letter

2018-01-23

Dear Prof. Boccaccini,

Thank you for giving us the opportunity to submit a revision of our manuscript MLBLUE-D-18-00536 "Pectin coatings on titanium alloy scaffolds produced by additive manufacturing: promotion of human bone marrow stromal cell proliferation"

We have prepared a point-by-point response to the reviewer comments (below). We have altered the manuscript according to the suggestions of the reviewer and marked changes with the “Track Changes” function.

Thank you for your attention and we look forward to hearing from you.

Yours sincerely,

Timothy E.L. Douglas

Corresponding Author

***Response to Reviewers**

Point-by-point response to reviewer comments

Reviewer #1: Fig. 2c, statistical analysis should be performed.
The labels for scale bars of Fig. 2 should be clearer.

AUTHORS: We have performed statistical analysis as recommended by the reviewer and modified the scale bars.

For coatings on implant, the adhesive strength is important. It is would be interesting to test and compare the adhesive strength of C-ALP and A-ALP on Ti6Al4V implant.
It is not clear how thick are the coatings.

AUTHORS: The reviewer is correct to point out the importance of adhesive strength and that the thickness of the coatings is unclear.

We would have liked to have tested adhesive strength. Unfortunately the Ti6Al4V substrate is rough and the coating is uneven, as shown on the optical microscopy and SEM images (Figures 2a and 2b). Hence, due to lack of a clear methodology to calculate adhesive strength and coating thickness directly, we chose to perform alternative tests, namely stability tests. We incubated the coatings upon incubation in an aqueous environment and analyse release of ALP and pectin. The results are shown in Figures 2d and 2e in the revised version.

Highlights

- Ti6Al4V discs were prepared by additive manufacturing (EBM)
- Discs were coated with pectins from citrus (C) and apple (A)
- Coatings also contained the enzyme alkaline phosphatase (ALP)
- Coatings promoted proliferation of human bone marrow stromal cells (hBMSC)
- C-ALP coatings promoted proliferation more than A-ALP coatings

*Manuscript

Pectin coatings on titanium alloy scaffolds produced by additive manufacturing: promotion of human bone marrow stromal cell proliferation

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Keywords: Biomaterials; Thin films; Biomimetic

Abstract

Ti6Al4V is a popular biomaterial for load-bearing implants for bone contact, which can be fabricated by additive manufacturing technologies. Their long-term success depends on their stable anchoring in surrounding bone, which in turn depends on formation of new bone tissue on the implant surface, for which adhesion and proliferation of bone-forming cells is a pre-requisite.. Hence, surface coatings which promote cell adhesion and proliferation are desirable.

Here, Ti6Al4V discs prepared by additive manufacturing (EBM) were coated with layers of pectins, calcium-binding polysaccharides derived from citrus (C) and apple (A), which also contained alkaline phosphatase (ALP), the enzyme responsible for mineralization of bone tissue.

Adhesion and proliferation of human bone marrow stromal cells (hBMSC) were assessed. Proliferation after 7 days was increased by A-ALP coatings and, in particular, by C-ALP coatings. Cell morphology was similar on coated and uncoated samples. In conclusion, ALP-loaded pectin coatings promote hBMSC adhesion and proliferation.

1. Introduction

The titanium alloy Ti6Al4V is a popular biomaterial for load-bearing implants for bone contact which can be fabricated by additive manufacturing technologies [1]. The adhesion and proliferation of bone-forming cells are pre-requisites for formation of new bone tissue on the implant surface, which guarantees implant stability and long-term success. Hence, surface coatings which promote cell adhesion and proliferation are desirable. Coatings containing certain polysaccharides have improved cell adhesion and proliferation [2-4].

Pectins are a family of complex, anionic, calcium-binding polysaccharides found in the primary cell wall and intercellular regions of higher plants, composed primarily of linear D-galactopyranosyluronic acids joined via α(1→4) glycosidic linkages (Homogalacturonan: HG). These are either partially methyl-esterified, acetylated or both [19]. In the Rhamnogalacturonan–I (RG-I) the linear galacturonic acid (GalA) chain is disturbed by α-(1,2)-linked L-rhamnose (L-Rha) units. Depending on plant source and isolation method, L-Rha residues are substituted with neutral saccharide side chains. The RG-II fragment is a substituted galacturonan being composed of a partially methyl esterified GalA backbone.

