Biocompatibility and antibacterial effect of silver doped 3D-glass-ceramic scaffolds for bone grafting

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ABSTRACT

A 3D-glass-ceramic scaffold for bone tissue engineering with an interconnected macroporous network of pores was doped with silver ions in order to confer antibacterial properties. For this purpose, silver ions were selectively added to the scaffold surfaces through ion-exchange using an aqueous silver nitrate solution. The silver-doped scaffolds were characterized by means of leaching, in vitro antibacterial, and citotoxicity tests. In particular, the silver effect was examined through a broth dilution test in order to evaluate the proliferation of bacteria by counting the colonies forming units. Moreover, cytotoxicity tests were carried out to understand the effect of silver-containing scaffolds on

KEY WORDS: glass-ceramic, scaffold, silver, antibacterial, biocompatibility.

INTRODUCTION

In the last few years, the antibiotic prophylaxis and the use of restricted hygienic-sanitary protocols have remarkably decreased the infections incidence. Nevertheless, infection development after a surgical implant remains one of the most serious problems connected to surgery treatment as it can cause prolonged hospitalization periods, patient diseases, and increased medical costs [1,2]. In particular, the introduction and the presence of an implant device within the body is known to increase the risk of infections [3], moreover implant associated infections are often resistant to antibiotics resultingin the removal of the infected implant.

Silver is a well known antimicrobial agent and it is already used in medical field as silver sulfadiazine cream for burn treatment, or barrier dressing for wounds treatment [4,5]. In orthopaedics, many research studies have been carried out to prevent infection on orthopaedic implants, in particular on fixation devices for osteosynthesis [6–9]. The major risk related to silver use is its toxicity which could affect all cells, both bacteria and tissue cells. Silver effect is defined as oligodynamicsince it depends on its concentration and on cells dimension. For this reason, it is possible to find a ‘safety range’ of Ag concentration with a toxic effect for bacteria and a not toxic one for tissue cells.

Another important issue in the field of orthopaedics is the need of synthetic bone substitutes able to replace the lost bone and to promote a chemical interaction with the natural bone supporting the osteointegrationand bone regeneration.

A suitable bone substitute should possess a pore volume greater than 50–60%, a macrostructure optimal for cell colonization and proliferation (interconnected macropores between 100 and 50 mm, micropores of about 10 mm), and mechanical properties similar to human bone. Moreover, a

synthetic bone substitute should be preferably easily shaped in order to conveniently fit into the bone defect.

Recently, glasses and glass-ceramics have been largely investigated as bone replacement materials for their ability to form in vivo an apatitelayer capable of promoting the osteointegration. Moreover, glasses and glass-ceramics are interesting materials since their composition can be tuned in order to obtain a tailored reactivity in the human body. Glasses and glass-ceramics are also able to release ions supportive for bone mineralization and can contain specific crystalline phases (i.e. apatite) similar to the inorganic phase of human bone.

In this research work, a bioactive glass-ceramic material has been used to realize macroporous 3D-scaffolds using the polymeric sponge replication technique [10] and a patented process [11] has been applied in order to introduce silver ions on the scaffold surfaces, to impart antimicrobial properties. The final aim consists of realizing a 3Dbioactive and antibacterial scaffold.The increasing phenomenon of bacterial antibiotic resistance development has led to the idea of introducing silver in glass-ceramic scaffold by means of ion-exchange process, without altering the basic material properties. Other metallic ions have well-known antibacterial properties, such as zinc and copper but silver has been selected since it has a good antimicrobial effect maintaining a limited cytotoxicity towards tissue cells. Moreover, several works confirmed the effectiveness of silver containing devices [7,9]. In a previous work [12], the scaffold preparation and characterization have been described, together with the ion-exchange technique used to introduce silver on the glass-ceramic superficial layers. The aim of this research is to investigate the silver release from the 3D-scaffold, its antibacterial behavior and its biocompatibility. Moreover, the effects of a dry hot sterilization have been evaluated, keeping in view its possible effects on the scaffold antimicrobial ability.

MATERIALS AND METHODS

Scaffold Preparation

The used glass-ceramic has the following molar composition: 50% SiO2, 18%CaO, 9%CaF2, 7%Na2O,7%K2O,6%P2O5, and 3%MgO. After melting and quenching, it is characterized by the presence of fluoroapatite crystals suitable for bone substitution and in particular for dentistry applications. The scaffolds were prepared with the polymeric sponge method following a procedure reported in previous works [10,12]. Briefly, a polyurethane sponge, cut in blocks (1.5x1.5x1.5 cm^3), was impregnated with a slurry containing 35wt% of prepared glass-ceramic powders, 6wt% of polyvinyl alcohol as a binder and the rest distilled water. The impregnated sponge was thermally treated at 700°C for 1 h. The sintering temperature was selectedon the basis of previous thermal

analysis and hot stage microscopy studies. The obtained scaffolds have been characterized in a previous work [12] in terms of structure (X-ray analysis), morphology and composition (Scanning electron microscopy – SEMPhilips 525 M, and energy dispersion spectrometry – EDS), and mechanical properties. Moreover, the micro and macro-porosity degree and interconnection have been evaluated by means of image analysis (software Qwin Leica) and capillary test. The ion-exchange technique was used in order to confer antibacterial properties to the obtained scaffolds. This process allows the silver introduction only into the external material surface without altering its characteristics, such as bioactivity and mechanical properties and was previously applied by the authors to bulk materials and to glass-ceramic coatings [11,13,14]. The technique is based on an exchange between modifier cations of the glass-ceramic and silver ions coming from the exchange solution. In this work, the treatment was carried out in an aqueous solution of silver nitrate using a 0.05M silver nitrate solutionmaintained at 378C for 8 h [12]. A moderate silver concentration and a low process temperature were selected in order to limit the Ag ions concentration on the scaffold surface and their diffusion on deeper atomic layers. In fact, the increasing of silver solution concentration and of the temperature process allow respectively an enhancement of the Ag concentration on the material surface and its diffusion inside the glass-ceramic. The macro and microporous structure of scaffolds involves a high specific surface area which can exchange and then release Ag ions, so it is crucial to use a low concentration silver nitrate solution to obtain a controlled silver release below the toxicity limit. For this reason and in order to obtain a homogenous and suitable silver ions distribution, a low temperature and a prolonged ion-exchange time was chosen. After the ions exchange, the scaffolds are rinsed in bi-distilled water in order to remove residual nitrate solution. The choice of the processing parameters (solution concentration, soaking time, and temperature) allowed modulating the amount of introduced silver and the thickness of the exchanged layer. From now on, the silver doped scaffolds will be labeled as Ag-scaffolds.

Sterilization

Sterilization is a mandatory process for all materials used in medical applications, but its effect on materials properties should always been investigated since it can produce important clinical impacts. For this reason, the scaffold sterilization was carried out in a furnace at 180°C for 3 h and afterwards, the scaffolds were tested again to verify if the silver efficacy was maintained after the treatment. The sterilized silver doped scaffolds will be named S-Ag-scaffolds and they have been characterized by EDS, SEM, and XRD analyses.

