

# PREPARATION OF TITANIA SOL-GEL COATINGS CONTAINING SILVER IN VARIOUS FORMS AND MEASURING OF THEIR BACTERICIDAL EFFECTS AGAINST *E. COLI*

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*The work describes titania coatings containing various forms of silver applied on a titanium substrate by a dip-coating sol-gel technique. Silver was added into the basic titania sol in form of colloid particles of Ag, crystals of AgNO<sub>3</sub>, particles of AgI, particles of Ag<sub>3</sub>PO<sub>4</sub> and Ag<sub>3</sub>PO<sub>4</sub> developed in situ (in the sol) by reaction of AgNO<sub>3</sub> with added calcium phosphate (brushite or monetite). Mechanically and chemically treated titanium substrates were dipped at a constant rate into individual types of sols. Subsequently, they were slowly fired. The fired coatings contained microcracks. All over the surface there were evenly distributed spherical nanoparticles of silver (Ag, AgNO<sub>3</sub>) or microcrystals of AgI and Ag<sub>3</sub>PO<sub>4</sub>. The prepared coatings were tested under static conditions for their bactericidal effects against gram-negative bacteria Escherichia coli (*E. coli*). The coated substrates were immersed into a suspension of *E. coli* in physiological solution for 24 and 4 hours. The basic titania coatings with no silver demonstrated no bactericidal properties. Very good bactericidal effect against *E. coli* in both types of bactericidal test showed the titania coatings with AgNO<sub>3</sub>, Ag<sub>3</sub>PO<sub>4</sub> crystals and Ag<sub>3</sub>PO<sub>4</sub> developed in situ.*

## INTRODUCTION

Titania coating containing various ions prepared by a sol-gel method on metallic, glass or other substrates find ever more use in photocatalysis, solar cells, sensors and biomaterials [1, 2]. The sol-gel method, which is based on a controlled mixing process of organic compounds in alcohols and water with an added catalyst, is suitable for formation of thin homogenous coatings using techniques like dip-coating [3], spin-coating [4, 5] or spray-coating [6]. Each of those techniques has its advantages and disadvantages. One advantage of dip-coating is the possibility to coat substrates of complicated shapes under constant conditions. By using suitable reagents and appropriate coating conditions it is possible to prepare coatings of various thickness [1] with bioactive [4, 5], photocatalytic [7, 8] and adhesive [9] properties. Many authors have investigated effects of silver in titania sol-gel coatings on antibacterial effects against various microorganisms, most frequently against *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) while in most cases silver was added in form of silver nitrate [10-12]. There are also other forms of

silver suitable for the purpose, e.g. silver acetate or silver phosphate [13, 14]. Nowadays, a lot of procedures are available for preparation of silver nanoparticles (NP) which also demonstrated antibacterial activity. Procedures for NP preparation focus mainly on reduction of AgNO<sub>3</sub>. The parameters which determine selection of a particular procedure include NP diameter and size, size distribution, shape, stability etc. [15]. Nanoparticles of silver (1 - 100 nm) in water solutions release silver ions that are biologically active and have antibacterial effects. The effect of silver ions on microorganisms is well known, however, the mechanism of the effect has not been fully explained. It is assumed that silver ions interact with three components of the bacterial cell to produce the bactericidal effect: with the peptidoglycan cell wall and plasma membrane, with bacterial (cytoplasmic) DNA and with bacterial proteins [15]. Due to the effect of silver ion, DNA may have lost its replication ability and cellular proteins became inactive [16-18]. Monitoring of effects of concentration of silver NP (10, 50 and 100 µg per cm<sup>3</sup>) on bacterial growth rate of *E. coli* strain B (10<sup>5</sup> CFU) has shown that inhibition of bacterial growth increases with the increasing concentration [19, 20]. Many authors

have also investigated effects of the size of silver nanoparticles on the antibacterial effects and they have agreed that small particles (~ 10 nm) have a better effect than big ones. Small silver nanoparticles were observed both in the membrane and inside the bacteria [21]. Small particles (with a larger active surface) have a stronger antibacterial effect than big particles because a higher number of silver atoms is exposed to the interaction. Moreover, small particles are able to penetrate bacteria better and to damage them [21-24]. An important role in antibacterial effects of the prepared silver nanoparticles may be also played by their shapes [25]. It has been determined that triangular nanoplate particles are more reactive than spheres or nanorods [26].

