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Surface silver-doping of biocompatible glass to induce antibacterial properties. Part I: massive glass

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Abstract A glass belonging to the system SiO_2–Al_2O_3–CaO–Na_2O has been subjected to a patented ion-exchange treatment to induce surface antibacterial activity by doping with silver ions. Doped samples have been characterized by means of X-Ray diffraction (XRD), scanning electron microscopy (SEM) observation, energy dispersion spectrometry (EDS) analysis, in vitro bioactivity test, Ag^+ leaching test by graphite furnace atomic absorption spectroscopy (GFAAS) analyses, cytotoxicity tests by fibroblasts adhesion and proliferation, adsorption of IgA and IgG on to the material to evaluate its inflammatory property and antibacterial tests (cultures with *Staphylococcus aureus* and *Escherichia coli*). In vitro tests results demonstrated that the modified glass maintains the same biocompatibility of the untreated one and, moreover, it acquires an antimicrobial action against tested bacteria. This method can be selected to realize glass or glass-ceramic bone substitutes as well as coatings on bio-inert devices, providing safety against bacterial colonization thus reducing the risks of infections nearby the implant site. The present work is the carrying on of a previous research activity, concerning the application of an ion-exchange treatment on glasses belonging to the ternary system SiO_2–CaO–Na_2O. On the basis of previous results the glass composition was refined and the ion-exchange process was adapted to it, in order to tune the final material properties. The addition of Al_2O_3 to the original glass system and the optimization of the ion-exchange conditions allowed a better control of the treatment, leading to an antibacterial material, without affecting both bioactivity and biocompatibility.

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1 Introduction

The need for prevention against bacterial infections is an essential target in orthopaedic surgery due to the significant damages to patients related to infections [1, 2]. Although severe hygienic protocols and preventive antibiotic prophylaxes have drastically reduced the percentage of post-operative infections, many bacterial species developed selective resistance against antibiotics that can cause serious infections, hard to recover and can lead to the need for re-operation. An alternative to the systemic delivery of antibiotics might be the use of orthopaedic devices

or prostheses realized using synthetic materials with antibacterial properties [3–7]. Glasses and glass-ceramics have been deeply studied as candidates for bone restoration [8–10] and many compositions have shown promising properties in terms of biocompatibility, bioactivity and controlled resorption kinetics [11–13]. Such materials are multitalented as they provide adequate mechanical properties combined with the opportunity of being prepared in different forms: massive devices, powders, coatings on different substrates, porous scaffolds and granules.

Several efforts have been focused on preparing silver-containing bioactive glasses by melt and quenching or sol–gel techniques [14–18]. However, these methods showed a lack in the silver content reproducibility and difficulty of tailoring the silver ion release. Silver is potentially cytotoxic if released in great amounts and thus, a strict control of the introduced amount is necessary. It is well-known that glasses can undergo ion-exchange treatments allowing their surface functionalization by means of ion substitution while maintaining unaltered their structure and main properties. In such a way silver, which is known to be an antimicrobial agent, can be introduced by ion-exchange in the outer layer of glass surfaces, conferring to them antibacterial properties while maintaining their peculiar characteristics [19, 20]. Moreover this method can be easily applied to final devices on an industrial scale. In this work, the ion-exchange technique was applied to a silica based glass to confer antibacterial properties in order to prepare bone substitutes or coatings on metallic devices characterized by long term stability. In comparison to previous works concerning the application of this method to glasses of the SCN system [19, 20], efforts have been focused on the opportunity of lowering the Ag content on the glass surface not only by a proper calibration of the exchange conditions [20] but also by modifying the glass composition. The glass composition was also optimized with the aim of using it for the development of vacuum plasma spray coatings on Ti6Al4V substrates, in order to apply the ion exchange technique on amorphous SCNA coatings, as described in Part II of the present work.