Leaching Tests

To evaluate the silver release, three Ag-scaffolds and three S-Ag-scaffolds were soaked in 30mL of simulated body fluid (SBF), incubated statically at 37°C up to 28 days. SBF is a solution with a pH of 7.45 that contains a series of powder reagents dissolved in bi-distilled water with a precise procedure proposed by Kokubo [15]; SBF has been selected in order to simulate the in vivo conditions. The experiment consisted of 1mL of SBF being picked out from the soaking solution with a pipette after 3 h, 1, 3, 7, 14, 28 days of soaking. The picked liquid has not been replaced by fresh SBF and the results were calculated considering the volume decrease over time. An untreated scaffold was used as control. Furthermore, three Ag-scaffolds were soaked in 15mL of hydrofluoric acid (HF) 5 M, to etch the glass-ceramic and to evaluate the total amount of silver loaded: after 28 days, 2mL of the etching fluid were picked out for analysis. Due to this operation, the scaffold collapsed due to theetching effect of HF and all Ag ionsreleased into the acid medium.All the spilled solutions were analyzed in a graphite furnace atomic absorption spectroscopy (GFAAS PERKIN ELMER, mod. 4100 ZL) employing a Zeeman-effect background corrector with an auto-sampler (mod. AS/71), programmed to dispense 20 µL of the sample. Two matrix modifiers have been employed in order to obtain a higher absorption signal: $Pd(NO_3)_2$ and $Mg(NO_3)_2$ have been directly added on the graphite tube by means of the auto-sampler. Standard solutions were daily prepared from AG MERCK standard solution traceable to SRM from NIST to calibrate the instrument. The calibration standard solutions were 25.0 and 50.0 µg/L. The limit of detection was 0.2 µg/L (CV53%). Noticeable data were obtained in µg/L from the instrument. Therefore to compare data with those of previous works, the results were multiplied by the amount of SBF or HF in which the samples were soaked and divided by the sample volume (1000mm^3) in order to obtain measurement unit in µg/mm^3. The sample volume is calculated without considering the porosity and can be considered as the apparent volume. Moreover the data in µg/L were divided for the SBF soaking time multiplied by the sample volume in order to extract the release speed values.

Antibacterial Tests

The antibacterial properties were evaluated through two microbial tests to investigate the adhesion and proliferation of bacteria. In particular, the test in broth was used to quantify the bacteria adhesion on the scaffold surfaces and their proliferation in the culture broth through the count of bacteria colonies forming units (CFU). The second type of test was the evaluation of broth turbidity through McFarland index measurements. These tests were performed in accordance to NCCLS standards [16,17] using *Staphylococcus aureus*standard stock (ATCC 29213). Bacterial broth was prepared dissolving a Staphylococcus aureuslyophilized disk in 5mL of brain-heart infusion; after

24 h of incubation at 35°C, 10 µl of suspension was spread on blood-agar plate and incubated for 24 h at 35°C in order to allow the bacterial colonies to grow. The incubation temperature has been selected since it allows an optimal bacterial proliferation. Afterwards, some bacterial colonies were spread in normal saline (0.9% P/V of NaClin distilled water) to obtain a standard 0.5 McFarland solution (turbidity has been evaluated by optical instrument at a fixed wavelength —Phoenix 237 Spec BD McFarland). Three Ag-scaffolds, three S-Ag-scaffolds and three as done scaffolds (control) were individually introduced in a test-tube containing 5 ml of Mueller Hinton broth and 25 µL of 0.5 McFarland solution, in order to obtain a suspension with a bacteria concentration of about $5x10^5$CFU/ mL (NCCLS standard). For further control, a test-tube containing only the broth was used. All test-tubes were incubated at 35°C and after 24 h the McFarland index of the broth was measured again by the optical instrument Phoenix 237 Spec BD McFarland in order to verify the turbidity increaseor decrease of the bacterial suspension. In case of bacteria proliferation,the broth turbidity will increase leading to a higher McFarland index. After the test, the samples were rinsed in 3mL of normal saline andvortexed in 3mL of normal saline for 1 min at 50 Hz, in order to remove the bacteria adhering on the samples surface. The bacterial broth (S), the solution of washing (W), and the solution of vortexing (V) were analyzed in order to count the CFU. In particular, S and W evaluation allowed quantifying the total bacteria amount considering also the liquid film surrounding the samples (W), whereas the bacteria adhering on the scaffolds surface were evaluated analyzing the vortexing solution (V). Serial dilutions of S, W, and V solutions were carried out to count the CFU and 100 µL of the last diluted solutions were spread on a blood-agar plate. The number of dilution was chosen on the basis of previous works [18,19]. The blood-agar plates were incubated at 35°C and after 24 h, the CFU were counted; the obtained number was multiplied by the dilution factor in order to get the total number of CFU of the starting solution.

Biocompatibility Tests

The biocompatibility of bulk materials and coatings of untreated and Ag-doped glass-ceramic, used in this work, has been already discussed in previous papers [18,19]. A 3D structure is characterized by a greater surface reactivity due to the presence of a large number of micro and macropores and also the difference in material morphology. For this reason, cells growth and viability were evaluated on control scaffolds and S-Ag-scaffolds using human osteoblast-like MG-63 cells. This was done in order to evaluate their behavior on a 3D-structure and to assess the effects of the introduced silver. Control scaffolds, previously dry sterilized at 180°C for 3 h, and S-Ag-scaffolds, all with an apparent volume about $1cm^3$, were placed in multi-wells and soaked in 12mL of MEM culture medium (Minimum Essential Medium, M5650 Sigma) added with 1mM sodium pyruvate,

1% antibiotics and antimycotics solutions, 2mM glutamine, and 10% fetal bovine serum. After 24 h, 90,000 cells were seeded on the scaffolds and maintained for 2, 4, 8, 10, and 20 days at 37°C in an atmosphere containing 5% of CO_2 and 95% of air. The medium was changed every 4 days. At the end of each incubation period, several tests were carried out to evaluate the material biocompatibility as follows. The culture medium was used to determine the number of floating MG-63 cells, to measure pH, to assess cellular metabolic activity, and to evaluate the release of lactate dehydrogenase (LDH), that is an appropriate marker of necrotic death [20]. After removal of medium, MG-63 cells present outside and inside the scaffolds were separately trypsinized, and counted using a Burkerchamber and a light microscope, to distinguish viable and dead cells by the Trypan blue exclusion test. The control and S-Ag-scaffolds colonized by MG-63 cells were used to evaluate cell morphology by SEM observation: the scaffolds were rinsed 4 times in PBS and fixed with 2.5% glutaraldehyde in 0.1M phosphatebuffer, pH 7.4, for 30 min at 48°C. Dehydration was performed with water replacement by a series of graded ethanol solutions with final dehydration in absolute ethanol. The determination of calcium deposits produced by MG-63 was performed on cells grown onto and inside the scaffolds. After trypsinization, MG-63 cells grown inside the scaffolds were fixed in 70% ethanol and washed with tris-buffered saline (TBS), stained with 1% alizarin red S solution for 2 min, washed with TBS and observed under light microscope. The cell ability to produce calcium deposits is a crucial factor for the in vivo bone regeneration. Data are expressed in all cases as mean ± S.D. The significance of differences between group means was assessed by variance analysis, followed by the Newman-Keuls test (p50.05). Samples references and tests performed in this work are summarized in Table 1.

