Arrangement techniques of proteins and cells using amorphous calcium phosphate nanofiber scaffolds

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ABSTRACT

We demonstrate arrangement techniques of proteins and cells using an amorphous calcium phosphate (ACP) nanofiber scaffold. It is well known that protein and osteoblastic cell are preferably adsorbed onto ACP surface. The ACP nanofiber scaffold was prepared by calcium phosphate mineralization on a polypeptide monolayer-coated mica substrate, and ACP nanofibers were oriented unidirectionally. In a protein system, the ACP nanofiber scaffold was soaked in a fluorescein isothiocyanate conjugated immunoglobulin G (IgG-FITC) aqueous solution. From fluorescence microscopic measurement, the adsorbed IgG-FITC was highly confined and arranged on the ACP nanofiber. In a cell system, a mouse osteoblast-like cell (MC3T3-E1) behavior on the ACP nanofiber scaffold was observed. The cell was elongated unidirectionally, and its cytoskeletal shape showed high aspect ratio. These results are clearly different from an ACP bulk template or bare mica substrate, and the arrangement technique enable to fabricate a fine-tuned biomaterial template.

1. Introduction

An arrangement technique of cells and proteins has potential for the development of tissue engineering and bio-sensing tools. In nature, a cell is the smallest unit of living organism, and macro anatomies such as organ and muscle are formed by cellular orientation and organization. Thus, we believe that the arrangement technique of biomaterials will be a technological bridge between and artificial (experimental) stage and a natural (functional) stage in the tissue engineering. The protein orientational technique has been researched for a bio-sensing using peptide, enzyme, antibody and polysaccharide [1-10]. The tissue engineering materials whose surface enable to assemble cells have advantage in bone-repairing ability.

In previous studies, it is clear that the cell recognizes surface geometry, and scaffold's surface characteristics control the cell behavior. A human gingival fibroblast cell adheres and orients along the groove direction of a titanium scaffold and also recognizes the difference of surface roughness [11]. Such contact guidance phenomenon is commonly noted about various cells [12-16], therefore, cell's adhesion, extension, propagation and orientation can be control with a surface-fabricated template. In addition, it is well known that a surface chemistry also controls the cell behavior [17-19]. Keselowsky et al. showed the cell adhesion on various functional group-modified templates [20]. A mouse osteoblast-like MC3T3-E1 cell adhere well on hydroxyl groups-modified surface more than other functional group-modified ones such as carboxyl, amino and methyl groups. Also, the cell behavior is related to a substrate's

1   crystal phase. An osteoblast cell adsorption onto several crystal phases of calcium

2   phosphate was investigated to simulate the bone regeneration system [21-24]. An

3   amorphous calcium phosphate (ACP) surface has highly cell adhesion ability more than

4   hydroxyapatite (HA) one. Although the ACP surface is great successful, its amorphous

5   phase is very unstable, and the ACP morphology is hard to control.

6   In our previous work, we produced the unidirectional ACP nanofiber scaffold by

7   peptide-based biomineralization [25,26]. We prepared the β-sheet conformational

8   peptide nanofiber on the mica substrate and applied the calcium phosphate

9   mineralization onto this peptide template. Although the ACP is unstable and does not

10   grow anisotropically in general, stable ACP nanofibers were formed on our peptide

11   template. Therefore, the ACP nanofiber template is ideal scaffold to establish the

12   fundamental arrangement technique of proteins and cells.

13   In this study, we present adsorption behaviors of immunoglobulin G (IgG) and

14   mouse osteoblast-like cell (MC3T3-E1) onto the ACP nanofiber scaffold, respectively.

15   We also investigate the adsorption onto ACP bulk scaffold, bare mica plate and peptide

16   monolayer template as control experiments and show the advantage of the ACP

17   nanofiber scaffold as the template for arrangement techniques of proteins and cells.