2 Experimental

2.1 Glass preparation

The glass used in this work has the following molar composition: 57% SiO_2, 34% CaO, 6% Na_2O, and 3% Al_2O_3 and will be named SCNA from now on. Its composition is based on previous characterizations carried out on a different bioactive glass composition belonging to the SiO_2–CaO–Na_2O ternary system (50% SiO_2, 25% CaO, and 25% Na_2O, named SCN) [21]. Results of preliminary characterizations led to select SCNA because it is chemically more stable than bioactive glasses belonging to the SCN system, due to the substitution of 3% Al_2O_3 instead of SiO_2. Due to the high silica content and the presence of Al_2O_3, SCNA can be placed in the group of glasses with a low bioactivity index [22], which means that it can be successfully used for the realization of devices characterized by long durability. A high durability is preferred for glasses which are designed to the realization of coatings on inert substrates (for example on metallic or ceramic devices) in order to introduce a certain degree of bioactivity without affecting the adhesion to the bone due to the formation of a thick gel-like reaction layer. SCNA composition was also selected to achieve a better control of surface ion leaching due to a higher stability of the glass network. SCNA glass has been prepared by melting reagent-grade reactants in a platinum crucible at 1,500°C for 1 h and then pouring the melt in a copper mould to obtain bars, or quenching the melt in water to obtain a frit. After drying, the frit was ball milled in a planetary miller and sieved down to 100 μm. The obtained glass powders have been subjected to thermal characterizations by means of differential thermal analysis (DTA) and differential scanning calorimetry (DSC) in order to establish SCNA characteristic temperatures. A glass transition temperature of 685°C was found and so glass bars have been annealed at 600°C for 10 h in order to release internal stresses; finally the bars have been cut and polished obtaining 10 × 10 × 1 mm samples. SCNA can be easily milled to produce powders suitable to realize plasma spray coatings on metallic or ceramic substrates, as will be discussed in Part II of this work.

2.2 Surface doping with silver

A set of samples has been subjected to a silver ion-exchange process to modify the glass surface composition by introducing a controlled amount of silver ions. This ion-exchange process is based on a thermo-chemical treatment of a glassy material in an opportune medium, able to exchange mono-valent ions coming from the glass with silver ions coming from the solution itself. The parameters that regulate such process are: silver ion concentration in the solution, time and temperature of the process; by tuning the ion-exchange parameters it is possible to modulate both the amount of silver ions introduced in the glass and their concentration profile along the glass material section [19]. Such process can be applied to virtually any material belonging to the class of glasses, glass-ceramics and ceramics by tuning the process parameters according to the material composition and characteristics. This silver-doping treatment of glasses, glass-ceramics and ceramics surfaces, allows imparting antibacterial properties to a wide

range of materials, without inducing any radical change in their peculiar properties [23].

The set of SCNA samples treated by silver ion-exchange process will be called from now on Ag-SCNA. Both untreated and treated glass samples have been characterized by means of different techniques to test the effects of the ion-exchange process.

2.3 Characterization methods

The structure of all samples has been evaluated by X-Ray diffraction analyses (XRD), using the Bragg Brentano camera geometry and the Cu-Kα incident radiation, to verify if any structural change or crystallization phenomena occurred due to the ion-exchange treatment. Scanning electron microscopy (SEM) observations and energy dispersion spectrometry (EDS) were performed to evaluate eventual morphological and compositional modifications both on Ag-SCNA surfaces and cross sections.

2.4 Silver release and bioactivity

The amount of released silver ions was investigated on sets of three Ag-SCNA and SCNA samples with a surface area of 100 mm^2 soaked into 30 ml of SBF at 37°C for up to 28 days. Graphite furnace atomic absorption spectroscopy (GFAAS) analyses were performed on 1 ml of SBF spiked from each soaking solution after 3 h, 1, 2, 7, 14 and 28 days. For comparative purposes also clean SBF has been analysed. The used GFAAS instrument is a PERKIN ELMER (mod. 4100 ZL) employing a Zeeman-effect background corrector with an auto sampler (mod. AS/71); the auto-sampler was programmed to dispense 20 µl of the sample. To obtain a higher absorption signal two matrix modifiers have been employed: $Pd(NO_3)_2$ and $Mg(NO_3)_2$ have been directly added on the graphite tube by mean 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 (CV $< 3\%$).

Ag-SCNA samples, soaked for 14 and 28 days have also been observed at SEM-EDS, to verify their degree of bioactivity compared to SCNA and to verify if any additional surface modification occurred.