RESULTS AND DISCUSSION

Figure 1 shows a 3D-cubic scaffold realized with the polymeric sponge method with a volume of about 1 cm^3. It can be observed that the scaffold is completely white in color because the burnt-out the polymeric skeleton and the binder did not leave residues. As reported in a previous work [12], the chosen thermal treatment led to a glass-ceramic material containing fluroapatite (already present in the as poured material) and canasite (JCDD 00-013-0553) which is a phase characterized by a high strength and well known biocompatibility [21]. Figure 2(a) and (b) report two SEM micrographs of the scaffold surface and its cross-section, respectively. As it can be observed, the scaffold is characterized by open macropores of a few hundred mm and the overall morphology is quite similar to cancellous bone [22–24]. The obtained 3D structure shows a highly interconnected network of trabecular-like struts and a lot of macropores, distributed homogeneously, both on the sample surface and along its cross-section, proving that this material is suitable for bone grafting

applications. The scaffold porosity has been reported in a previous work [12] and it is about 75% which is an optimal value for an effective vascularization of the scaffold. As it can be observed in Figure 2(a), many micropores (10–50 mm) are present due to the removal of the polymeric phase in a gaseous form. The presence of a bimodal distribution of the pores size is a very positive feature as macropores are crucial for cell penetration and migration, while micropores are essential for nutrient flow and protein adhesion. Micropores are also responsible for the high up-take ability of these scaffolds due to capillary absorption and their presence is essential for a rapid impregnation of scaffold by the surrounding biological fluids [25,26]. A porosity of about 60 vol.% is a fundamental requirement for artificial grafts [21,22], as well as sufficient mechanical competence. For the 3D-scaffold, a compressive strength of 2 ± 0.6MPa was obtained [12] and considering the fact that, when implanted in vivo, the integration and regeneration processes will involve new bone apposition, this value can be considered as satisfactory.The high and bimodal scaffold porosity and their compressive strength represent a good balance for bone grafts if compared to data reported in literature and in the ISO Standard (ISO 13779-1:2000) proving that these scaffolds can be proposed as graft materials. In order to confer antibacterial properties to the scaffolds, they were doped with silver using the ion-exchange technique [11]. In a very preliminary study, where only the presence of silver and an inhibition halo were investigated [12], the EDS analysis showed the presence of Ag in the scaffold after the ion-exchange. The Ag amount, detected both on the scaffold surface and on its cross-section, was 1.7% on the surface and 0.6% in the inner part of the scaffold (about 5mm depth), respectively; these amounts should be considered only approximate due to the low accuracy of EDS. In any case, the Ag content is in a range which, according to literature [13,14], should impart an antibacterial behavior without affecting cell vitality. XRD analysis and SEM observations on S-Ag-scaffolds (not reported) showed that the sterilization process did not induce new crystallization phenomena or morphological modifications.

Leaching Tests

The silver release trend is similar to those obtained in the previous works for sintered samples and coatings [18,19]. Previous data refer only to dense exposed surfaces and so they can not be compared with the present work in which a complex 3D structure is investigated. When in contact with an aqueous medium, silver ions will diffuse from the whole exposed surface leading to a great amount of Ag release that has to be carefully controlled in order to induce an antibacterial effect, without affecting the scaffold biocompatibility. Therefore this work was focused on investigating the Ag release from a porous structure and to assess the maintenance of the antibacterial effectiveness after sterilization. HF etching of Ag-scaffolds for 28 days allowed to evaluate the

global amount of loaded silver. The obtained results showed that about 0.274±0.0018 µg/mm^3 of silver was loaded; the observed low standard deviation is an evidence of the high reproducibility of the proposed method. The amount of silver released in SBF from Ag-scaffolds and S-Ag-scaffolds have been quantified with the GF-AAS technique, analyzing the solution picked out at different times of soaking in SBF. As it can be observed, silver is gradually released during the soaking in SBF and major differences between not sterilized and sterilized samples have not been found either for the global silver release or its release rate. These results are in good accordance with the XRD and SEM observations on Ag-Scaffolds and S-Ag-scaffolds. As far as Figure 3(a) is concerned, the observed trend shows a continuous increase with respect to any plateau found for the tested time frames (up to 28 days). This feature indicates that a certain silver amount remains on the scaffold's pore wall surface and would be released at longer times; only about 14 wt.% of the loaded silver wasreleased after 4 weeks and so the remaining silver is noticeable. Futuretests will investigate this feature and will evaluate the release kinetics for longer times. The slope of the observed release trend (Figure 3(a)) showed that a higher amount of silver is released during the first hours and days of immersion: 0.010 µg/mm^3 during the first hours, a further 0.010 µg/mm^{3}within the first week and a further 0.010–0.015 µg/mm^{3}during the following three weeks. This trend is interesting as, an important silver release during the first days after the implant is desirable since, after a surgical treatment, this time frame is the most critical for infection development. Subsequently, a slower but protracted Ag release can be important in order to maintain an antibacterial effect in the case of latent infections.

Antibacterial Tests

The antibacterial behavior of the Ag doped scaffolds was assessed by the CFU counts and by the turbidimetric evaluation of antibacterial activity (McFarland index). The graph reported in Figure 4 shows the McFarland values obtained for the control and for Ag- and S-Ag-scaffolds; the test was carried out in triplicate. As it can be observed, both Ag and S-Ag-scaffolds are able to significantly limit the S. aureus proliferation and in fact the McFarland values of Ag- and S-Ag scaffolds is noticeable-lower than the control one. The antibacterial feature can be immediately seen by observing the test-tube containing the different samples that are shown in Figure 5; the liquid in the test-tubes containing the control scaffold is more turbid than the one in the test-tubes of Ag- and S-Ag-scaffolds. Due to the low difference in the McFarland indices, the difference within Ag and S-Ag-scaffold can not be observed. A more precise quantification of the bacteria growth and adhesion on the scaffold surfaces was carried out by using the CFU count test. The graphs reported in Figure 6 show the count of CFU proliferated in the broth surrounding the scaffolds (S – 6a), in the liquid

film in direct contact with the samples (W – 6b), and the ones adhered onto the scaffolds (V – 6c). In each case, the silver presence decreased the bacteria number by 2–3 magnitude orders with respect to the control scaffolds and thus about 99.9% (3-Log reduction) of bacterial colonies did not proliferate. It is assessed that a 2-Log reduction in the CFU count is an index of a bacteriostatic behavior and thus Ag-scaffolds can be classified as bacteriostatic [27], but with the present results it is not possible to assess that the Ag-scaffolds have a bactericidal effect. We believe that, during the first minutes, the released silver amount is not high enough to prevent the CFU proliferation (the replication time for Staphylococcus aureus is estimated to be about 20 min), but after a few hours, the total silver released is probably sufficient to kill most of the bacterial colonies. The aim of this work was to induce a marked reduction of the bacteria proliferation and of the related risk of infection onset: the observed effect satisfied both requirements. As far as the CFU count is concerned, significant difference betweenAg-scaffolds and S-Ag-scaffolds was observed for the S and W solutions. Instead on the V, a difference on the CFU count was observed between Ag and S-Ag-scaffolds, and the observed CFU number is more than an order of magnitude lower for S-Ag-scaffolds. The test was carried out in triplicate and, as it can be observed, the bar error is very narrow: on this basis it can be stated that the sterilization process seems to improve the antibacterial effect on the bacteria adherent to the scaffolds. If these results are combined with the observed release trend reported in Figure 3(a) and in which, a slightly lower release of silver was observed for the S-Ag-scaffolds, it is possible to hypothesize a different diffusion profile of silver ions in the scaffolds. In fact, the sterilization treatment is carried out at 1808C for 3 h and thus during this treatment Ag ions are likely to diffuse towards the deeper layers of the scaffolds due to the presence of a concentration gradient and the temperature effect. For this reason, the Ag doped layer, in the sterilized samples will show a less sharp profile and the Ag release in SBF will be slower. The images reported in Figure 7 show the plates with CFU from S (Figure 7(a), W (Figure 7(b)) and V (Figure 7(c)) of the control scaffold on the left and of the S-Ag scaffolds on the right with a dilution of order of 6. The effect of silver in reducing the bacterial colonies adhesion and proliferation is unquestionable.

