Adhesion and Colonisation Intensity of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* on a Composite Material Surface of Hydroxyapatites and Titanium Dioxide

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Abstract

Introduction: The use of biomaterial implants in medicine is becoming ever more popular, and on many occasions the use of biomaterial implants becomes a lifesaving procedure. A primary obstacle to a wider use of biomaterial implants is their risk of infection and unsuccessful tissue integration with the surface of biomaterials. For the creation of new substances various combinations of materials are used nowadays, thus forming composite materials. Composite materials are materials which consist of two or more components or phases, which according to their characteristics vary considerably, but they are mutually dissolvent or little solvent, and they have a distinct border surface.[1] Most often titanium (Ti) and titanium fused implants are covered, as they are bioinert materials.[2-4] HAp/TiO_2 composite ceramic layer shows better linkage strength with the material if compared with pure HAp. By increasing TiO_2 amount in the composite, its strengths also enhances, as well as TiO_2 addition can decrease attachment of bacteria to the biomaterial, thus decreasing the possible infection risk of the implant; however infection risk around TiO_2 containing implant is still a problem in medicine.[5]

Objective: The objective of the study was to determine *Staphylococcus epidermidis (S.epidermidis)* and *Pseudomonas aeruginosa (Ps.aeruginosa)* adhesion and colonisation intensity on hydroxyapatite (HAp) and titanium dioxide (TiO_2) commercial composite material surfaces originally synthesised by Biomaterial Development and Innovation Centre of Riga Technical University. Materials used: Composite material samples used:

No 1 100% HAp – burned 1000^{o}C, No 2 50% HAp and 50% TiO_2 – burned at 1000^{o}C, No 3 80% HAp and 20% TiO_2 – burned at 1000^{o}C, No 4 100% TiO_2 – burned at 1000^{o}C, No 5 20% HAp and 80% TiO_2 – burned at 1000^{o}C, No 6 100% TiO_2 – burned at 1200^{o}C, No 7 20% HAp and 80% TiO_2 – burned at 1200^{o}C, No 8 80% HAp and 20% TiO_2 – burned at 1200^{o}C, No 9 50% HAp and 50% TiO_2 – burned at 1200^{o}C, No 10 100% HAp – burned at 1200^{o}C

Method: *Ps.aeruginosa* ATCC 27853, *S.epidermidis* ATCC 12228 reference cultures were used in the study. Bacterial suspensions were prepared from bacterial pure cultures of 1 ml TSB (Trypto-Casein-Soy Broth) volume with a concentration of 10, 10^2 and 10^3 CFU/ml (*colony forming units*). Samples were cultivated at 37^{o}C temperature for 2 h in order to determine adhesion intensity, and a sample with a concentration of 10^2 CFU/ml for 24 h – to determine colonisation intensity. For adhesion evaluation and determination of colonisation amount, sonication and culture method was used. For determination of colonisation intensity sonication-culture method was used as well as scanning electron microscope.

Result: In general, adhesion intensity on HAp and TiO_2 composite material surface is not big. *S.epidermidis* adhesion starts at 10 CFU/ml/2h/37^{o}C exposition only for biomaterial types 2, 3, 4 (from 0.0027-0.003 CFU/mm^2), at 10^2 CFU/ml/2h/37^{o}C – better adhesion is observed on surfaces of biomaterials 3 and 4, and worse adhesion on surfaces of biomaterials 6, 7, 8. At 10^3 CFU/ml/2h/37^{o}C exposition the greatest adhesion is observed on surfaces of biomaterials 3 and 10 (0.093 and 0.094 CFU/mm^2 respectively).

Ps.aeruginosa adhesion intensity was lower than *S.epidermidis* and at 10 CFU/ml/2h/37^{o}C exposition happened only on biomaterial surfaces 1 and 4 (0.028 and 0.001 CFU/mm^2 respectively). At 10^2 CFU/ml/2h/37^{o}C exposition also demonstrated a low adhesion level, and at 10^3 CFU/ml/2h/37^{o}C exposition the lowest adhesion intensity was demonstrated on biomaterials 3, 6, 9, 10, and the greatest one on biomaterial 1.

Conclusion: Used bacteria have low adhesion abilities on HAp and TiO_2 containing biomaterials. *Pseudomonas aeruginosa* show even lower adhesion ability on biomaterials with HAp and TiO_2 mixture.

Optimal HAp/TiO_2 composite ceramics composition, which ensures the lowest contamination risk of microorganisms is with a 50% and 80% TiO_2 content after a thermal processing at 1200 ^{0}C.