The objective of our work was to prepare thin titania sol-gel coatings containing silver on titanium substrate by dip-coating technique. Antibacterial coatings prepared in this manner on implants might be in the future used for orthopedic applications in order to reduce the risk of post-surgery infection. Several forms of silver were used in the coatings both, commercially available or prepared by ourselves. Subsequently, we monitored and compared their bactericidal effects against gram-negative bacteria *Escherichia coli* (strain DBM 3138) after the interaction periods of 24 and 4 hours.

## EXPERIMENTAL

Substrates made of pure titanium (Grade 2, ASTM B265) sized 30 × 10 × 1 mm were ground with SiC paper No. 500 and washed with acetone in an ultrasonic bath and then 3 times in demineralized water. After drying the substrates were dipped into diluted hydrofluoric acid (HF:H<sub>2</sub>O = 1:5) for 30 s and rinsed in demineralized water.

Titania sol was prepared by gradual mixing of tetra-*n*-butyl-orthotitanate (Merck), acetylacetonate (p.a. Penta), triton X-100 (Roth), ethanol and 1 mol·l<sup>-1</sup> HNO<sub>3</sub>. The resulting solution was mixed with a magnetic stirrer until the following day at the laboratory temperature. This basic titania sol was divided into seven samples and various forms of silver were added into six of them. The concentration of silver in the sol samples was always the same ( $c_{Ag} = 0.015 \text{ g}\cdot\text{ml}^{-1}$ ). Silver was added in form of colloid particles or crystals of AgNO<sub>3</sub> (p.a. Lach-Ner), Ag<sub>3</sub>PO<sub>4</sub> (Sigma-Aldrich) and AgI. Colloid particles of silver were prepared in a generator of colloid silver (GKS Forte). Silver electrodes (under the voltage of 24 V) were submerged into distilled water to form a colloid suspension after 7 - 12 hours. After the suspension was completely dried we obtained colloid particles of Ag. Crystals of AgI were prepared by reaction of AgNO<sub>3</sub> and KI with subsequent separation. Powders of brushite (CaHPO<sub>4</sub>·2H<sub>2</sub>O, puriss, Sigma-Aldrich) and monetite (CaHPO<sub>4</sub>, puriss, Merck) were added into selected sols containing AgNO<sub>3</sub> in order to chemically produce

Ag<sub>3</sub>PO<sub>4</sub> *in situ* in the sol. In the future we are planning to monitor bioactivity of the coatings as well. Identification and compositions of the individual titania sols and coatings prepared from them, with or without Ag, are provided in Table 1.

Table 1. Composition of the titania sols and coatings prepared from them.

Type of the sol (coating)	Composition of the sol
T	Titania sol
TAN	Titania sol + AgNO <sub>3</sub>
TANB	Titania sol + AgNO <sub>3</sub> + brushite
TANM	Titania sol + AgNO <sub>3</sub> + monetite
TAP	Titania sol + Ag <sub>3</sub> PO <sub>4</sub>
TAI	Titania sol + AgI
TAC	Titania sol + Ag Colloid

Crystals of AgNO<sub>3</sub>, micro-particles of Ag<sub>3</sub>PO<sub>4</sub> and AgI, colloid particles of Ag and brushite and monetite powders were added into the sols immediately before the coating process. The coatings were developed by means of the dip-coating technique. The mechanically and chemically treated substrates were dipped once into the individual sols at the rate of 20 cm·min<sup>-1</sup> and remained in the sols for 30 s. Then the substrates were pulled out from the sols at the rate of 6 cm·min<sup>-1</sup>. Substrate coating was performed by continual mixing on a magnetic stirrer at the laboratory temperature to ensure homogenous distribution of powders in the sols and subsequently in the coatings. All the coated substrates were fired at 400°C for 2 hours, using the heating rate 2°C·min<sup>-1</sup>. Cooling was performed in an oven until the following day. Identification of the coatings was identical as that of the sols from which they were prepared (Table 1).