2.5 Assessment of biocompatibility

A preliminary biological test has been carried out by culturing cells on materials surfaces to evaluate their behaviour in the first 24 h. The cells behaviour on the materials has been studied using a fibroblast cell line; the cells have been cultured at 37°C in 95% air/5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS, Sigma), penicillin (100 U $ml^{-1)}$, streptomycin (100 µg $ml^{-1)}$ and L-glutamine 0.03%. Specifically, fibroblasts adhesion and proliferation studies were performed using a starting cell density of 10^4 cells/cm^2; incubated cells have been counted in a Burker camera after being removed with SDS solution from samples surface, respectively after 6 h (adhesion), and 24 h (proliferation) culture. Some samples were also prepared, at the same incubation conditions previously described, to be observed by SEM for cell morphology evaluation. Moreover IgG and IgA proteins adsorption onto SCNA and Ag-SCNA surfaces has been evaluated to verify if the two materials are able to properly react to inflammatory processes, that naturally occur when a material is implanted in a living organism. For all biocompatibility tests, three sets of SCNA and Ag-SCNA samples have been used and compared with a control material (PS).

2.6 Assessment of antibacterial performances

Antibacterial tests have been performed on Ag-SCNA and, for comparative purposes, also on SCNA, in triplicate. *Staphylococcus aureus* (ATCC 29213-BD Microtrol discs) and *Escherichia coli* (ATCC ATCC 25922-BD Microtrol discs) have been selected for this study: both qualitative analyses and quantitative counts have been performed. Mc Farland indexes (i.e. measurement of solution turbidity) of the culture media and colonies forming unity (CFU) counts have been carry out, as well as *zone of inhibition* tests (in accordance to NCCLS standards for antimicrobial susceptibility [24]) have been carried out. Bacterial adhesion and proliferation onto glass surfaces have been evaluated using the procedure described below. All samples have been sterilized at 380°C in dry air for 3 h and then a bacterial culture broth has been prepared from lyophilized tablets which have been dissolved in a brain-heart medium (BBL™ Brain Heart Infusion-BD) and incubated at 35°C for 24 h. 10 µl of this bacterial medium has been diluted in 5 ml of Mueller–Hinton broth (Biolife) in which Ag-SCNA and SCNA samples have been finally soaked and incubated at 35°C for 24 h. For the adhesion evaluation, samples have been removed from the Mueller–Hinton solution, rinsed and stirred for 1 min at 40 Hz with 2 ml of physiological solution; both the rinsing solution and the stirred one have been subjected to serial dilutions and 100 µl of the last dilution have been spread on blood Agar plates (Columbia Agar with 5% sheep blood-BD) and incubated at 35°C for 24 h to count the colony forming unities (CFU). For the bacterial proliferation evaluation, the procedure has been the same as described above but without rinsing the solution: in this way both bacteria adherent onto material surface and bacteria proliferated in the solution around the

material could be counted. The discrimination between the bacteria adhesion and proliferation is important, especially in the case of bacterial species showing low adhesion onto surfaces, such as *E. coli*. In fact, *E. coli* does not adhere on PE control samples or on the glass, but this behaviour is an intrinsic property of the bacterium and it is not due to an antimicrobial activity of the materials. For this reason, the realization of both adhesion and proliferation tests allows to verify the effective antibacterial behaviour of a material. Mueller–Hinton solutions containing samples have been also subjected to turbidity evaluation by means of Mc Farland index measurement: the turbidity of a bacterial solution increase with the bacteria proliferation. The zone of inhibition evaluation is a qualitative standard test normally used to characterize antibiotics: a 0.5 Mc Farland solution, prepared dissolving some bacteria colonies in a physiological solution, has been spread on Mueller–Hinton Agar plate. Afterwards, the samples have been put in contact with Agar and incubated 24 h at 35°C and subsequently the zone of inhibition has been observed and measured.

3 Results and discussion

3.1 Glass characterization

Ag-SCNA and SCNA XRD data have been collected and all patterns showed the same feature: an amorphous halo without defined diffraction peaks, demonstrating the glassy nature of all materials. This means that the parameters selected for the silver ion-exchange process did not induce changes in the glass structure. The amount of introduced silver is low enough to avoid the formation of any crystalline silver compound that could induce a cytotoxic effect for direct contact with cells. Also SEM observations showed an unchanged morphology of Ag-SCNA and, as any significant difference have been found, the results were not reported. The introduced silver is confined in the outer surface layers as no evidence of Ag presence has been detected by EDS analyses on sample cross section (not reported); nevertheless, EDS analyses allowed finding trace of silver on glass surface. Ag was present below the instrumental detection limit and for this reason is was not possible to estimate the exact weight percentage (<1%) of introduced Ag ions.