Biocompatibility Tests

The biocompatibility of the used glass-ceramic was tested on sintered samples and coatings doped or not with silver [18,19], but it was not studied before on porous materials doped with silver (S-Ag-scaffold). Thus, cell cultures were necessary to assess the biological properties of S-Ag-scaffolds, utilizing the as done scaffolds as control. Silver effect on cell proliferation was evaluated by counting the number of cells grown inside and outside the control and S-Ag-scaffolds, and those

floating in the medium. Specifically, cells grown inside the scaffold were counted removing the samples from the multi-wells and treating them with trypsin, whereas outside cells were counted after detaching with trypsin the cells attached to multi-wells. Figure 8 shows that control scaffolds were able to allow the cell proliferation inside and outside the scaffold reaching the highest peak of proliferation at 10 and 8 experimental days, respectively. Then, cell proliferation decreased for both inside and outside cells, with an important difference: the inside cells were all alive, as evidenced by Trypan blue exclusion test, whereas the outside cells were dead for 4% at 10 days and 20% at 20 days (data not shown). The presence of dead cells outside the scaffold was confirmed by the increased number of cells detached from the monolayer and floating in the medium (Figure 8 SPT). Therefore, it is possible to hypothesize that the cells inside the scaffold showed an arrest of the proliferation because of the increased activity in bone deposition. Unfortunately, as far as silver is concerned, the concentrations, usedin this experimental protocol, affected cell proliferation inside and outside the scaffold, detaching the cells from the monolayer already in the first experimental stages (Figure 8 IN, OUT, SPT). Both cells present inside and outside the scaffold, obtained by trypsinization, were dead for 60% in comparison with control, as determined by Trypan blue exclusion test (data not shown). This detrimental effect was likely due to the accumulation of release of silver in the culture medium until 4 days of treatment. After this time, the medium was renewed, but the cells were not able to recover cell proliferation. On the contrary to Trypan blue exclusion test results, the determination of the lactate dehydrogenase released from the cells in the medium, expressed as enzymatic activity per mL of medium, showed an increase during the experimental time only in the controls, whereas it was not found in the presence of Ag (Table 2). This feature seems unusual as cells die, but in the literature it was described that Ag is an inhibitor of lactate dehydrogenase [28] and this could be the reason for the lack of enzymatic activity. The pH evaluation did not evidence differences in the control and S-Ag- scaffolds with exception for 10 days (Figure 9). Alizarin red S staining, that evidences calcium deposits, was evaluated on cells grown in the scaffold to determine the ability of MG-63 cells to synthesize bone. Figure 10 shows great differences between as done and Ag-scaffolds. In fact, cells cultured on control scaffolds were very active as they induced the deposition of calcium especially at 20 days, whereas in the presence of Ag the calcium deposits were very few or even absent. Moreover, the calcium deposits in the presence of Ag had a smaller size than the ones produced on the control. This result is in good accordance with the data concerning cell number. Figure 11 reports SEM micrographs of cells cultured on the control and on the S-Ag-scaffolds at different times. The low number of cells attached on S-Ag-scaffolds was confirmed by SEM observation, where it was very difficult to find attached cells. On the other hand, SEM micrographs of cells cultured on control scaffolds showed a

lot of well spread MG-63 cells with many filopode bridges on the surfaces at all the studied culturing times. On these bases, it is possible to state that, at the concentrations used in this research, Ag is toxic not only for the bacteria, but also for the cells. The observed cytotoxic effect could be related to the settings of in vitro tests that did not simulate the fluid renewal; for this reason, future experiments should concentrate on a better setting of cells culture experiments that would take into account the renewal of physiological fluids also in the first experimental times.

CONCLUSIONS

3D glass-ceramic scaffolds characterized by an highly interconnected structure of large pores and satisfactory mechanical properties were obtained through the sponge replication method. Ion exchange technique permits to dope the 3D-scaffolds with a controlled silver quantity. In fact the Ag-doped scaffolds were able torelease silver in a controlled and prolonged way, up to 1 month, on the basis of the selected ion-exchange parameters; Ag ions were released in greater amount during the first hours and days which are considered the most critical for infections after a surgical treatment. The hot dry sterilization process applied to the Ag-doped scaffolds did not affect the material ability to release silver and its antibacterial effect; the sterilization treatment seems only to slightly increase the bactericidal for the bacteria in contact with the scaffolds. This effect is likely due to Ag diffusion due to the temperature and concentration gradient. Microbiological tests confirmed the good antibacterial effect of the Ag-doped scaffolds towards Staphylococcus aureusstock and the doped scaffolds were able to inhibit both the bacteria adhesion and proliferation. About 99.9% of bacterial colonies did not proliferate and adhere both on Ag- and S-Ag-scaffolds surface. Nevertheless, cell culture tests showed that the Ag presence negatively affected osteoblast-like cell proliferation, limiting cell growth and viability although the total released silver was well below the toxic limits. A cells reduction of 60% has been observed inside and outside the Ag-doped scaffold, in comparison with control, especially during the first 4 days of treatment in which the culture medium was not renewed. Future work will focus on scaffolds doped with lower Ag amounts through a careful optimization of the ion-exchange parameters in order to reach a concentration toxic for bacterial but not for tissue cells. The cell culture experiments will also be modified to take into account the physiological fluid renewal.

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FIGURES

Fig. 1 Image of a 3D-scaffold thermally treated at 700°C for 1hour

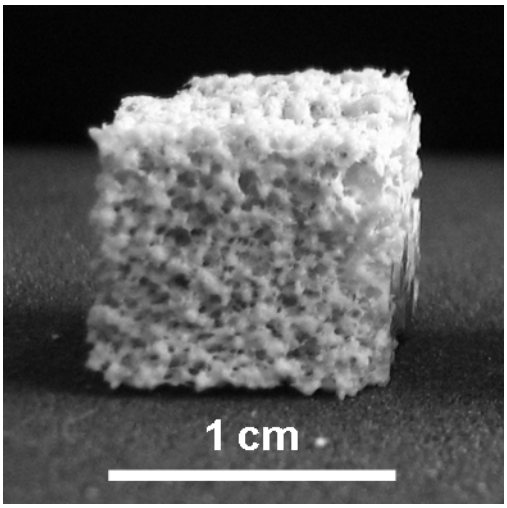


Fig. 2 SEM micrographs of the 3-D scaffold surface (a) and cross-section (b)