Commercial pectins from apple and citrus pomace are extracted under hot acidic conditions and therefore, many regions containing high proportions of neutral sugars are hydrolysed. Thus, extracted pectins are mainly composed of HG containing free or genuine esterified carboxylic groups that are more acid-stable. They form hydrogels and are thus widely applied in viscous, hydrated foods such as jams. They are also inexpensive and widely available and have been applied as scaffolds for tissue regeneration [5]. Pectic RG-I nanocoatings have been used to tailor surface properties of tissue culture polystyrene (TCPS) [6, 7] and titanium [8]. However, pectin coatings on Ti6Al4V remain relatively unexplored.

The enzyme alkaline phosphatase (ALP) plays an important role in hard tissue mineralization. ALP catalyses hydrolysis of organic phosphate monoesters to yield inorganic phosphates. ALP has been used as a coating material on Ti6Al4V surfaces [9, 10], which has improved the osteogenic response.

Here, Ti6Al4V discs prepared by additive manufacturing (electron beam melting, EBM) were coated with pectins derived from citrus (C) and apple (A) and ALP. Coatings were characterized physicochemically and cell biologically with human bone marrow stromal cells (hBMSC). Cell adhesion and proliferation were assessed.

2. Materials and methods

All materials, including alkaline phosphatase (P7640) and BCA Assay (BCA1) were acquired from Sigma-Aldrich, unless stated otherwise. Pectins C (degree of esterification (DE) 35%, Galacturonic acid content (GalC) 86%) and A (DE 34%, GalC 74%) were obtained from Herbstreith & Fox KG Pektin-Fabriken, D. Rough Ti6Al4V discs of diameter 2 cm were prepared using additive manufacturing techniques in an ARCAM EBM A2 machine as described previously [1]. Ti6Al4V discs and 0.8% (w/w) C (degree of esterification (DE) 35%, Galacturonic acid content (GalC) 86%) and A (DE 34%, GalC 74%) pectin solutions were autoclaved (121ºC, 15 minutes). Sterile-filtered ALP solution (1.6% (w/v)) and pectin solution were mixed 1:1 (v/v). 250 µl of this solution was spread on Ti6Al4V and allowed to air-dry under sterile conditions.

Morphologies of uncoated and coated samples were examined by digital optical microscopy (KEYENCE VHX-5000) and scanning electron microscopy (SEM, TableTop 3030PLUS, Hitachi).

Surface contact angles were determined using a drop shape analysis system (DSA 10Mk2, KRÜSS). UHQ water droplets (approximate volume 0.2 µl) were deposited on sample surfaces. Images captured by video camera were analyzed to calculate the contact angle. Results (30 drops per sample) were expressed mean ± standard deviation.

To test coating stability, uncoated and coated samples were immersed in 4 ml ddH_2O for 1 and 15 h. ALP release was quantified by the BCA Assay according to the manufacturer's instructions. Pectin release was quantified by the method of van den Hoogen et al. [11].

For cell experiments, hBMSC from two different donors (ethical approval granted by ethics committee of Technische Universität Dresden, No. 466112016).were seeded at a density of 7000 cells/cm². Cells were seeded onto the samples in 400 µl of cell culture medium (DMEM with 10% heat-inactivated fetal calf serum, and antibiotics (penicillin and streptomycin). After 2 h the medium was filled up to 4 ml and culture proceeded at 37°C in a humidified CO_2 incubator. Proliferation was assessed by the MTS assay. Cells were treated with 10% dye solution in DMEM for 2 h. The formed formazan amount was measured photometrically at 490 nm. Analyses were performed 24 h and 7 days after seeding. Statistical significance was analyzed by one-way ANOVA and Bonferroni post-test (prism graph pad software). Cell morphology was assessed after 24 h. Cells were fixed with 4% paraformaldehyde and stained with Alexa488-phalloidine to visualize F-actin cytoskeleton (green fluorescence) and with DAPI to stain the nuclei (blue fluorescence). The images (three from each sample) were taken with Axiophot microscope (Zeiss) using a digital camera and Axiovision software. Focusing of cells of samples was complicated by the roughness of the sample surfaces.