18

19   **2. Experimental Section**

20   *2.1 Preparation of ACP Nanofiber Template*

21   We prepared the ACP nanofiber template by calcium phosphate mineralization on the

peptide monolayer (Scheme 1). In our previous study, we succeeded to fabricate a

monolayer template of orientational peptide nanofibers [25-26]. This peptide has

hydrophobic leucine (L) – hydrophilic glutamic acid (E) alternate sequence $(LE)_8$ that is

easy to form a β-sheet conformation, moreover, polyethylene glycol (PEG, degree of

polymerization = 70) is placed at the C-terminal of $(LE)_8$ to prevent a formation of

amyloid-like aggregate. Therefore, this peptide was named $(LE)_8$-PEG_{70} and

synthesized by combinatorial solid phase peptide synthesis [27]. Dipping the fresh mica

substrate in 1.0×10^{-5} M $(LE)_8$-PEG_{70} aqueous solution (pH 11.0) at 25 ^{o}C for 18 h,

peptides adsorb on the mica surface and self-assemble to unidirectional nanofibers. On

this peptide monolayer template, we carried out the calcium phosphate mineralization

by what we call the alternate dropping method. For the calcium and phosphate sources,

we used aqueous solutions of calcium acetate ($Ca(CH_3COO)_2$) and diammonium

hydrogen phosphate (($NH_4)_2HPO_4$). 20 μL of a 50 mM $Ca(CH_3COO)_2$ aq solution was

dropped onto the peptide-template over the course of 1 min. Then, the template was

rinsed and dried. The washing process removes the surplus calcium ions on the peptide

surface and prevents homogeneous nucleation when the phosphate source is dropped.

Then, 20 μL of a 30 mM $(NH_4)_2HPO_4$ aq solution was dropped onto the template in the

same way. After 10 min, the template was washed and dried again. An ACP bulk

template as a control was prepared by alternate soaking method that bare mica substrate

was soaked in 50 mM $Ca(CH_3COO)_2$ aq and 30 mM $(NH_4)_2HPO_4$ aq solutions

alternately.

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2 *2.2 Protein Adsorption*

3   Protein orientational adsorption was carried out on the ACP nanofiber template. First,

4 to confirm that proteins could adsorb and orient on ACP nanofiber, IgG-FITC

5 adsorption was applied. The ACP nanofiber scaffold was soaked in 0.5 or 0.05 wt%

6 IgG-FITC aqueous solution for 1 h. After adsorption, the template was rinsed with

7 distilled water to remove surplus adsorbed IgG-FITC. The ACP bulk scaffold, bare

8 mica substrate and peptide monolayer template were used as control experiments.

9 Second, to better the MC3T3-E1 cell adhesion onto the template, fibronectin that is

10 commonly known as an adhesive protein of cells was adsorbed on the ACP nanofiber

11 scaffold. The template was soaked in 0.05 or 0.5 wt% fibronectin aqueous solution and

12 incubated at 37 ^{o}C under 5 % CO_2 atmosphere for 2 h. After incubation, the template

13 was rinsed with phosphate-buffered saline (PBS (-)).

14

15 *2.3 Cell Culture*

16   Mouse osteoblast-like cell MC3T3-E1 (Riken Bio Research Center, Japan) was

17 cultured in the minimum essential alpha medium (α-MEM) containing 10wt% fetal

18 bovine serum (FBS) and 1wt% penicillin-streptomycin. During the cell culturing, the

19 experimental system was maintained in 5% CO_2 at 37 ^{o}C using a gas jacket incubator.

20 The MC3T3-E1 cell was trypsinized and 3 mL of a suspension (5.0×10^3 cells/cm^2) was

21 added to each template placed into the well of a 6-wells plate. Templates of ACP

1   nanofiber, ACP bulk, bare mica and peptide monolayer were incubated for 24 h under

2   standard culture conditions (5% CO_2, 37 ^{o}C), respectively. After incubation, the

3   suspension was removed, and cells were rinsed with PBS (-) and were fixed with 3.7%

4   formaldehyde in PBS (-) for 10 min. The fixed cell membrane was permeabilized with

5   0.1% Triton-X-100-containing PBS (-) at room temperature for 5 min and incubated

6   with 400 µL PBS (-) containing 10 µL rhodamine-phalloidin, 1% arubuminn for 20 min

7   to stain the actin filament. We observed the IgG-FITC protein and the actin filament

8   stained with rhodamine-phalloidin by fluorescence microscope (BX51, OLYMPUS Co.