Key words: S.epidermidis, Ps. aeruginosa, Titanium Dioxide, Composite Material, Hydroxyapatite, Adhesion, Colonisation.

Introduction

For the creation of new substances various combinations of materials are used nowadays, thus forming composite materials. Composite materials are materials which consist of two or more components or phases, which according to their characteristics vary considerably, but they are mutually dissolvent or little solvent, and they have a distinct border surface. The use of composites in medicine is a relatively new discovery and many of these materials are still being studied.[1] However, already now their use and manufacturing in implants and medical appliances, e.g. catheters, is much wider, if compared with simple homogenous materials. It is important to note that bacterial adhesion and colonisation intensity must be determined in vitro and in vivo as each component and biomaterial must be biologically compatible[6] and there's a question of how they will interact with the organism of a living being (including humans)[7] as well as how great is the risk of implant infection.[8-11] Within the last 10 years metallic implants have been widely used and they have been covered with a bioactive HAp/TiO_2 layer in order to enhance fixation between the living bone and implant, and the formation of new bone cells, as well as in order to prevent metal corrosion. Most often titanium (Ti) and titanium fused implants are covered, as they are bioinert materials.[2-4] HAp/TiO_2 composite ceramic layer shows better linkage strength with the material if compared with pure HAp. By increasing TiO_2 amount in the composite, its strengths also enhances,[12] as well as TiO_2 addition can decrease attachment of bacteria to the biomaterial,[13] thus decreasing the possible infection risk of the implant; however infection risk around TiO_2 containing implant is still a problem in medicine.[5]

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Material and Method

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No 8 80% HAp and 20% TiO_2 – burned at 1200^oC

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Ps.aeruginosa ATCC 27853, *S.epidermidis* ATCC 12228 reference cultures were used in the study. Bacterial suspensions were prepared from bacterial pure cultures of 1 ml TSB (Trypto-Casein-Soy Broth) volume with a concentration of 10, 10^2 and 10^3 CFU/ml (*colony forming units*). Samples were cultivated at 37^{o}C temperature for 2 h in order to determine adhesion intensity, and a sample with a concentration of 10^2 CFU/ml for 24 h – to determine colonisation intensity. For adhesion evaluation and determination of colonisation amount, sonication and culture method was used.[14-15] For determination of colonisation intensity sonication-culture method was used[14-15] as well as scanning electron microscope.

Sonication-culture method:[14-15] After incubation unattached microorganisms were rinsed off. In order to detach bacteria that had not attached to the surface of biomaterials, discs were processed for 1 minute in ultrasonic bath (at 45 kHz frequency) and for 1 minute at a maximum velocity in a *Vortex* centrifuge. In order to determine the total amount of microorganisms, cultures were prepared from each sample on a TSA (Trypto-Casein-Soy agar) culture and they were cultured for 24 hours in a temperature of 37^{o}C.

Scanning electron microscope: *TESCAN* manufactured SEM *Mira LMU* was used. Prior to analysis, biomaterial samples were dried and fixated in ethyl alcohol ether (1:1) mixture, and then a thin layer of gold was dusted onto the samples for better electron conduction in order to make qualitative SEM micrographs.

Acquired data was processed, using *Microsoft Office Excel.*

Results

In general, adhesion intensity on HAp and TiO_2 composite material surface is not so high. *S.epidermidis* adhesion starts at 10 CFU/ml/2h/37^{o}C exposition only for biomaterial types 2, 3, 4 (from 0.0027-0.003 CFU/mm^2), at 10^2 CFU/ml/2h/37^{o}C – better adhesion is observed on surfaces of biomaterials 3 and 4, and worse adhesion on surfaces of biomaterials 6, 7, 8. At 10^3 CFU/ml/2h/37^{o}C exposition the greatest adhesion is observed on surfaces of biomaterials 3 and 10 (0.093 and 0.094 CFU/mm^2 respectively).

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Adhesion intensity results are shown in diagrams 1a, 1b, 2a, 2b and table 1.

Bacterial colonisation on composite materials used in the study is various. Very low colonisation intensity was observed in HAp material burned at 1000^{o}C, and a very great colonisation intensity on HAp material burned at 1200^{o}C. Various colonisation intensities were observed on materials with a various HAp and TiO_2 composition, and preparation technology (Table 2, Diagram 3a and 3b).