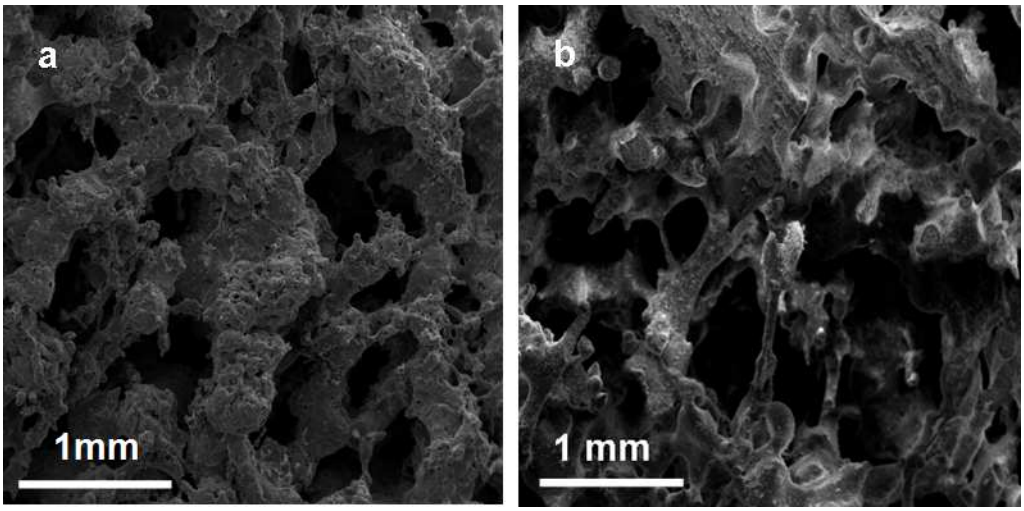


Fig. 3 Leaching tests: amount of released Ag (a) and speed of Ag release (b)

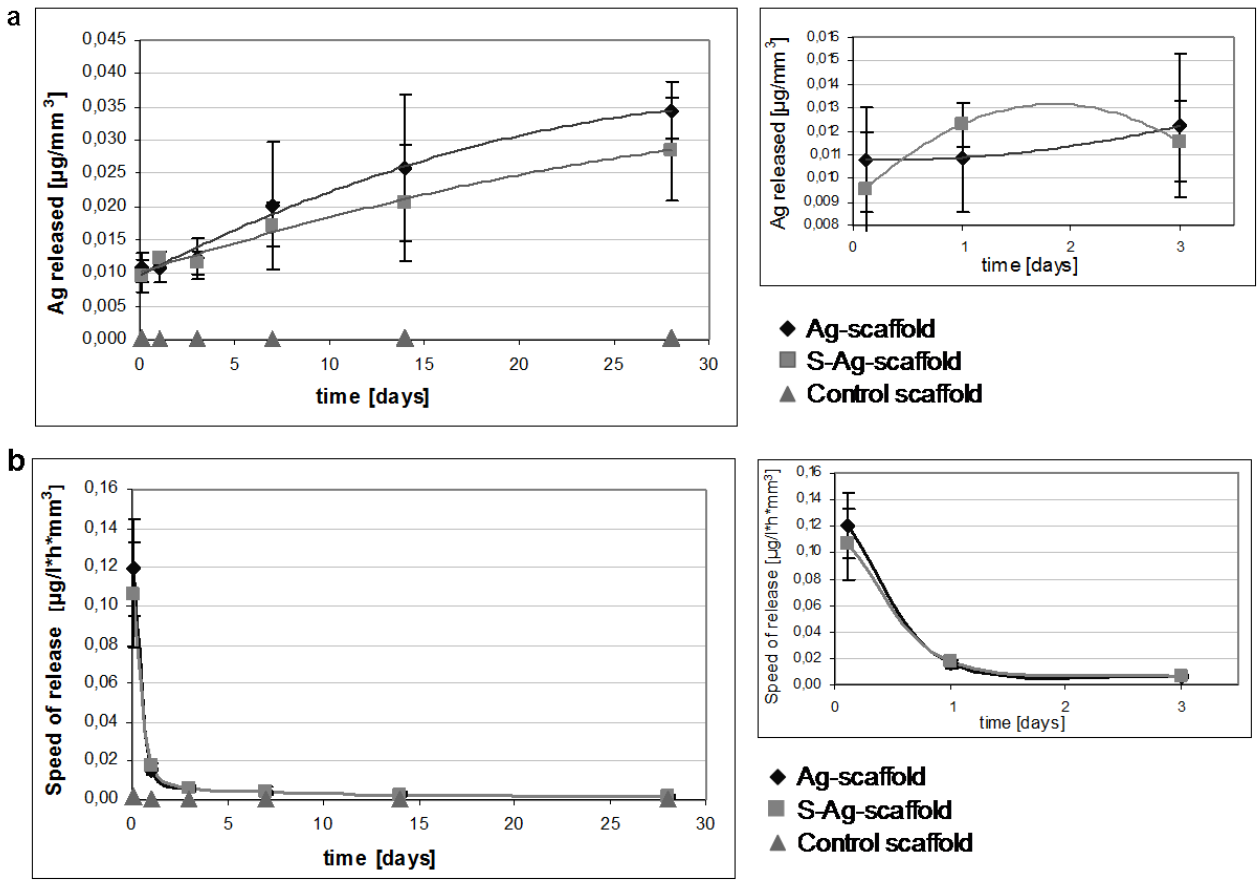


Fig. 4 McFarland indexes of control and Ag, S-Ag-scaffolds

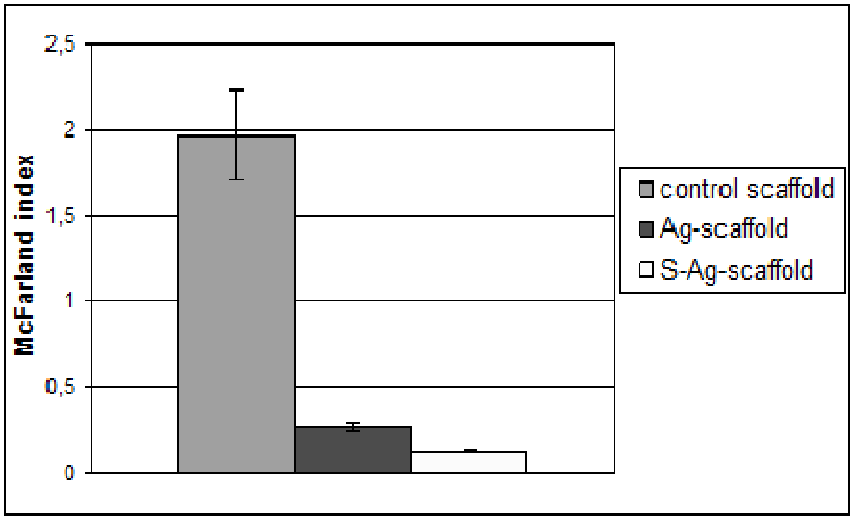


Fig. 5 McFarland index: Test-tubes of control (left), Ag (middle) and S-Ag- (right) scaffolds respectively

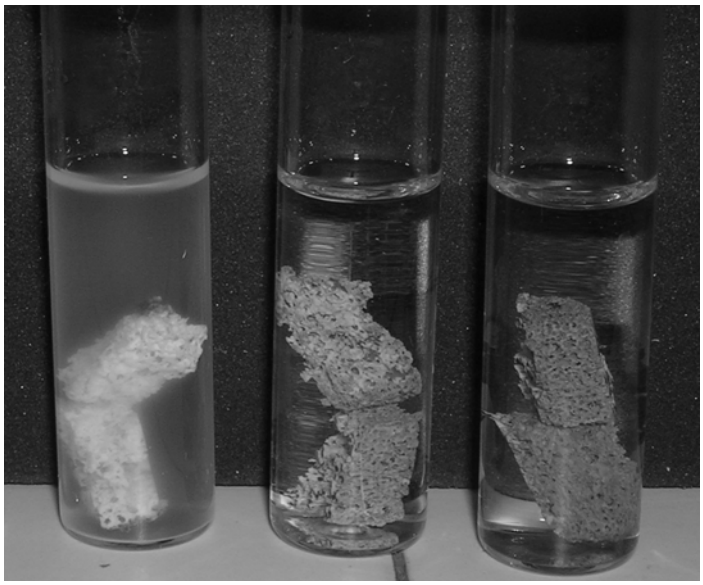


Fig. 6 CFU counts in Broth (a), Washing solution (b) and vortexing solution (c)