9   Japan).

10

11   **3. Results and Discussion**

12   *3.1 Morphology of ACP Nanofiber Scaffold*

13   We show the field-emission scanning electron microscopic (FE-SEM) image of the

14   ACP nanofiber scaffold in Fig. 1. Unidirectional nanofibers were observed on the

15   peptide-coated mica surface and formed at regular intervals. Formation of the

16   orientational morphology relates to the peptide monolayer that consists of β-sheet

17   nanofibers. At the outermost layer of the peptide monolayer, carboxyl groups that are

18   side chain of glutamic acid orient two-dimensionally and induce the calcium phosphate

19   mineralization. Therefore, the calcium phosphate precipitation which cannot be

20   normally controlled their morphology is confined by the orientational peptide

21   monolayer. The crystal phase of the calcium phosphate nanofiber was ACP assigned by

1   electron diffraction measurement [25].

2

3   *3.2 Protein Adsorption*

4   The ACP nanofiber, ACP bulk, bare mica and peptide monolayer templates that were

5   soaked in the 0.05 wt% IgG-FITC aqueous solution were observed by fluorescence

6   microscopy, respectively (Fig. 2). On the ACP nanofiber scaffold, highly confined and

7   oriented adsorption of IgG-FITC protein was observed along the ACP nanofiber

8   direction (a : fluorescence image, f : optical image). On the other hand, IgG-FITC was

9   adsorbed randomly on the ACP bulk scaffold (b), bare mica substrate (c) and peptide

10   template (d) as the control experiments. On the ACP nanofiber fiber template, the

11   peptide monolayer was exposed between the ACP nanofibers. At the surface of peptide

12   monolayer, the PEG part that is inhibitor of protein adsorption is also exposed [28-30].

13   Moreover, calcium phosphate generally has highly protein-adsorbed ability and is used

14   as a protein adsorbent reagent [31,32]. Therefore, the IgG-FITC was selective adsorbed

15   onto the ACP parts. IgG was able to adsorb onto the mica surface because mica surface

16   also had hydrophilic group such as hydroxyl group. At the result of 0.5 wt%

17   solution-treated to ACP nanofiber template, IgG-FITC was adsorbed randomly (e).

18   Therefore, the ACP part reached critical limit to adsorb the protein, and IgG was also

19   adsorbed onto the peptide monolayer region.

20

21   *3.3 Cell Adhesive Behavior*

1   The MC3T3-E1 cell adhesion was applied onto ACP nanofiber, ACP bulk, peptide

2   monolayer and bare mica templates, respectively, and their

3   rhodamine-phalloidin-stained actin filaments were observed by fluorescence

4   microscopy (Fig. 3). On ACP nanofiber (a) and peptide monolayer templates (b), actin

5   stress fibers elongated anisotropically, respectively. Especially, the actin fibers on the

6   ACP fiber template were longer than on the peptide template (average of filament

7   length, on ACP nanofiber: 430±12 μm; on peptide monolayer: 332±7 μm) and were

8   highly aspect ratio (on ACP nanofiber: 50.2; on peptide monolayer: 32.4). It is well

9   known that the MC3T3-E1 adhesion is promoted on ACP surface [33]. At the outermost

10   surface of ACP, calcium ion may elute slowly, and Ca^{2+} concentration is locally

11   increased at the scaffold's surface. These calcium ions promote a cellar motor protein

12   such as myosin, which is driven with energy of adenosine triphosphate hydrolysis.

13   Comparing ACP bulk and bare mica templates, although the cell's extensional direction

14   was not controlled, the stress fiber was also more elongated on the ACP bulk template

15   than on the bare mica substrate. From the viewpoints of the surface geometry and

16   chemistry, ACP nanofiber scaffold is ideal template to control the cell orientation. To

17   better the cell adhesion, we additionally immobilized the fibronectin that is adhesive

18   protein on the ACP nanofiber. Fig. 4 shows the actin filament of M3CT3-E1 on 0.05

19   wt% (a) and 0.5 wt% (b) fibronectin solution-treated ACP nanofiber scaffolds. As the

20   fibronectin concentration was increased, the number of cell was increased, but the

21   filament length and aspect ratio were decreased. Fibronectin was adsorbed enough on

both the ACP fiber parts and non-ACP parts (peptide part) at 0.5 wt%. Therefore, surface specific pattern was buried, and cells were omnidirectionaly elongated.