3. Results and Discussion

The presence of A-ALP and C-ALP coatings was confirmed by optical microscopy (Figure 2a) and SEM (Figure 2b). Coatings lowered contact angle, and A-ALP coatings were more hydrophilic than C-ALP coatings (Figure 2c). ALP release was more pronounced from C-ALP coatings (Figure 2d) and increased from 1 to 15 h. No differences in pectin release from A-ALP and C-ALP coatings after 1 and 15 h were observed (Figure 2e). Cells retained viability and proliferated over 24 h and 7 days (Figure 3a). Proliferation was significantly higher on C-ALP coatings than on A-ALP coatings after 24 h, and higher than on both uncoated samples and A-ALP coatings after 7 days. A-ALP coatings were significantly superior to uncoated samples after 7 days. Cells on all substrates displayed a spread morphology and distinct, well organized F-actin fibers, characteristic for good adhesion (Figure 3b).

Several authors have reported that polysaccharides, including pectins, have promoted adhesion and proliferation of cells [12]. Coatings of RG-I derived from plant pectins on tissue culture polystyrene have promoted proliferation of primary osteoblasts and osteoblast-like MC3T3-E1 cells [13]. Conversely, other studies by the same group (Gurwaszka K et al) on different RG-I preparations on titanium surfaces revealed no positive effect or, in some cases, an inhibitory effect of the coatings on the proliferation of SaOS-2 osteoblast-like cells [8]. It is possible that the effect on proliferation is cell-type dependent; in this study, hBMSC were used, while Gurwaszka K and colleagues used primary osteoblasts, MC3T3-E1 and SaOS-2 cells. The reasons for the stimulatory effect of pectin coatings on proliferation, and the differences between the C-ALP and A-ALP coatings remain unclear. It can be speculated that the differences in GalC (C: 86%, A:74%) may play a role, as may the differences in wettability. Pectin-coated samples were considerably more hydrophilic than uncoated surfaces (Figure 2c). A stimulatory effect of ALP on cell proliferation is not ruled out. The morphology of hBMSC did not seem to be affected by the C-ALP and A.ALP coatings (Figure 3b). This seems to be in agreement with previous work by Svava et al, where coatings of different RG-I preparations did not influence morphology of SaOS-2 cells [7]. Since the A-ALP and C-ALP coatings improve cell proliferation, future work should focus on the ability of these coatings to promote osteogenic differentiation of hBMSC.

4. Conclusions

hBMSC proliferation after 7 days was increased by A-ALP coatings and, in particular, by C-ALP coatings. Cell morphology was similar on coated and uncoated samples.

5. Acknowledgement

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7. **Figure captions**

Figure 1. Schematic description of coating of Ti6Al4V discs with pectin and ALP.

Figure 2. a) optical microscopy images of bare Ti6Al4V disc (uncoated), Ti6Al4V coated with C + ALP and Ti6Al4V coated with A + ALP. Scale bar: 1000 µm. b) SEM images of bare Ti6Al4V disc (uncoated), Ti6Al4V coated with C + ALP and Ti6Al4V coated with A + ALP. Scale bars: 500 µm (top row), 100 µm (bottom row). c) Contact angle measurements on bare Ti6Al4V disc (uncoated), Ti6Al4V coated with C + ALP and Ti6Al4V coated with A + ALP. Differences between all groups were statisitically significant ($p<0.001$ in all cases). d) Results of BCA Assay. Significances: *:$p<0.05$;***:$p<0.001$. A1: significant relative to A + ALP after 1 h incubation. A15: significant relative to C + ALP after 15 h incubation. C1: significant relative to C + ALP after 1 h incubation. C15: significant relative to C + ALP after 15 h incubation. e) Results of pectin assay. No statistically significant differences were observed between any sample groups. Error bars show standard deviation.

