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In situ reduction of antibacterial silver ions to metallic silver nanoparticles on bioactive glasses functionalized with polyphenols

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ABSTRACT

The realization of surfaces with antibacterial properties due to silver nanoparticles loaded through a green approach is a promising research challenge of the biomaterial field.

In this research work, two bioactive glasses have been doubly surface functionalized with polyphenols (gallic acid or natural polyphenols extracted from red grape skins and green tea leaves) and silver nanoparticles deposited by in situ reduction from a silver nitrate aqueous solution. The presence of biomolecules – showing reducing ability to directly obtain in situ metallic silver – and silver nanoparticles was investigated by means of UV–vis spectroscopy, X-Ray Photoelectron Spectroscopy (XPS) and Field Emission Scanning Electron Microscopy (FESEM). The antibacterial activity of the modified surfaces was tested against a multidrug resistant *Staphylococcus aureus* bacterial strain.

1. Introduction

Silver is known from ancient times for its broad spectrum of antibacterial activity and is widely investigated as multi-purpose antibacterial agent. The raising diffusion of bacterial resistance to common antibiotics, recently defined as global threat [1], increases the interest in alternative antibacterial substances and in particular inorganic ones, such as silver. The development of nanotechnologies focuses the attention on silver nanoparticles as promising antibacterial agents [2,3] and numerous silver nanoparticles-loaded products come into the market in various application fields (e.g. drugs, personal care/cosmetics, textiles/shoes, electronics, household products, filtration/sanitization, medical devices) [4]. Despite the wide diffusion of silver nanoparticles, its potential toxicity for health and environment is not completely known up to now [4]. The antibacterial mechanism of silver nanoparticles is not yet fully understood, however a multiple action was proposed: i) interaction with the bacterial cell wall and its consequent damage, ii) induction of oxidative stress by Reactive Oxygen Species (ROS) production and iii) sustained release of silver ions [5,6]. Thanks to the high surface to volume ratio and the probable multiple mode of action, silver nanoparticles present superior antibacterial activity compared to bulk metallic silver.

Silver nanoparticles can be produced by top down approaches, which foresee the dimensional reduction of larger structures (e.g. mechanical, ball milling, chemical etching, thermal/laser ablation, sputtering) or bottom up approaches, based on aggregation processes (such as chemical/electrochemical precipitation, vapor deposition, atomic/molecular condensation, sol-gel, spray pyrolysis, laser pyrolysis or aerosol pyrolysis) [7,8]. The main drawbacks of common synthesis routes for silver nanoparticles are the employment of toxic chemicals and high temperature, pressure and energy, so that new green and environmentally friendly strategies are needed. Various green synthesis approaches have been investigated such as those using microorganisms (bacteria, fungi, yeasts) rather than algae or polysaccharides and plants extracts [8–11]. Among them, the use of natural substances such as plant extracts seems more promising for the industrial application because it is easily scaled up and does not require complex systems for microorganisms' culture and related biohazard concerns [8]. Moreover, reducing agents can be obtained from vegetal products coming from the wastes of food and wine production chains, opening the opportunity for a sustainable use of resources obtained from vegetal residues that would become wastes.

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Table 1
Glass composition (molar percentages), melting temperatures and annealing conditions.

	Glass composition (mol%)				Tmelt [°C]	Annealing
	SiO_2	Na_2O	CaO	Al_2O_3		
SCNA	57.0	6.0	34.0	3.0	1550	10h@600 °C
SCN1	57.0	9.0	34.0	0.0	1500	12h@550 °C

Numerous experimental strategies have been reported in the recent scientific literature for the green synthesis of colloidal silver nanoparticles [8–21] although few papers consider specifically in situ reduction of silver nanoparticles on substrates [22–24].

In the present paper, for the first time, silver nanoparticles (Ag-NPs) have been obtained by in situ reduction on bioactive glasses functionalized with gallic acid (used as a simple model molecule to represent the polyphenols reactivity) or with natural polyphenols extracted from red grape skin and green tea leaves. The effect of glass surface reactivity on the ability to graft biomolecules and, subsequently, to induce in situ reduction of silver ions was investigated by means of UV–vis photometry, X-Ray Photoelectron Spectroscopy (XPS) and Field Emission Scanning Electron Microscopy (FESEM). The antibacterial activity of Ag-NPs doped bioactive glasses was evaluated against a multidrug resistant strain of *Staphylococcus aureus* by means of bioactive coating derived metabolic reduction evaluation.