Surfaces of the individual types of the coatings after firing were investigated with the electron microscope (SEM) Hitachi S4700 with an SDD detector.

The test of bactericidal properties was performed with gram-negative bacteria *Escherichia coli* (*E. coli*, strain DBM 3138). Bacterial culture was incubated in liquid LB medium at 37°C and 230 rpm for 18 hours and then diluted in physiological solution (0.15 mol l<sup>-1</sup> NaCl) to the bacteria concentration 10<sup>4</sup> CFU ml<sup>-1</sup>. The test was performed by dipping of the coated substrate into suspension of *E. coli* in physiological solution with the volume of 2.5 ml for 24 or 4 hours at the laboratory temperature. Then the substrate was removed from the suspension and 100 µl of the suspension was spread on a Petri dish with LB agar. The dish was placed into a biological thermostat set up at 36.5°C for 24 hours. After the incubation period the dish was photographed and the quantity of surviving *E. coli* was counted with NIS Elements AR. 3 software [27]. 2 samples were used for each type of coating. This procedure was performed two times independently and therefore the results indicating

the number of surviving *E. coli* colonies include 4 results. The reference sample was a suspension which was not in contact with the coated substrate.

## RESULTS AND DISCUSSION

The mechanical and chemical treatment of substrates was performed to clean and to roughen the surfaces as the coatings adhere better to a rough surface. The chemical treatment with hydrofluoric acid enlarged the reaction surface of the substrate. Roughening of the substrate was supposed to improve adhesion of the prepared coatings to the substrates.

The previous experiment [27], in which silica sol-gel coatings were prepared containing silver (in form of  $\text{AgNO}_3$ ) and brushite and monetite powders, showed that before the coating both types of the white calcium phosphate powders reacted in the sol with silver nitrate and produced yellow silver phosphate *in situ*. The presence of  $\text{Ag}_3\text{PO}_4$  in the silica sol was confirmed by XRD. Both types of silica coatings (SANB and SANM) had very good antibacterial effects against *E. coli*. The *in vitro* test in simulated body fluid also confirmed bioactive behavior of the coatings.

In case of titania sols the white powders of calcium phosphates were proved to react with silver nitrate. The XRD analysis did not confirm any *in situ*-developed  $\text{Ag}_3\text{PO}_4$  (visibly yellow) in the sol. The detected phases included only brushite, monetite and silver. This can be explained by a small quantity of the newly formed  $\text{Ag}_3\text{PO}_4$  or its low crystallinity.

Figures 1b-g show surfaces of the substrates with fired titania coatings with silver in various forms. For comparison purposes Figure 1a shows the fired substrate with the basic titania coatings without silver.

The figures indicate that all titania coatings (with/without silver) after firing were partly cracked. The titania coatings without silver (Figure 1a) contained mostly isolated cracks ca.  $10\mu\text{m}$  long. Spherical silver particles in all the other types of coatings were nearly evenly distributed all over the surface. The size of silver particles added into the sol as  $\text{AgNO}_3$  (Figure 1b-d) or colloid particles prepared by us (Figure 1g) was in units of nanometers and for particles added as  $\text{Ag}_3\text{PO}_4$  (Figure 1e) and  $\text{AgI}$  (Figure 1f) in units of micrometers. For TANB (Figure 1c) and TANM (Figure 1d) coatings the surface featured particles of calcium phosphates and spherical particles of silver. SEM/EDS did not make it possible to determine which particles of brushite and monetite present in the coating are non-reacted and which are reacted (into  $\text{Ag}_3\text{PO}_4$  generated before the *in situ* coating in the sol). It was because all particles of calcium phosphate and probably also of  $\text{Ag}_3\text{PO}_4$  (*in situ*) were bound in the sol which also contained distributed particles of silver alone. Irregular cracks developed in TANB and TANM coatings after firing appeared