3.2 Leaching test

On the other hand, leaching tests in SBF showed a not negligible silver ions release, which is higher immediately after contact between Ag-SCNA sample and SBF medium and became gradually lower with increasing time of soaking (Fig. 1). In previous works [19, 20] the same

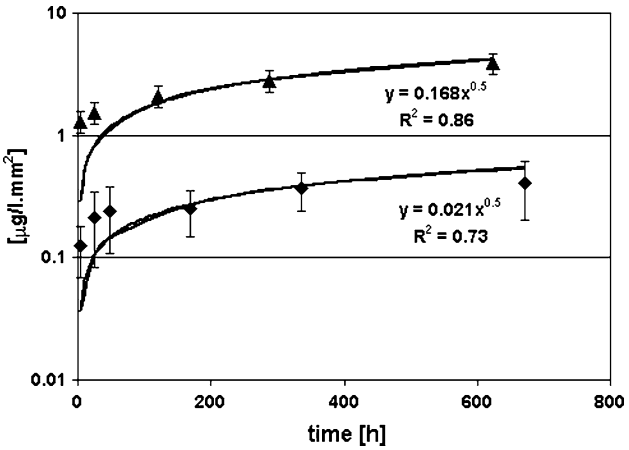

Fig. 1 Ag^+ (µg/l mm^2) leaching test in SBF during time: comparison between SCNA glass (◆) and SCN glass (▲) [21]

characterization was performed on a different glass composition (50% SiO_2, 25% CaO, and 25% Na_2O, named SCN), subjected to the ion-exchange treatment, and the authors found out a good agreement between experimental data and the equation:

$$q = kt^{1/2} \quad (1)$$

where q is the released silver ions amount, t the soaking time and k a multiplying coefficient. Equation 1 shows the dependence of released silver from the square root of time of treatment. Also for Ag-SCNA the agreement with Eq. 1 was good and k coefficient have been reduced from 0,168 [19] to 0,021, corresponding to a lower silver release. The silver ion-exchange parameters have been maintained the same as in Ref. [21], but the Na amount has been reduced in the starting glass composition from 25% to 6% molar, lowering the total amount of introducible silver by Na/Ag exchange. Moreover, the addition of 3% of Al_2O_3 can affect the bioactivity mechanism and the ability of the glass of exchanging ions [25] and thus can be responsible for a further reduction of the introduced silver amount. These results let us state that by controlling both the ion-exchange parameters and/or the composition of the glass it is possible to tailor the introduced silver amount at degrees not obtainable by conventional glass-syntheses.

3.3 In vitro bioactivity

SEM observations of soaked samples showed that silver introduction did not significantly change the glass reactivity towards SBF, thus bioactivity remains equivalent for SCNA and Ag-SCNA samples. After 14 days of SBF dipping, neither silica gel nor hydroxyapatite presence have been detected on samples surface, while some hydroxyapatite-like agglomerates can be seen on the surface of both SCNA and Ag-SCNA samples, after 28 days of immersion

