GUIDE

How to perform biofilm investigations in vitro
― Biofilm formation and prevention on P3D Scaffolds

Application
This guide is for seeding bacteria and thereby forming biofilm on P3D Scaffolds to mimic microbe attachment and interactions in relation to human bone and bone implants.

The P3D Scaffold is a porous, bone-like structure made of β-tricalcium phosphate. To understand biofilm formation mechanisms, the P3D scaffold can be used to investigate microbe attachment and how biofilm-residing cells interact with 1) each other, 2) synthetic bone implants, and 3) bone cells on and within the porous structure. Moreover, the structures can be used to test the efficacy of antimicrobial treatments in relation to biofilm prevention and treatment.

For more guides and protocols, please visit our Resources Platform.

Guidelines

- How to seed *Staphylococcus aureus* (S. aureus) onto the scaffold to investigate bacterial biofilm formation.

- How to pretreat the scaffold with antimicrobial drugs prior to seeding to investigate their effect on biofilm formation.

- How to examine the influence of biofilm microbes on bone cells and bone homeostasis.
Notes before starting and preparation of the P3D Scaffolds

- All handling of the P3D Scaffolds should be performed using gloves, according to standard aseptic methods.

- The P3D Scaffolds are supplied sterile by dry heat sterilization and remains sterile until opened.

- **Optional:** Wash the disinfected or sterilized P3D Scaffolds in PBS x3 for one minute to remove any particles. Note: the material and structure of the P3D Scaffold are not compromised during sterilization or disinfection, but loose particles may occur. This can be done by placing one sterile P3D Scaffold in each well of a suitable multi-well plate. The choice of container depends on the scaffold format you are using.

![Figure 1: Optional: place the scaffolds in a suitable container for pretreatment.](image)

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P3D Scaffolds Guide for biofilm investigations in vitro (Rev. 2-2021)
Biofilm formation: How to seed S. aureus onto the P3D Scaffold

Biofilm formation on the P3D Scaffold can be conducted by Staphylococcus Aureus (S. aureus).

1. Create a Herapin Plasma containing GFP-producing S. aureus and fluorescent fibrogen.
   a. Herapin Plasma is created from Full blood from a pool of at least five people. Centrifuge the blood to separate the plasma from the cells.
   b. Dilute the plasma in PBS to create a Medium with a concentration of 10% heparin plasma.
   c. Add 2 μg/mL of fluorescent fibrinogen to the medium.
   d. Add 5 mL of the medium to a 50 mL tube together with 250 μL of an OD = 0.1 suspension of GFP-producing S. aureus to the Herapin Plasma.

2. Place each P3D Scaffold in an individual 50 mL tube so that it is submerged in the Heparin Plasma containing GFP-producing S. aureus and fluorescent fibrinogen.

3. Wrap the tubes in aluminum foil and incubate them at 35°C for 20 hours.

4. After incubation, place the scaffolds between glass slips and assess each scaffold in a microscope to determine biofilm formation.

To assess variations in biofilm formation on synthetic bone implant materials, the same procedure could be used with scaffolds of different materials for comparison with the β-tricalcium phosphate scaffold.
Biofilm prevention: How to treat the P3D Scaffold with antimicrobial drugs

To prevent biofilm formation, it is important to treat the surface they are growing on. *S. aureus* cannot form biofilm if the immune system is allowed access to the implant or if antibiotic drugs inhibit the *S. aureus*.

Antibiotic treatment of the scaffold could be performed as a pretreatment in preparation for implantation or for treating significant damage to the bone tissue, e.g., in the case of severe bone fractures with open wounds.

For example, the prevention of biofilm formation by *S. aureus* can be obtained by rifampicin:

1. Place the P3D Scaffolds in a suitable container. You can use the multi-well plate or other container that was used during the preparation of the scaffolds. For optimal results, only place one P3D Scaffold per well or container.

2. Dissolve 100 mg of rifampicin in 10 mL ethanol under stirring and low heat (37°C).

3. After dissolving the rifampicin, pour the solution onto the scaffolds and set them to load for 24 hours. **Note:** Each well or dish could be loaded with different antibiotic treatments to test their respective efficacy.

4. After 24 hours, remove the scaffolds from the loading solution to dry.

5. Once subjected to the treatment, submerge the pretreated scaffold in Heparin Plasma containing GFP-producing *S. aureus* and fluorescent fibrinogen in individual 50mL tubes. (See above, item 1.a.-d.)

6. Wrap the tubes with Heparin Plasma and scaffold in aluminum foil and incubate them at 35°C for 20 hours.
7. Asses each scaffold in a microscope to determine biofilm formation.

**Note:** The same antibiotic loading method / pretreatment of the scaffolds can also be used with other antibiotics such as Vancomycin etc.

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**How to examine the influence of biofilm microbes on bone cells and bone homeostasis.**

If you wish to examine the effect of biofilm on bone cells and bone homeostasis, you can create a human bone 3D culture on the scaffold, using bone cells, prior to the above tests.

To create the 3D bone culture, seed human mesenchymal stem cells (hMSCs) onto the scaffold. Then, differentiate the stem cells using osteogenic media to develop a natural diversity of osteocytes and osteoblasts on the scaffold to mimic native bone tissue.

By subsequently adding bacteria to the 3D culture containing scaffold, you can create an osteomyelitis model.

If you want to know more about how to grow human bone 3D cultures on the P3D Scaffolds, please see our detailed protocol: 'How to 3D Culture Human Mesenchymal Stem Cells'.

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