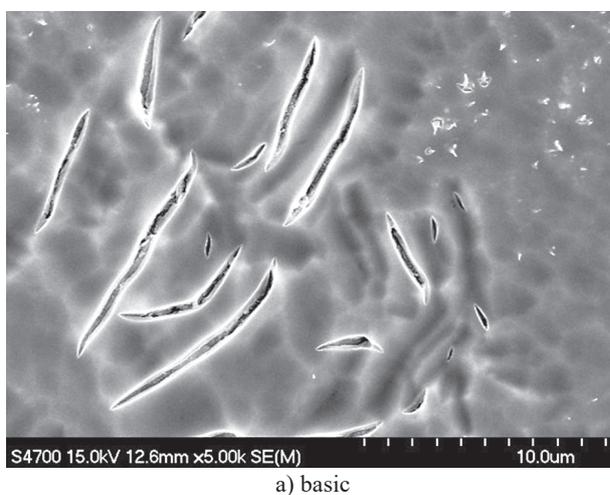
mainly around the calcium phosphate powders. In case of the titania coatings with added  $\text{Ag}_3\text{PO}_4$  (Figure 1e) the cracks propagated always from particles of the mentioned compound.

Bactericidal effects of all coated substrates were tested with *E. coli* using a 24-hour bactericidal test. Results of the test are shown in Figure 2a-h in representative photographs of Petri dishes with LB agar and number of colonies of surviving bacteria *E. coli*.

After the 24-hour tests the bactericidal effect was proved for the TAN, TANB, TANM, TAP coatings (Figure 2b-e) as very good (the number of surviving colonies was 0-25) and in case of the TAI coating (Figure 2f) as weak (the number of surviving colonies was 400 - 600). In case of the TAC coating (Figure 2g) and the basic coating T (Figure 2a) no bactericidal effect was demonstrated (the number of surviving colonies was 800-1200 which is comparable with the reference sample). The results indicate that silver is the most effective in the coatings in the form of dissolved  $\text{AgNO}_3$  and in the form of  $\text{Ag}_3\text{PO}_4$  added into the sol in the form of particles or developed *in situ* in the sol.

Based on the positive results of the 24-hour test we performed also a 4-hour bactericidal test to determine effects of various forms of silver in the coatings if the interaction with the suspension is significantly shorter.

The results after 4 hours of interaction of titania coatings with/without silver with the suspension are very similar to those of the 24-hour bactericidal test. They again confirmed that the most effective form of silver in the coating is  $\text{Ag}_3\text{PO}_4$  developed *in situ* (TANB, TANM) and  $\text{Ag}_3\text{PO}_4$  particles (TAP) with the number of surviving bacteria colonies 0-25, followed by silver nitrate (TAN) where the number of surviving bacteria was 0-50. As the number of surviving bacteria colonies is by two or three orders lower in comparison with the reference sample we can speak of a very good antibacterial effect. The TAI coating containing silver in the form of silver iodide had



a) basic

Figure 1. (SEM) Titania coatings after firing: a) basic (*continue on next page*).