Figure 3. a) MTS assay 24 h (left) and 7 days (right) after seeding of hBMSC on bare Ti6Al4V disc (uncoated), Ti6Al4V coated with C + ALP and Ti6Al4V coated with A + ALP. a and c indicate significant differences (a:$p<0.05$, c: $p<0.001$). b) Fluorescence microscopy images of hBMSC after 24 h. Blue: cell nucleus. Green: F-actin fibres. Scale bar: 50 µm.

Figure 1

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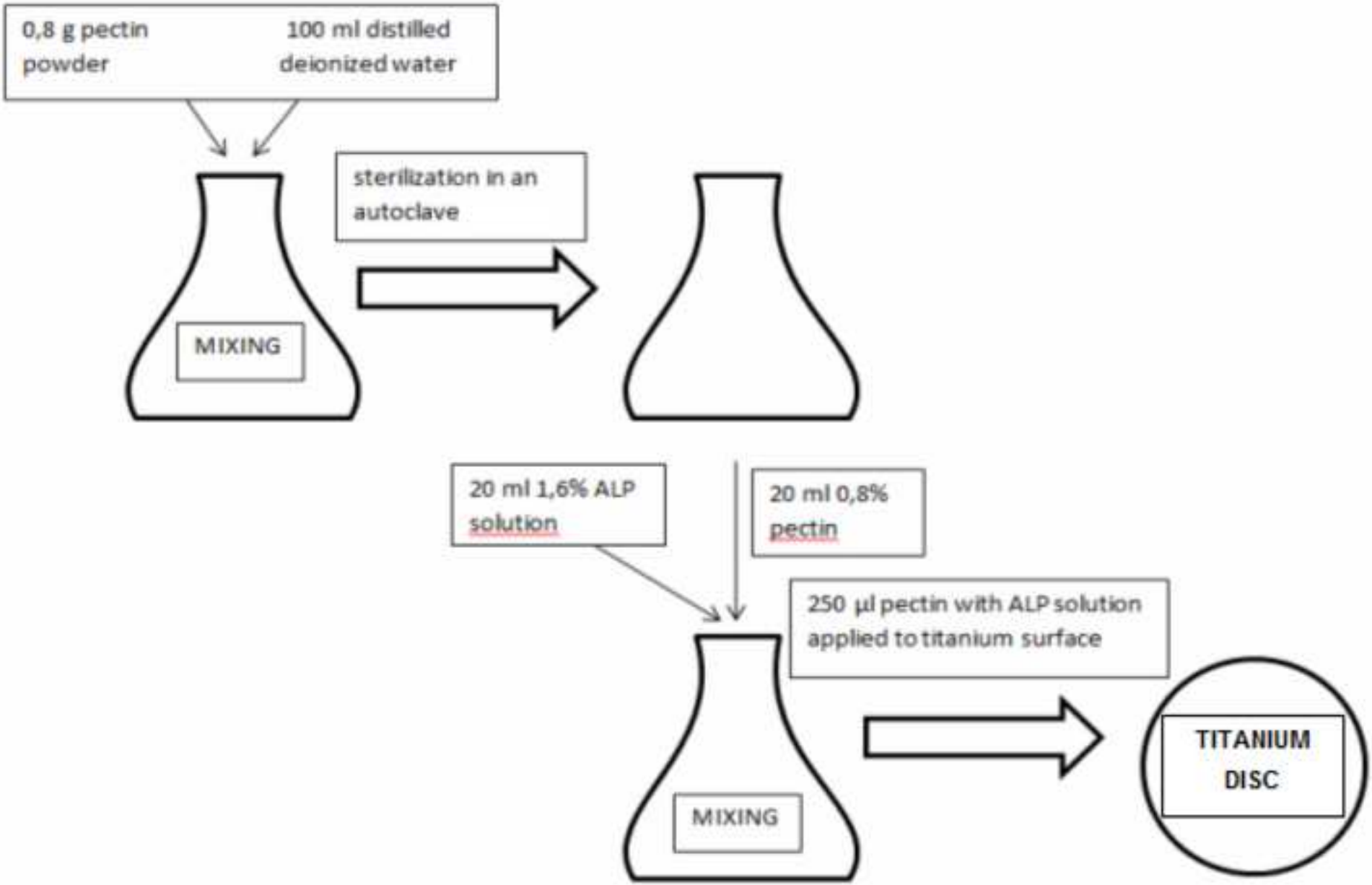