4. Conclusion

We demonstrate the arrangement techniques of protein and cell on our ACP nanofiber scaffold. Both IgG protein and MC3T3-E1 osteoblast-like cell were attached well along the ACP nanofiber direction, and these results were clearly different against their behaviors on peptide monolayer and bare mica substrate. At the present, we are able to fabricate various morphological calcium phosphate nano-patterned scaffolds, in addition, the nanofiber consisted of hydroxyapatite crystal phase is also prepared on the various kinds of peptide monolayers. The peptide monolayer controls the calcium phosphate crystal phase and morphology. Therefore, we believe that protein and cell orientational systems that enable to comply the various needs in the fields of tissue engineering and bio-sensing technology will be produced using this technique.

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3 **Scheme caption**

4 **Scheme 1** Preparation of the ACP nanofiber on the peptide self-assembled-monolayer.

5 (a) β-sheet peptides self-assemble to the nanofiber. (b) Peptide nanofibers self-organize

6 to the monolayer. (c) Calcium ions are trapped and confined by hydroxyl groups of the

7 peptide monolayer surface. (d) Calcium phosphate precipitation is formed along the

8 peptide nanofiber direction.

9

10 **Figure captions**

11 **Figure 1** FE-SEM image of the ACP precipitate on the peptide monolayer. The

12 unidirectional ACP nanofibers were formed along the peptide nanofiber direction.

13 **Figure 2** Fluorescence microscopic images of IgG-FITC adsorbed onto (a) ACP

14 nanofiber scaffold, (b) ACP bulk scaffold, (c) bare mica substrate and (d) peptide

15 monolayer, respectively. Templates were soaked in 0.05 wt% IgG-FITC aq solution. (e)

16 ACP nanofiber scaffold was soaked in 0.5 wt% IgG-FITC aq solution. (f) Optical

17 microscopic image of (a).

18 **Figure 3** Fluorescence microscopic images of rhodamine-phalloidin-stained actin

19 filaments onto (a) ACP nanofiber scaffold, (b) ACP bulk scaffold, (c) bare mica

20 substrate and (d) peptide monolayer, respectively.

21 **Figure 4** Dependence of the cell behavior on the fibronectin concentration. The ACP

1   nanofiber scaffolds were soaked in (a) 0.05 wt% and (b) 0.5 wt% fibronectin aqueous

2   solutions at 37 ^{o}C under 5% CO_2 atmosphere for 2h. (c) Original ACP nanofiber as the

3   control.

1   Scheme 1

Peptide self-assembly

Glutamic acid side

Leucine side

$(LE)_8$-PEG_{70} Peptide   (a) Self-assemble to nanofiber   (b) Self-assemble to monolayer