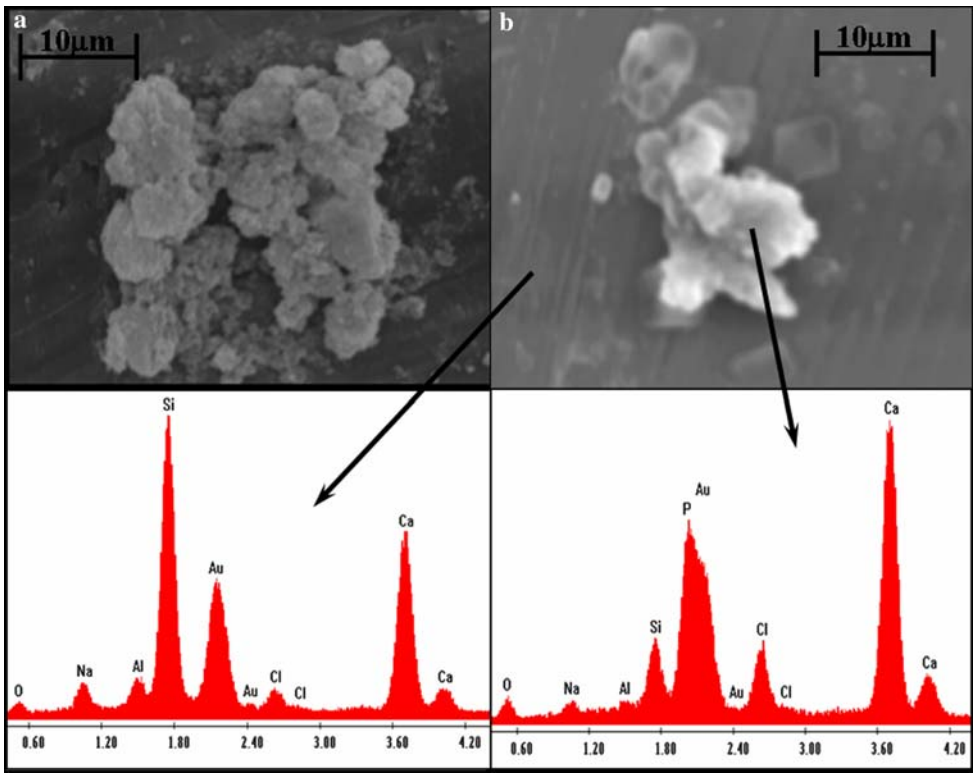

Fig. 2 SEM micrographs of HAp agglomerates on SCNA (**a**) and Ag-SCNA (**b**) surfaces. Comparison of EDS analyses on HAp agglomerate and on glass surface

in SBF (Fig. 2), confirming the moderate bioactivity of this glass. In fact, as previously mentioned, the high silica content and the presence of 3% of Al_2O_3 confine SCNA in the glasses group characterized by a low bioactivity index.

3.4 Biocompatibility test

Biocompatibility tests confirmed the material safety towards cells: both counts (Fig. 3) and morphology (Fig. 4) of cells incubated onto Ag-SCNA and SCNA samples did not reveal any significant difference between the two materials. There are no significant variations of counted alive fibroblasts and their morphologies among silver doped sample and untreated ones; moreover cells number, in both cases, is similar to control one. At this step, cells biocompatibility has been investigated till one day, because, as it can be observed from the release trend (Fig. 1), in the first 24 h Ag-SCNA releases most of the Ag^+ ions. We do expect that the amount of introduced, and consequently released, silver will not cause cytotoxic response for longer culture period, considering the lower release kinetic, as well as the clearance effect which occurs in vivo. As reported in literature [26, 27] IgA and IgG are respectively known to be, alternative and classical pathway complement activators. Their binding to biomaterials might influence complement activation at the material interface [28, 29]. For this reason, proteins adsorption assays are frequently performed, collecting more precise information about the modalities of interaction of biomaterials in vivo. In particular high IgA and IgG binding to surfaces is index of complement-activating materials. Protein adsorption of IgG and IgA (Fig. 5) resulted to be the same for SCNA, Ag-SCNA and control, thus demonstrating that both SCNA and Ag-ACNA provide a surface capable to properly react against inflammatory processes.

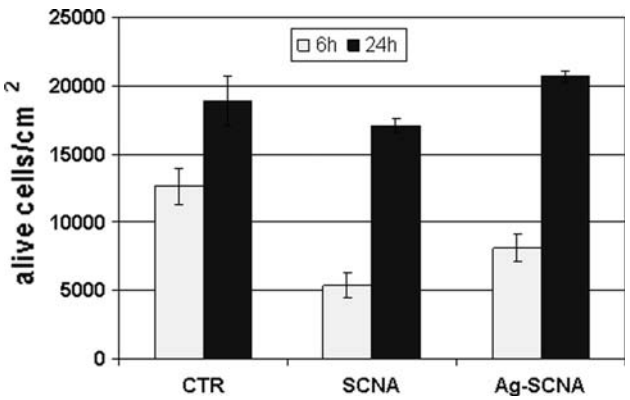

Fig. 3 Counts of alive cells adhered (6 h) and proliferated (24 h) onto SCNA and Ag-SCNA compared to PS control

3.5 Antibacterial test

Qualitative antibacterial tests, performed by Mc Farland index measurements, showed the efficacy of the silver