Analysing SEM Figures it was determined that on the surface of biomaterial 1 *S.epidermidis* was practically not observed, whereas on the rest of composite materials *S.epidermidis* did not form its characteristic biofilm, instead colonising material surface in a dispersed manner, in types of colonies (Figure 1)

Ps.aeruginosa colonised composite materials similarly as staphylococci, colonising, without forming a biofilm, rather forming a dispersed bacterial film (Figure 2).

Discussion

From the experimental data you can see that a clean TiO_2 has very low bacterial adhesion compared to other samples, which coincides with *A. Pavlovas* study carried out in 2011. This can be explained by the fact that TiO_2 has a hydrophilic surface, whereas both bacterial surfaces are hydrophobic. Since microorganisms with hydrophobic characteristics attach better to hydrophobic surfaces, composite materials with a greater amount of TiO_2 contain less adhesion. It was observed that *S.epidermidis* bacteria has greater tendency to attach to synthesised HAp. Sample surfaces burned at 1200^{0}C demonstrate lower levels of adhesion, because bacteria find it easier to attach to porous materials, therefore adhesion intensity of microorganisms on denser samples is much lower, because microstructure of materials burned at 1200°C is less porous.

In diagrams 2a and 2b you can see that composite materials HAp with 50%TiO_2 and HAp with 80% TiO_2 burned at 1200 °C have the lowest microorganism adhesion. Tendency of *Ps.aeruginosa* bacteria to attach to composite material surfaces is lower than that of *S.epidermidis*.

Experimental data in diagram 3 shows that tendency of *Ps.aeruginosa* bacteria to colonise ceramic surfaces of the samples is slightly less than that of *S.epidermidis*. Lower microorganism colonisation can be observed on sample surfaces burned at 1200^{0}C because of decrease of pores.[16] Therefore in case of *S.epidermidis* composite ceramics with HAp composition of 80% TiO_2 chances of colonisation are lower at 1200^{0}C, whereas in case of *Ps.aeruginosa* it is characteristic of all composites at both burning temperatures.

Conclusion

Used bacteria have low adhesion abilities on HAp and TiO_2 containing biomaterials. *Pseudomonas aeruginosa* show even lower adhesion ability on biomaterials with HAp and TiO_2 mixture.

Optimal HAp/TiO_2 composite ceramics composition, which ensures the lowest contamination risk of microorganisms is with a 50% and 80% TiO_2 content after a thermal processing at 1200 ^{0}C.

For future studies and applications - biomaterials that demonstrated low bacterial adhesion will be implanted in laboratory animals to determine the minimal infective dose of biomaterial, as well as research expression of bacterial-biomaterial interactions on inflammatory mediators (IL-10, TNF-α, β-defensin-2) in tissues.

Acknowledgment

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Conflict of Interest: None declared.

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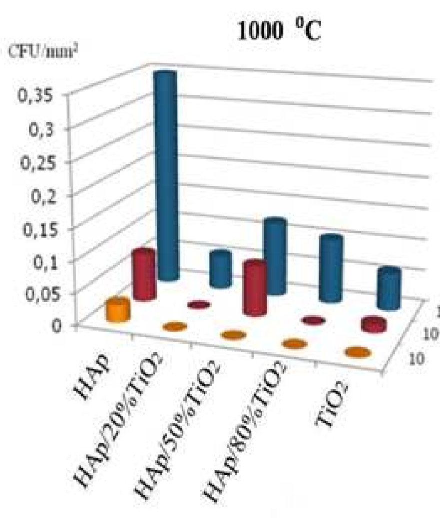
Table 1: Table 1 Bacterial adhesion intensity on surfaces of HAp and TiO_2 biomaterials ($P<0.05$)

B/m	Adhasion intensity					
	S.epidermidis CFU/mm^2			*Ps.aeruginosa* CFU/mm^2		
	10^1	10^2	10^3	10^1	10^2	10^3
1.	Adhesion not observed	0.028	0.078	0.028	0.077	0.347
2.	0.0027	0.0057	0.056	Adhesion not observed	0.08	0.122
3.	0.003	0.009	0.093	Adhesion not observed	Adhesion not observed	0.055
4.	0.003	0.009	0.014	0.001	0.013	0.06
5.	Adhesion not observed	0.008	0.073	Adhesion not observed	Adhesion not observed	0.103
6.	Adhesion not observed	0.002	0.018	Adhesion not observed	Adhesion not observed	0.047
7.	Adhesion not observed	0.003	0.069	Adhesion not observed	Adhesion not observed	0.036
8.	Adhesion not observed	0.005	0.134	Adhesion not observed	0.016	0.064
9.	Adhesion not observed	0.004	0.067	Adhesion not observed	Adhesion not observed	0.042
10.	Adhesion not observed	0.014	0.094	Adhesion not observed	Adhesion not observed	0.057