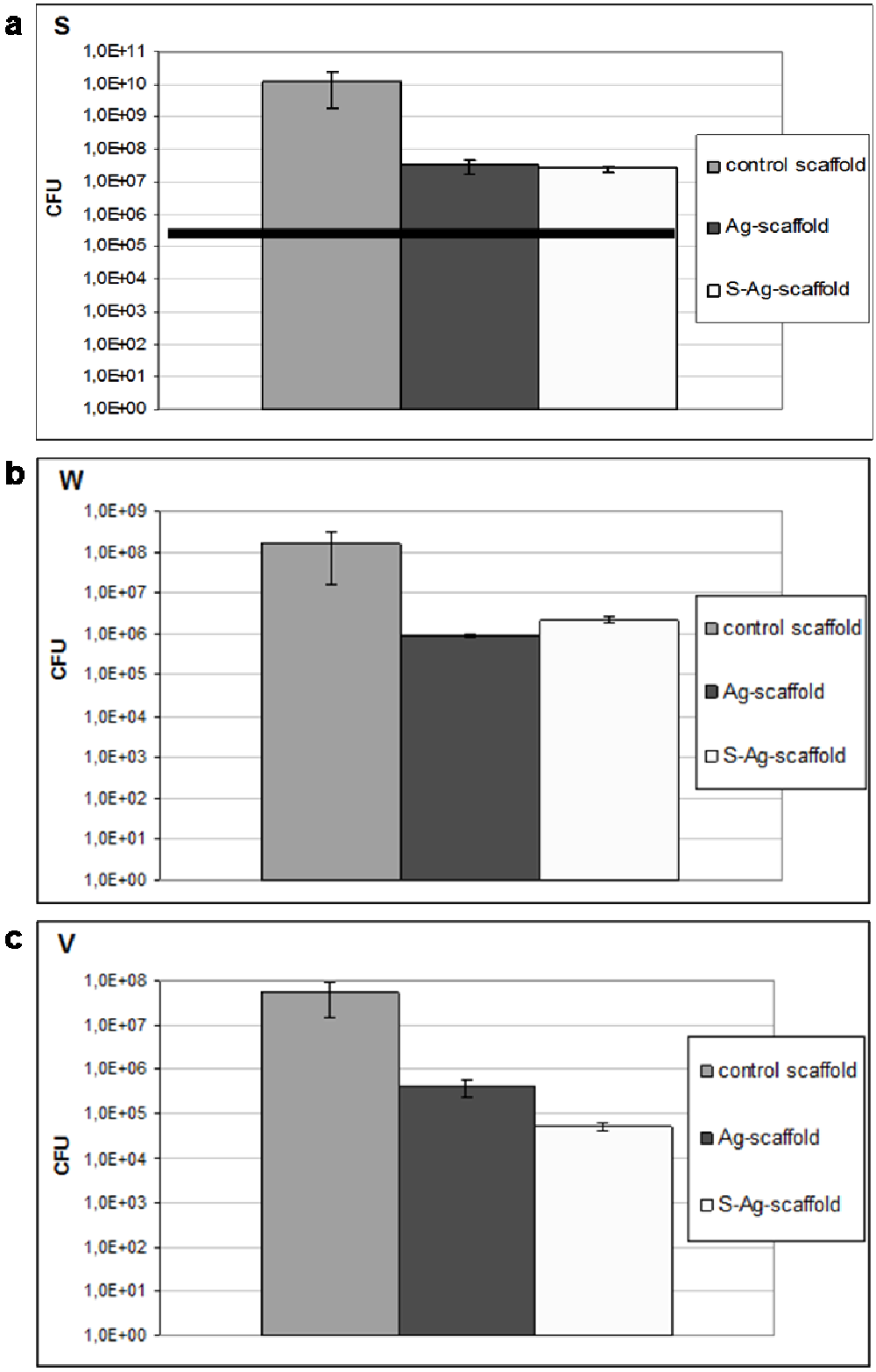


Fig. 7 Plates for the CFU evaluation of control and S-Ag-scaffold in the broth (a), in the washing solution (b) and in the vortexing solution (c).

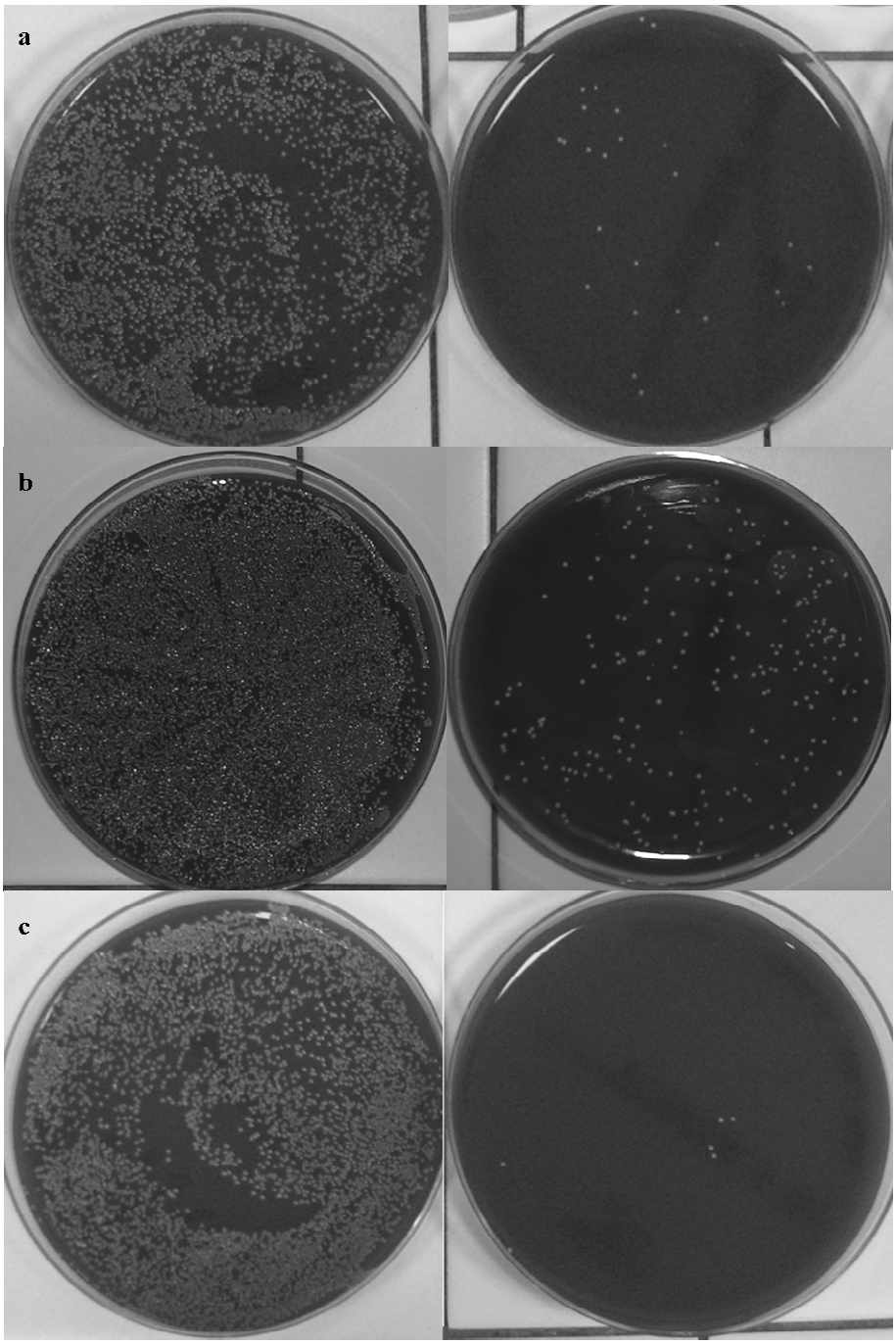


Fig. 8 Number of cells present inside the scaffold (IN), outside the scaffold (OUT) and in the culture medium (SPT).