Figure 2

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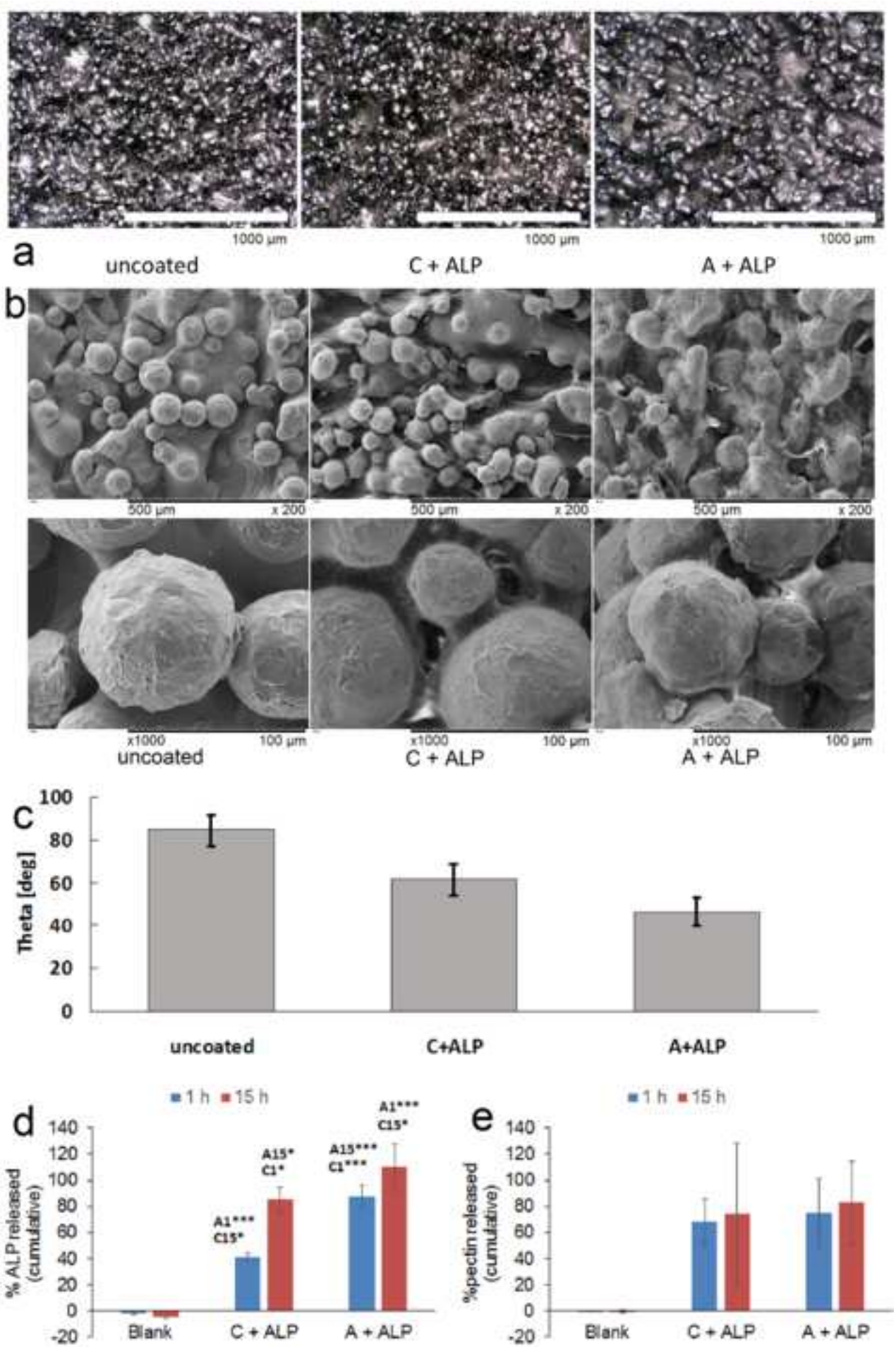

Figure 3

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