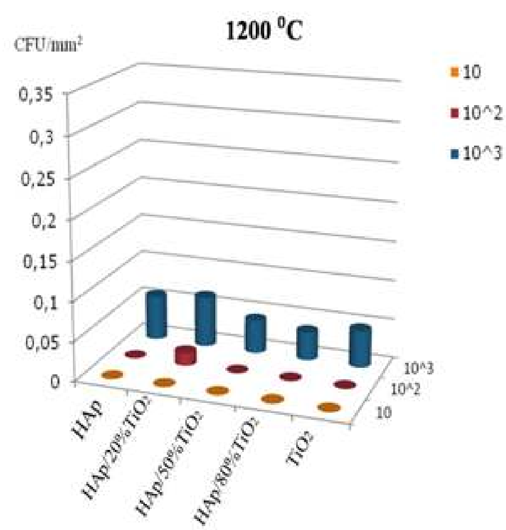
Table 2: Table 2 Bacterial colonisation intensity on surfaces of HAp and TiO_2 biomaterials. ($P<0.05$)

B/m	Colonisation intensity	
	S.epidermidis CFU/mm^2	*Ps.aeroginosa* CFU/mm^2
1.	1.6	627
2.	2168	487
3.	10109	61
4.	13223	362
5.	13607	270
6.	3591	1044
7.	4459	1266
8.	5839	476
9.	5362	780
10.	18652	1677

Diagram 1 and 2: Bacterial adhesion intensity on surfaces of HAp and TiO_2 biomaterials.

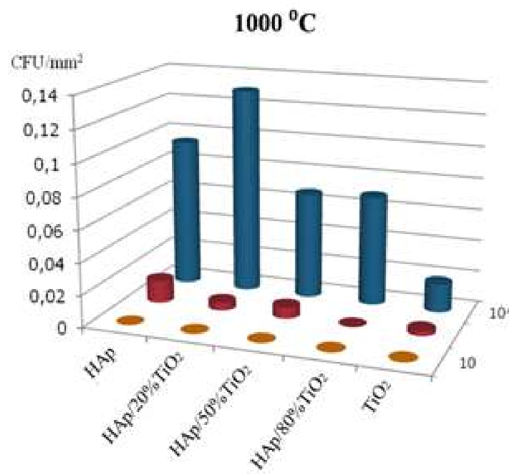


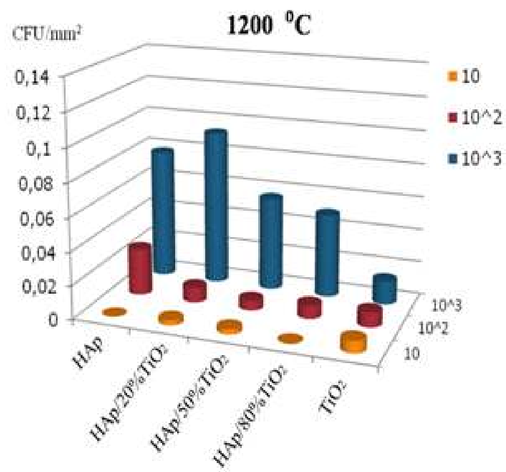
a)



b)

Diagram 1a and 1b. *Ps.aeruginosa* adhesion results on samples obtained after thermal processing in a) 1000 ^{0}C and b) 1200 ^{0}C.