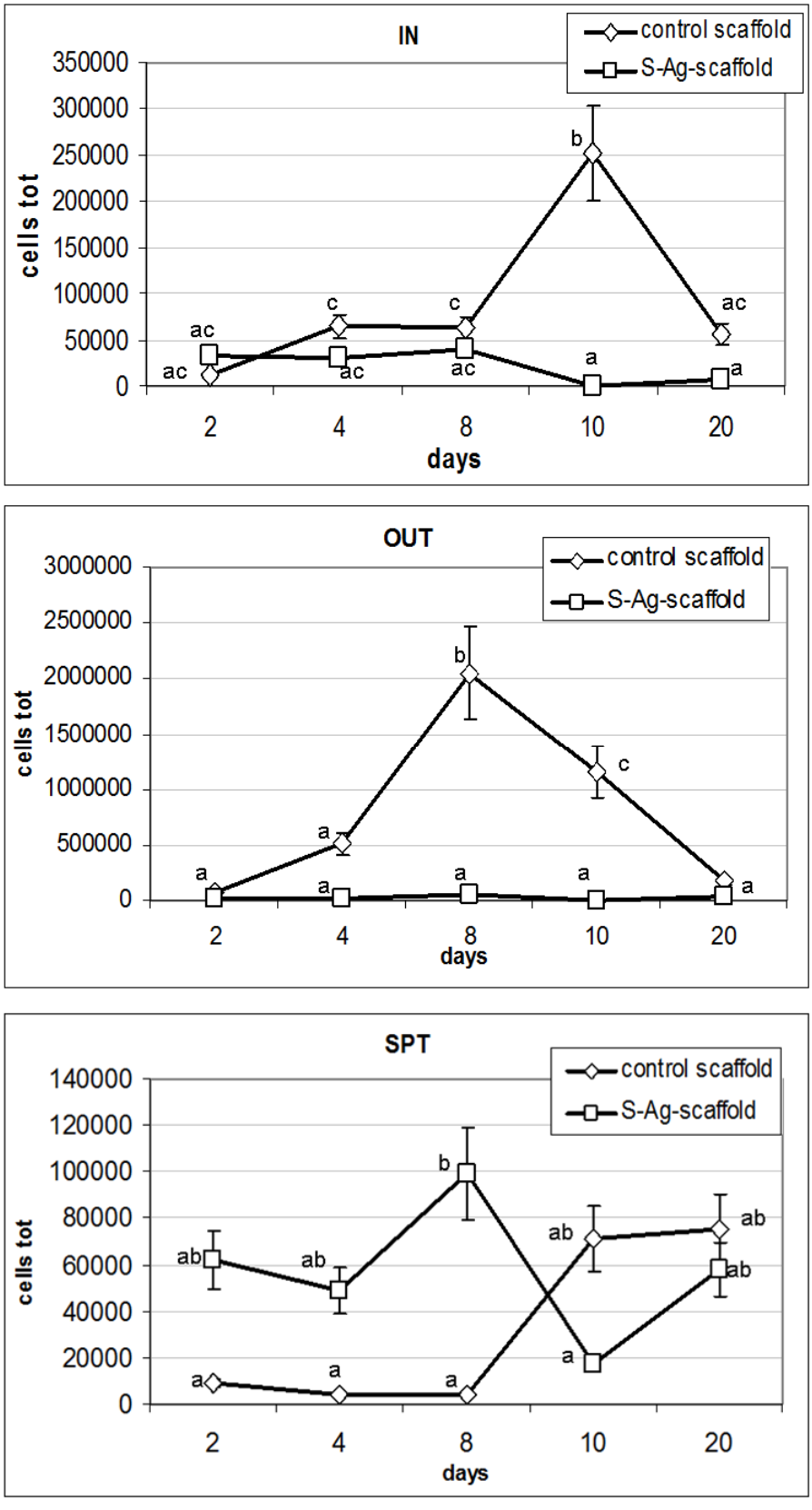


Fig. 9 Evaluation of pH in the culture medium after 2, 4, 8, 10 and 20 days after seeding the cells on the scaffold.

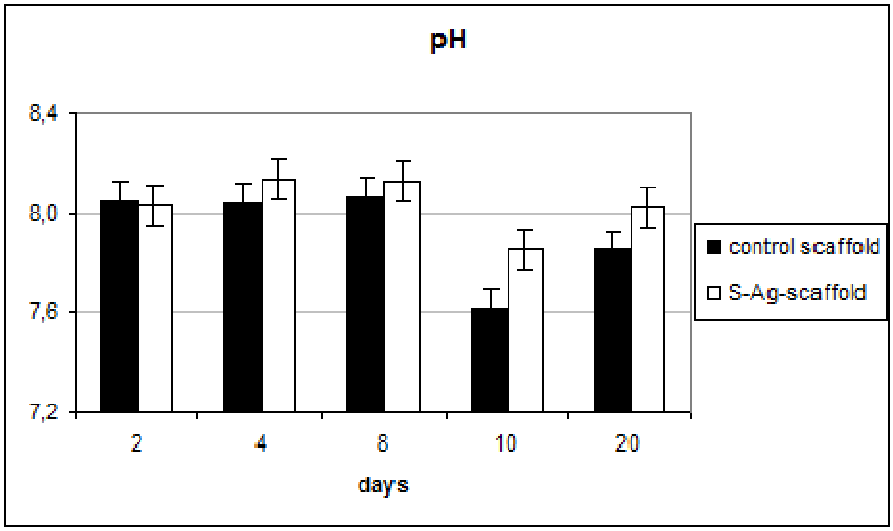


Fig. 10 Alizarin red S staining of cells detached from the scaffolds with trypsin after 2, 4, 8, 10 and 20 days after seeding the cells on the scaffold.