CaP mineralization

$(NH_4)_2HPO_4$ aq

$Ca(CH_3COO)_2$ aq

Absorbed Ca ions

(c) Calcium source dropping   (d) Phosphate source dropping   ACP nanofiber scaffold
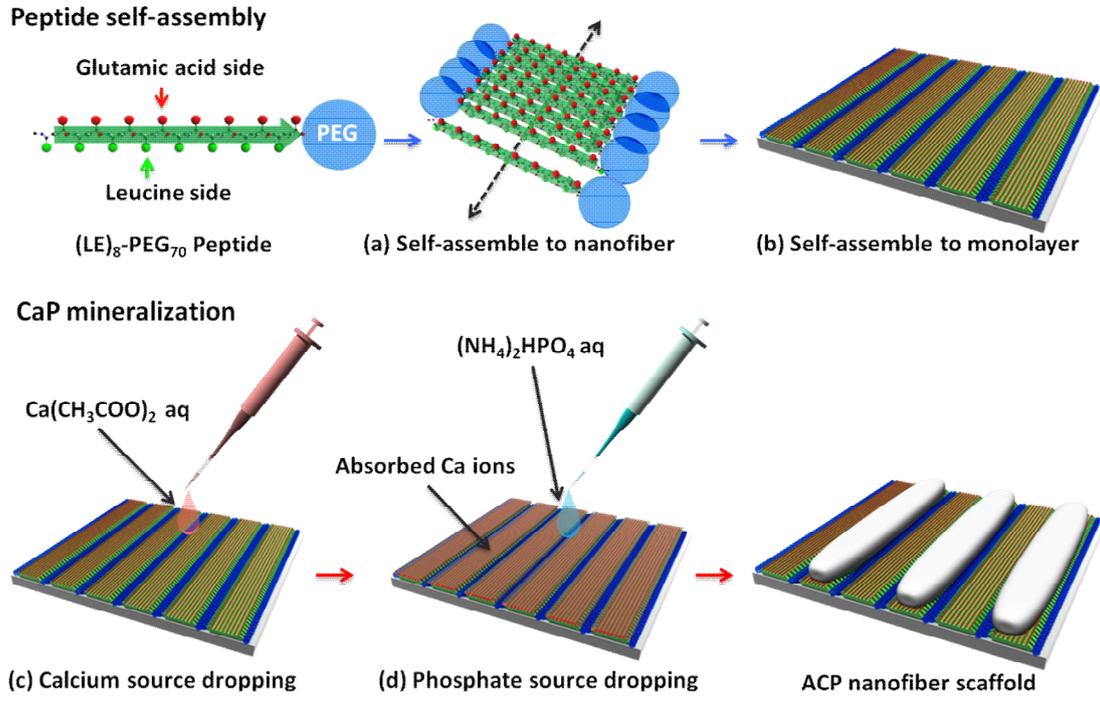
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