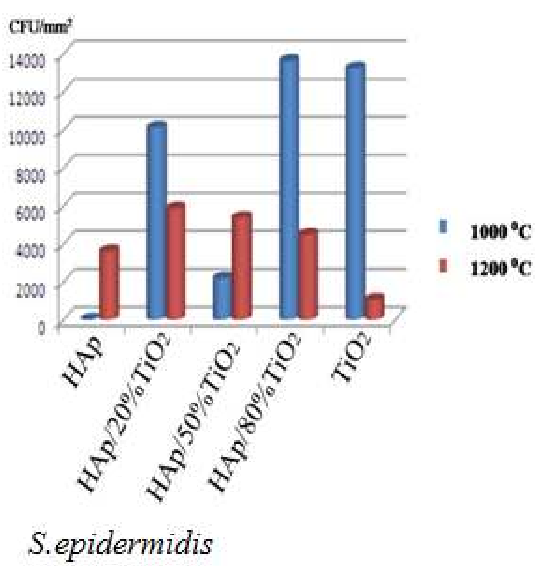


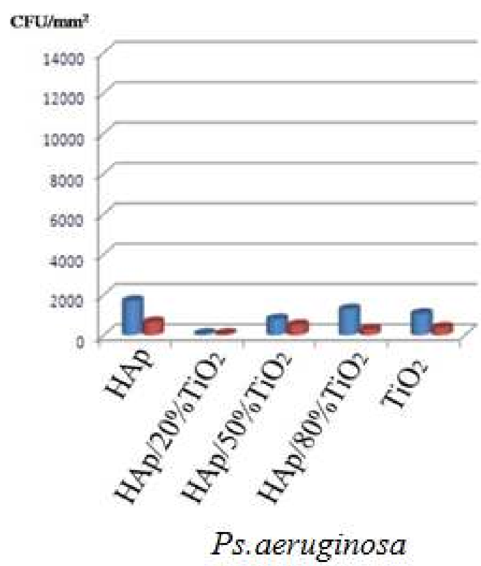


a) b)

Diagram 2a and 2b *S.epidermidis* adhesion results on obtained samples after thermal processing in a) 1000 ^{0}C and b) 1200 ^{0}C.

Diagram 3





a) b)

Diagram 3 Colonisation study a) *S.epidermidis* and b) *Ps.aeruginosa* on sample surfaces after thermal processing at 1000^{0}C and 1200^{0}C of temperature.