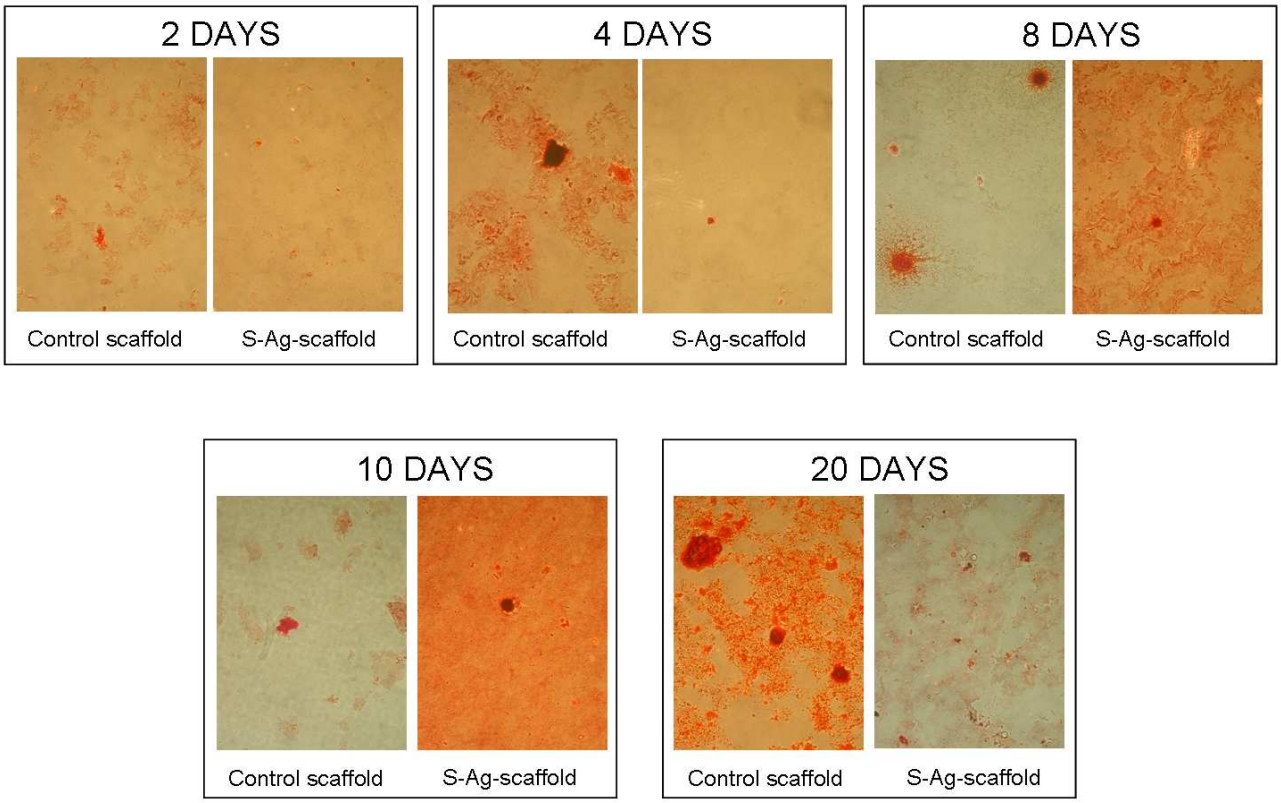
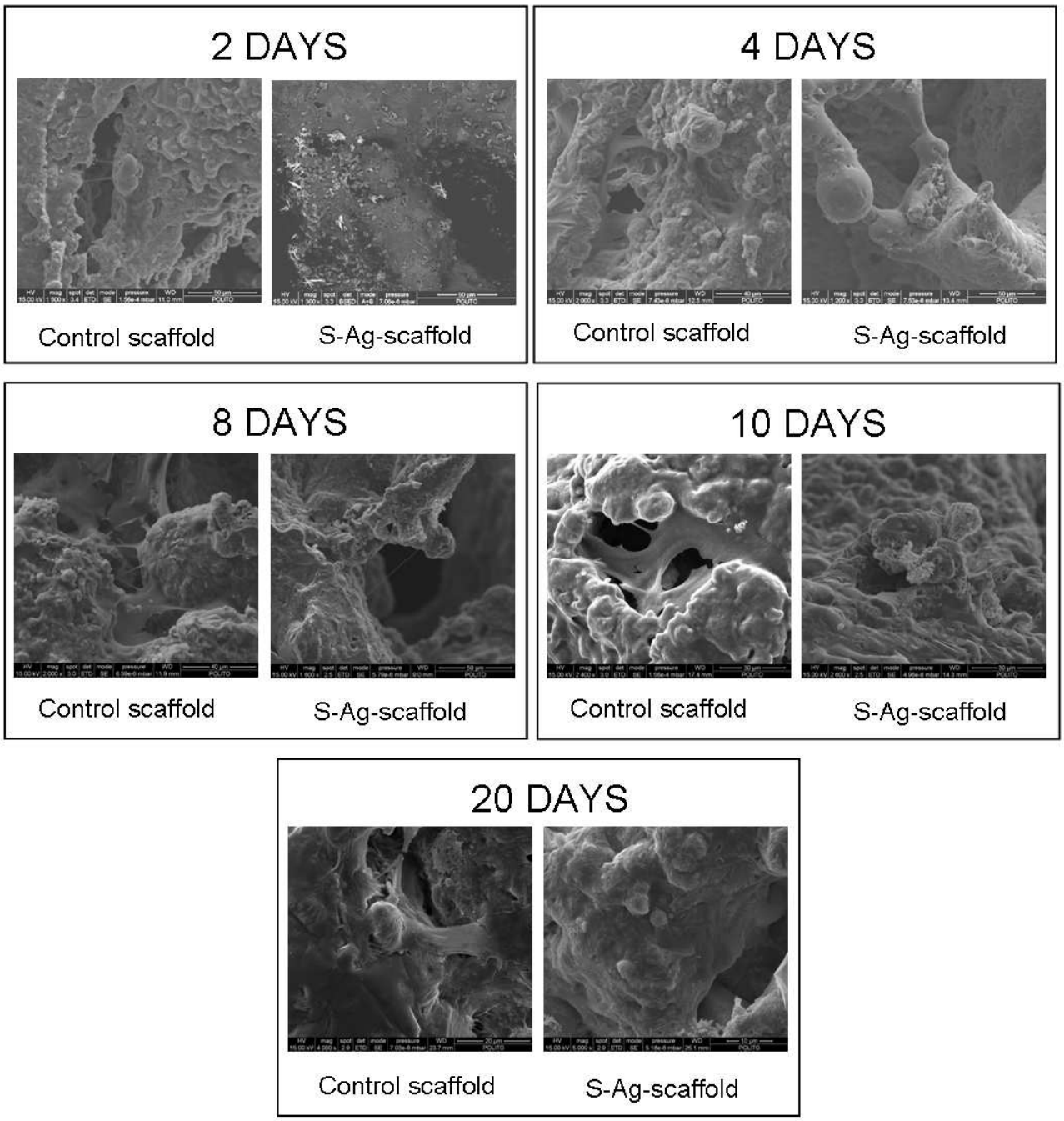


Fig. 11SEM evaluation of cells present on the scaffold after 2, 4, 8, 10 and 20 days of culture



TABLES

Table I: Lactate dehydrogenase **(**LDH) release in culture medium for control scaffold and S-Ag-scaffold, after 2, 4, 8, 10 and 20 days of culture.

LDH (nmoles NADH consumed/min/ml medium)					
Days	**2**	**4**	**8**	**10**	**20**
control	0.061	5.28	59.57	30.31	146.26
S-Ag-scaffold	0	0	0	0	0

Table II. Lactate dehydrogenase analysis

Lactate dehydrogenase (nmoles NADH consumed/min/ml medium)					
DAYS	**2**	**4**	**8**	**10**	**20**
Control scaffold	0.061	5.28	59.57	30.31	146.26
S-Ag-scaffold	0	0	0	0.10	0.26