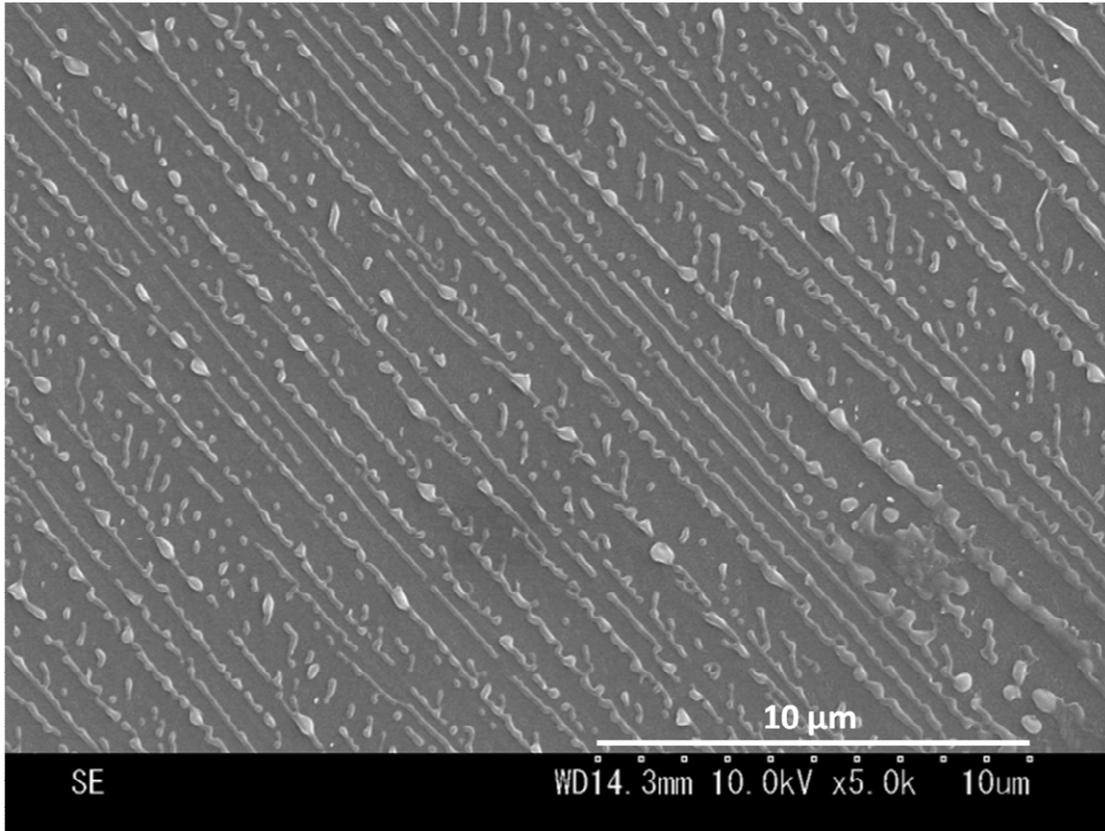
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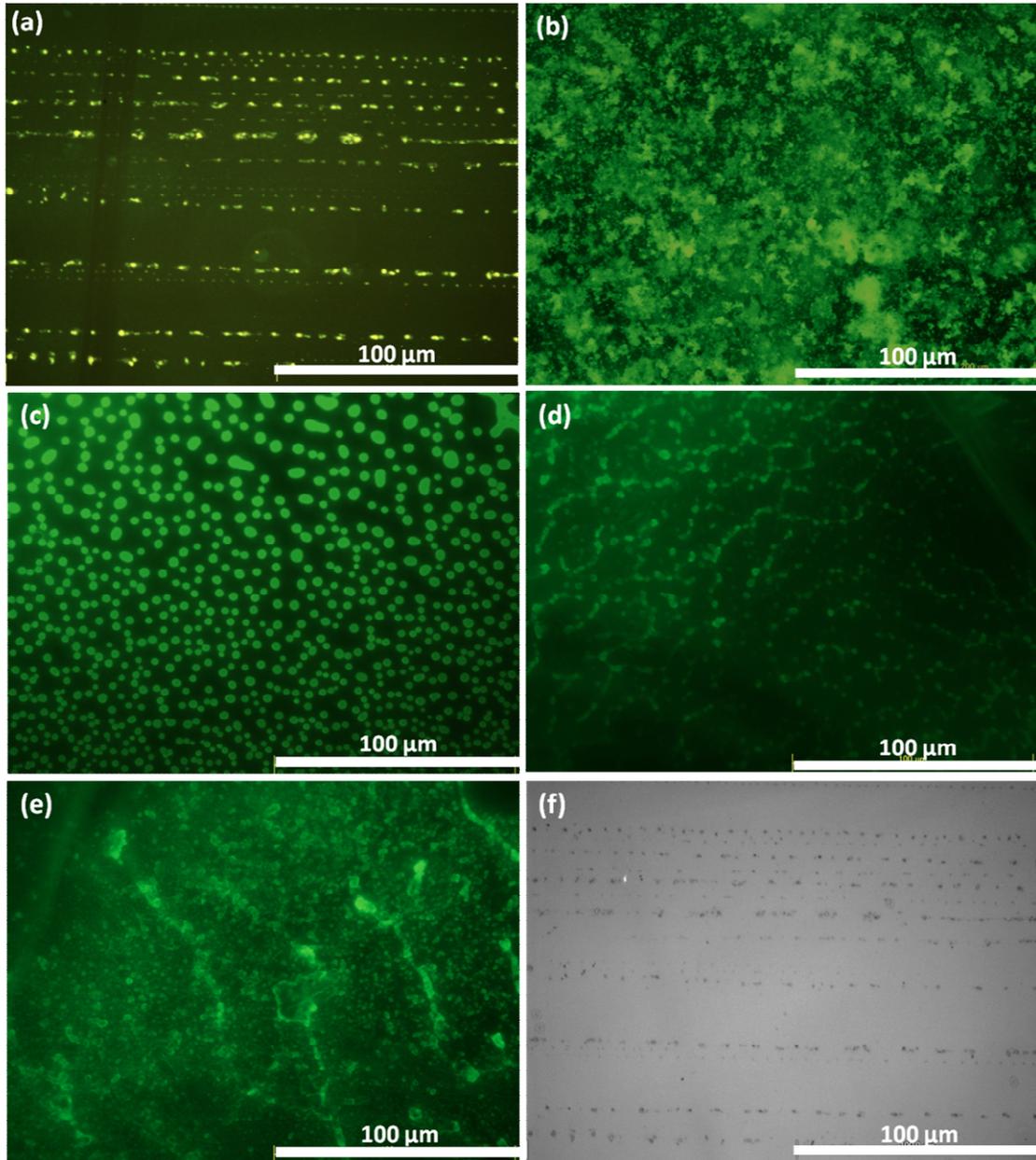
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Figure 4

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