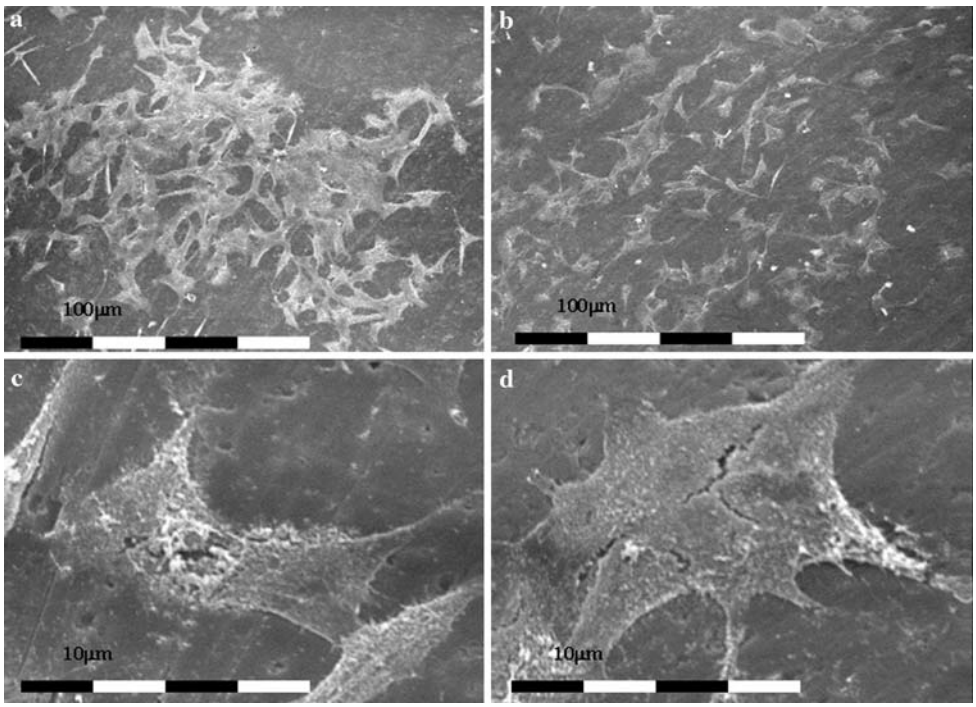

Fig. 4 SEM micrographs and magnifications of cells adhered on SCNA samples (**a** and **c**) and on Ag-SCNA samples (**b** and **d**) after 24 h culturing

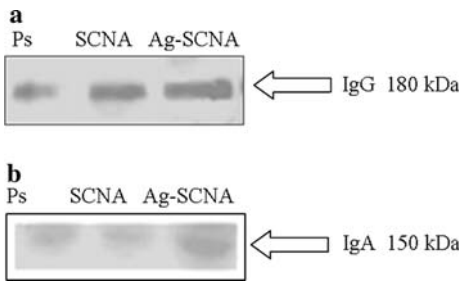

Fig. 5 Western Blot of **a** IgG and **b** IgA protein adsorption onto polystyrene, SCNA sample and Ag-SCNA

ion-exchange treatment to impart antibacterial properties to SCNA glass: the difference between Ag-SCNA and SCNA Mc Farland index is about 4.6 for *S. aureus* and 4 for *E. coli*. The zone of inhibition test revealed that the field of action of silver-modified glass is about 2 mm while bacteria proliferated close to SCNA glass (Fig. 6). Bacteria counts confirmed these qualitative data, providing a reduction higher than 99.9% (3 log-reduction), both for bacteria adhesion onto material surface and proliferation in the culture medium (Fig. 7). It can be noted that *E. coli* does not adhere on Ag treated sample nor on