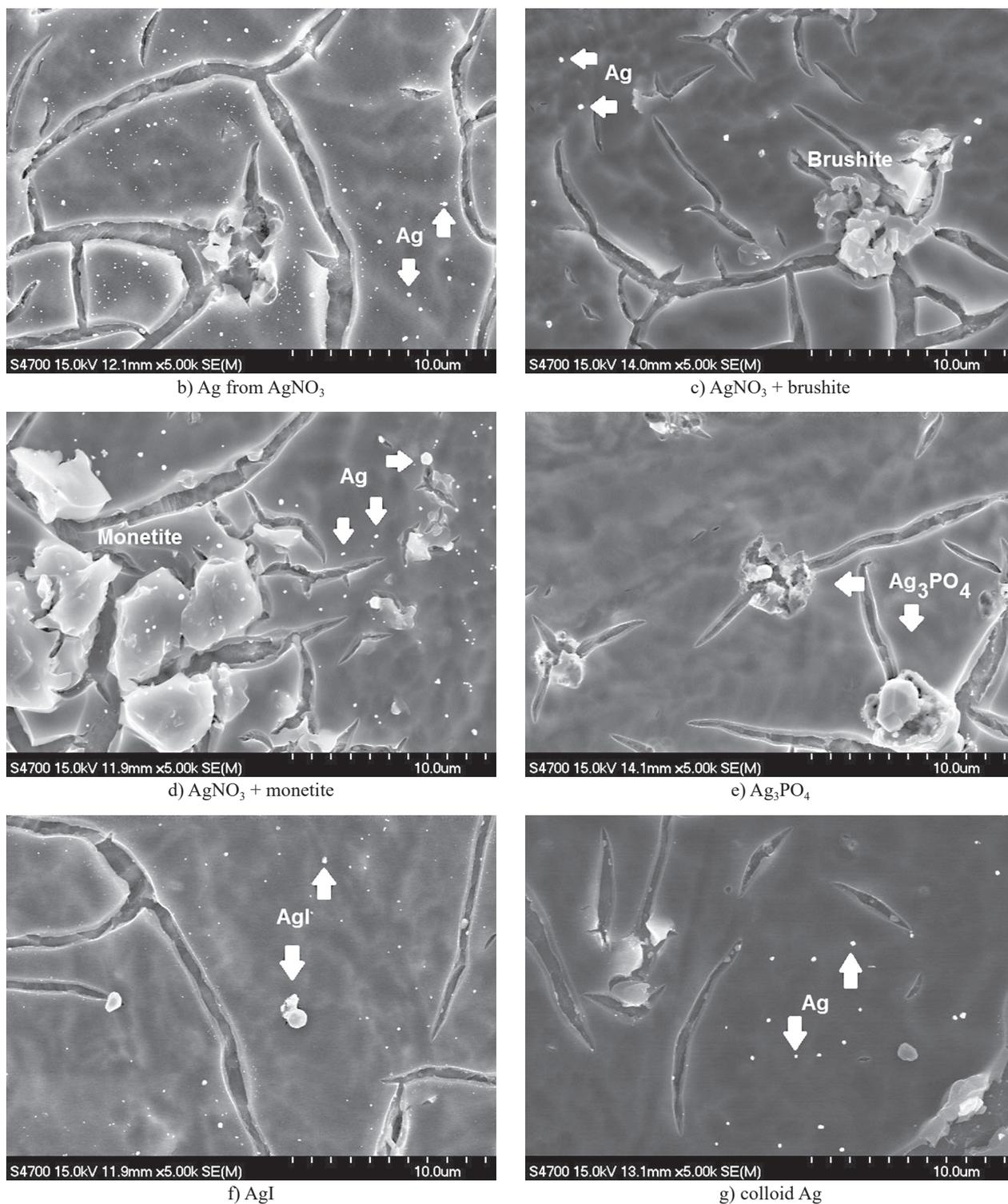


Figure 1. (SEM) Titania coatings after firing: b) with Ag from AgNO<sub>3</sub>, c) with AgNO<sub>3</sub> + brushite, d) with AgNO<sub>3</sub> + monetite, e) with Ag<sub>3</sub>PO<sub>4</sub>, f) with AgI, g) with colloid Ag.

again a weak bactericidal effect because one half of the bacteria colonies survived the test. The basic titania coatings T and the coating containing colloid silver TAC had no bactericidal effect because the number of surviving colonies was approximately the same as that on the reference sample. Despite the fact that the

conditions and types of the coatings (silica vs. titania) were different from the previous experiment [27] we can conclude that the selected forms of silver (AgNO<sub>3</sub> and Ag<sub>3</sub>PO<sub>4</sub> developed *in situ* in the sols) in both types of the coatings had very similar antibacterial effects against *E. coli*.