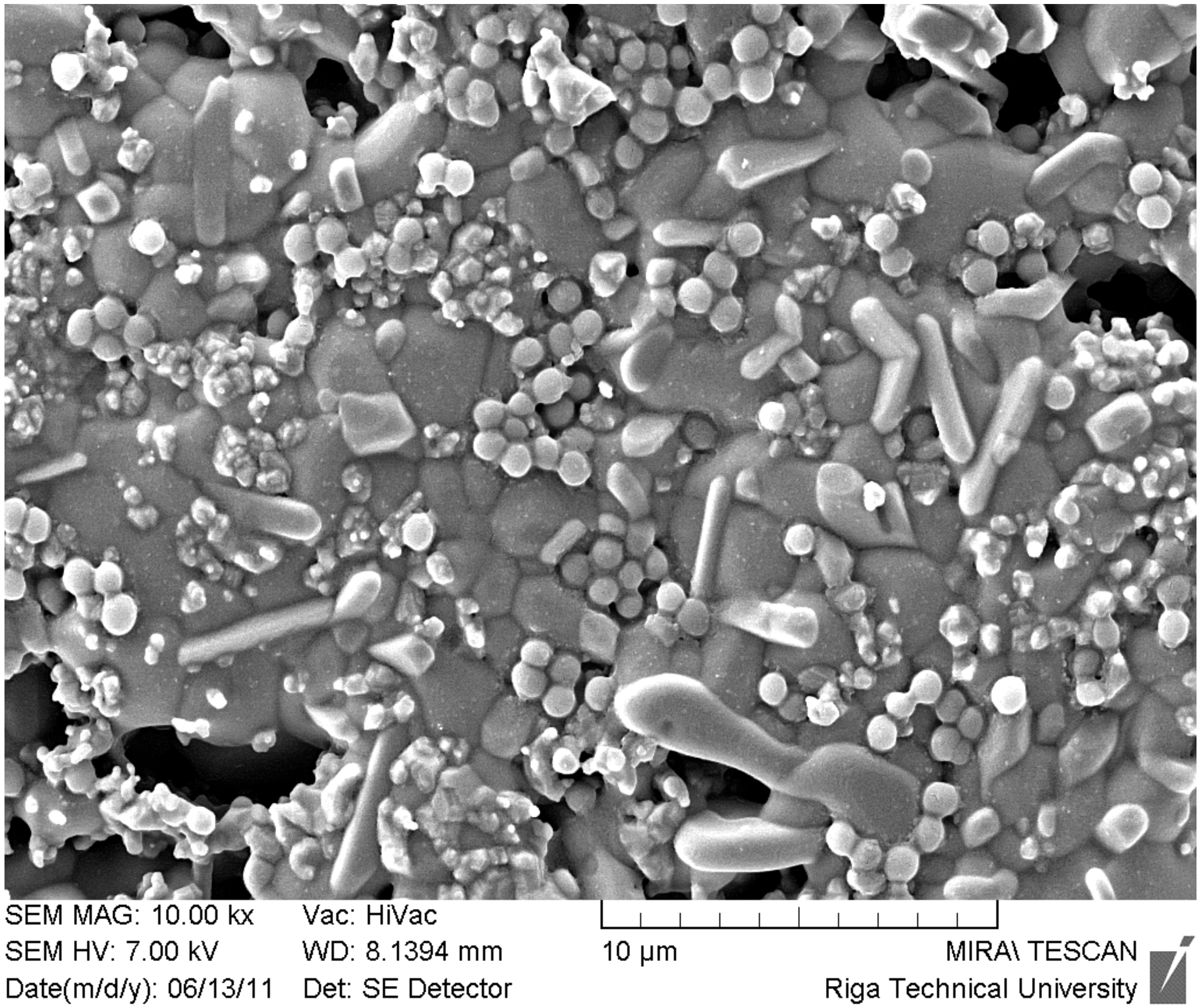


Figure 1: Rare groups of *S.epidermidis* on 80% HAp and 20% TiO_2 surface burned at 1200^oC.

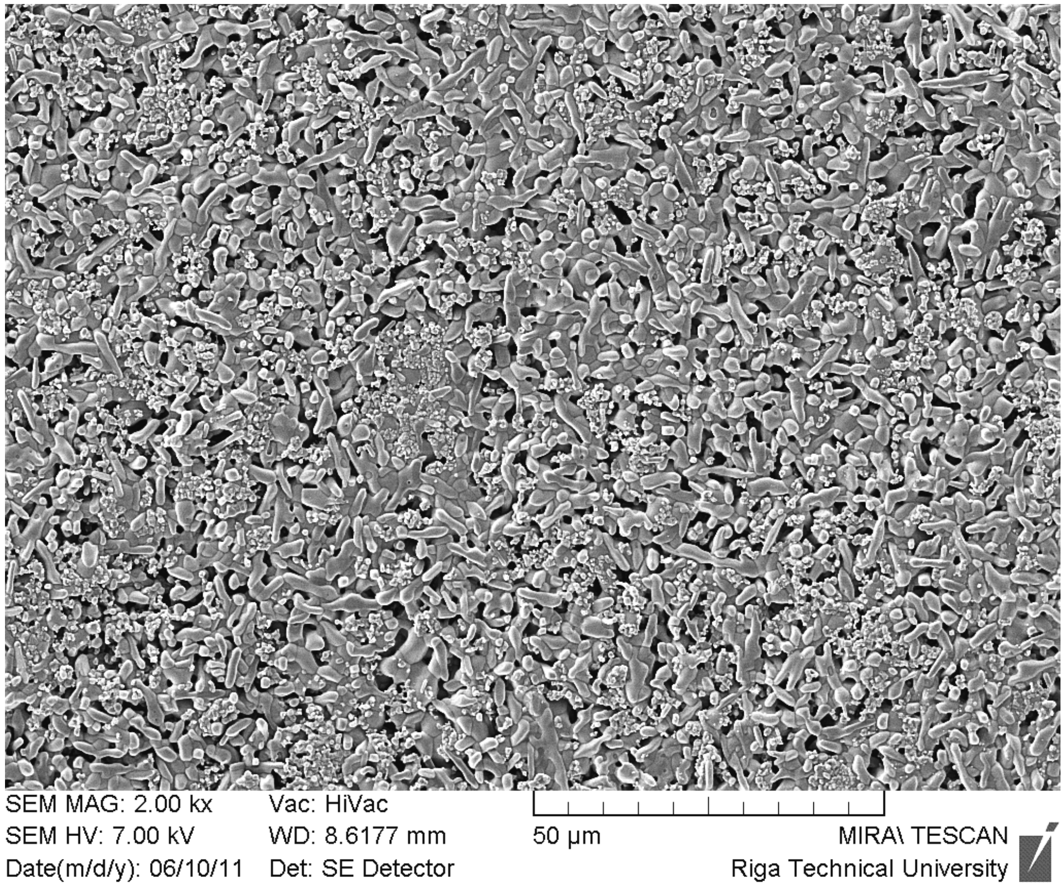


Figure 2: Thin *Ps.aeruginosa* bacterial layer on 50% HAp and 50% TiO_2 surface burned at 1200^{o}C.