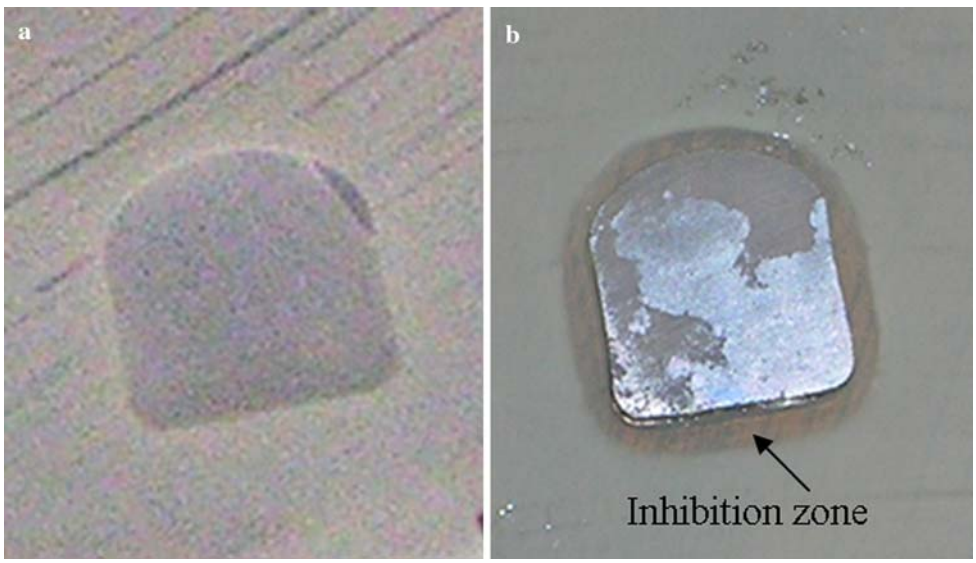

Fig. 6 SCNA (**a**) and Ag-SCNA (**b**) sample on Mueller–Hinton agar plate. It is evident a zone of inhibition around silver doped samples, while no sign of bacteria inhibition is observable around untreated sample

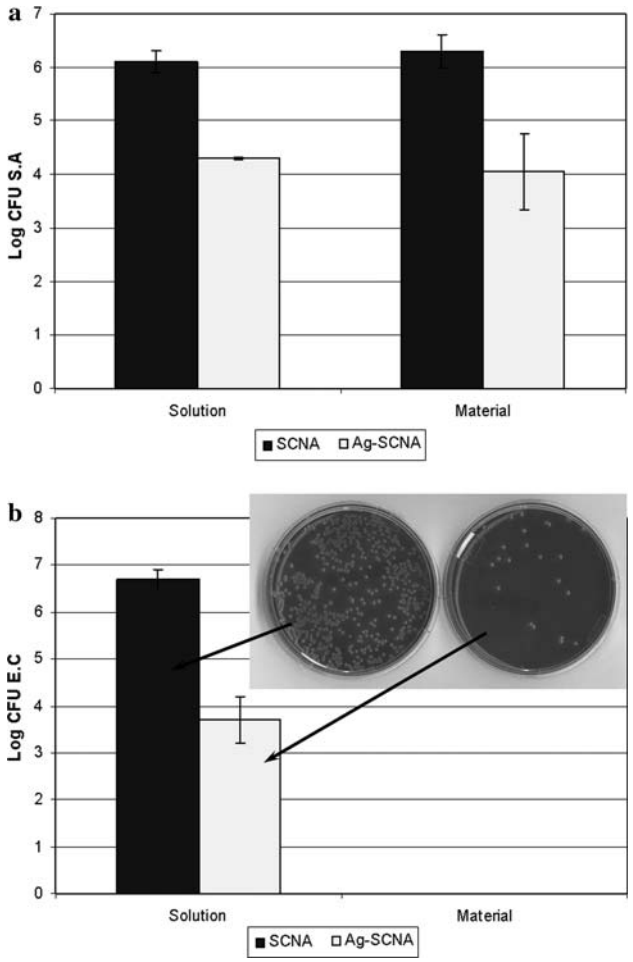

Fig. 7 *Staphylococcus aureus* (**a**) and *Escherichia coli* (**b**) CFU adhered on SCNA and Ag-SCNA (stirred solution) and proliferated in a Muller–Hinton solution containing the same samples (rinsed solution)

untreated glass surface (Fig. 7b), nevertheless this behaviour is a peculiar quality of this bacterium, as demonstrated by adhesion test on polyethylene sample (not reported). These antibacterial tests clearly demonstrated that the introduced silver is effective in reducing both bacteria adhesion onto Ag-SCNA surface and their proliferation close to the material itself. Ag-SCNA can be definitely considered a bacteriostatic material because it is able to inhibit the bacteria adhesion and proliferation. In order to define Ag-SCNA a bactericidal material an in-depth study to verify if the silver amount introduced and released is able to kill some starting bacteria colonies, inoculated in Mueller–Hinton broth should be done. Nevertheless, the obtained results meet the goal of this work: the realization of a biocompatible and antibacterial material able to inhibit the proliferation of bacteria usually present in human body without cytotoxic effect on the surrounding cells.

4 Conclusions

The present work had demonstrated that the silver ion-exchange process can be successfully applied to low reactivity bioactive silica based glasses, thus permitting to confer antibacterial properties to medical devices, partially or totally realized with a glass, without any adverse effect in terms of biocompatibility towards tissue cells and/or bioactivity. New implant devices and prostheses can be produced by using biocompatible or bioactive glassy materials, thus allowing to improve bone healing processes while reducing the risks of infection, due to the added value of antibacterial action induced by the silver ion-exchange treatment.

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