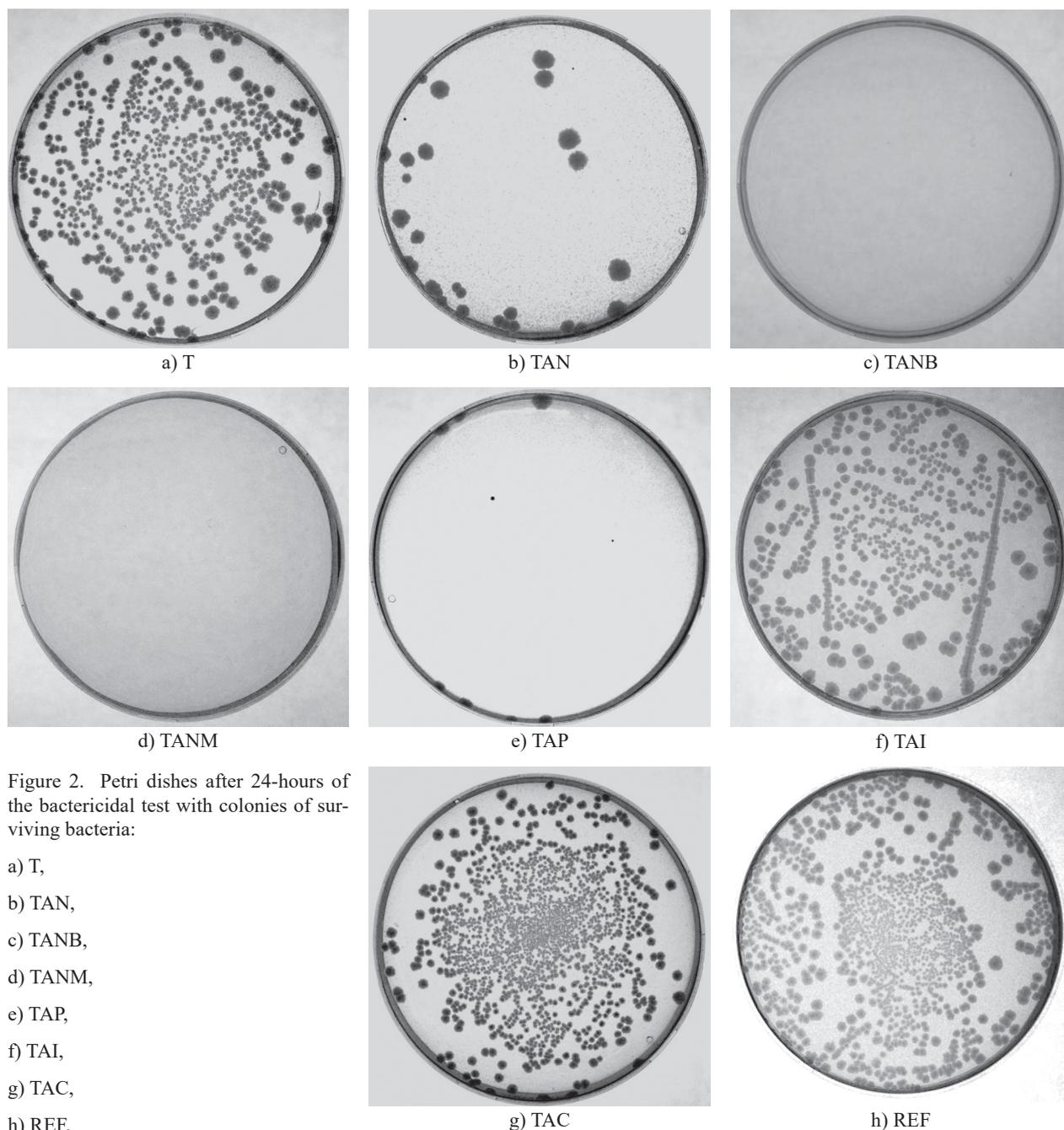


Figure 2. Petri dishes after 24-hours of the bactericidal test with colonies of surviving bacteria:

- a) T,
- b) TAN,
- c) TANB,
- d) TANM,
- e) TAP,
- f) TAI,
- g) TAC,
- h) REF.

#### CONCLUSION

- Titania sol-gel coatings prepared by dip-coating technique contained silver in form of colloid particles, AgI, dissolved AgNO<sub>3</sub>, Ag<sub>3</sub>PO<sub>4</sub> particles and Ag<sub>3</sub>PO<sub>4</sub> developed *in situ* on mechanically and chemically treated titanium substrates.
- Bactericidal effects against gram-negative bacteria *E. coli* were tested for all the coatings (with/without Ag) for 24 and 4 hours at the laboratory temperature.
- Both types of the bactericidal tests confirmed significant bactericidal effect of titania coatings containing AgNO<sub>3</sub>, Ag<sub>3</sub>PO<sub>4</sub> particles and Ag<sub>3</sub>PO<sub>4</sub> developed *in situ*.

- In the future we are planning to test cytotoxicity, antibacterial effects against *S. epidermidis* and bioactivity using *in vitro* tests in simulated body fluid.

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