This research work presents a promising strategy to obtain innovative smart polyfunctional biomaterials combining the peculiar features of i) bioactive glasses (bioactivity, ion release ability), ii) polyphenols (antioxidant, antibacterial, vascular protective, bone stimulating activities) and iii) silver (antibacterial). In this route, Ag-NPs results embedded on the glass surface reducing risk concerns related to the free metallic nanoparticles.

2. Materials and methods

2.1. Glass synthesis

Two bioactive glasses, designed in the authors' laboratories, were considered: SCNA and SCN1. The molar composition and melting/annealing conditions are reported in Table 1. The reactivity of bioactive glasses strongly depends on their composition. In particular, it was evidenced that the silica content (for melt derived bioactive glasses) should not exceed 60% (mol), in order to obtain a bioactive behavior [25] and that the addition of alumina in the glass composition reduce the glass bioactivity because it inhibits the ion exchange improving the material stability [25,26]. Moreover sodium is involved in the ion exchange process, which is the first step in the bioactivity mechanism [25–27]. The two bioactive glasses considered in this research work present the same silica content and differs for the presence/absence of alumina and for the sodium content. SCNA is a highly stable glass due to the presence of alumina among its constituent oxides. SCN1 presents a larger reactivity because of a higher Na content and the absence of alumina. These glasses were chosen for their simple compositions and controllable reactivity in order to investigate their ability to graft polyphenols and induce in situ reduction of silver nanoparticles.

2.2. Surface functionalization with polyphenols

Gallic acid (GA), used as simple model molecule, or polyphenols extracted from red grape skin (GPH) and green tea leaves (TPH) were considered for the organic surface functionalization.

Gallic acid was purchased from Sigma Aldrich (GA 97.5–102.5% titration, G7384, Sigma Aldrich, Milan, Italy) while natural polyphenols were extracted by conventional solvent extraction method, as previously described by the authors in [28,29].

Table 2
Samples names, treatments and surface features.

Sample name	Treatment	Surface feature
SCNA-wash	Acetone and water washing	OH groups
SCNA + GA	Acetone and water washing + GA grafting	GA molecules
SCNA + GPH	Acetone and water washing + GPH grafting	GPH molecules
SCNA + TPH	Acetone and water washing + TPH grafting	TPH molecules
SCN1-wash	Acetone and water washing	OH groups
SCN1 + GA	Acetone and water washing + GA grafting	GA molecules
SCN1 + GPH	Acetone and water washing + GPH grafting	GPH molecules
SCN1 + TPH	Acetone and water washing + TPH grafting	TPH molecules
SCNA-wash + Ag	Acetone and water washing + in situ reduction Ag nps	OH groups/Ag NPs
SCNA + GA + Ag	Acetone and water washing + GAgrafting + in situ reduction Ag NPs	GA molecules/Ag NPs
SCNA + GPH + Ag	Acetone and water washing + GPHgrafting + in situ reduction Ag NPs	GPH molecules/Ag NPs
SCNA + TPH + Ag	Acetone and water washing + TPHgrafting + in situ reduction Ag NPs	TPH molecules/Ag NPs
SCN1-wash + Ag	Acetone and water washing + in situ reduction Ag nps	OH groups/Ag nps
SCN1 + GA + Ag	Acetone and water washing + GAgrafting + in situ reduction Ag NPs	GA molecules/Ag NPs
SCN1 + GPH + Ag	Acetone and water washing + GPHgrafting + in situ reduction Ag NPs	GPH molecules/Ag NPs
SCN1 + TPH + Ag	Acetone and water washing + TPHgrafting + in situ reduction Ag NPs	TPH molecules/Ag NPs

The above cited biomolecules were directly grafted on the surface of SCNA and SCN1 after hydroxyls exposition on the glass surface. Reactive OH groups were exposed by means of acetone and water washings in ultrasonic bath, as described in [28–32]. Washed samples were named SCNA-wash and SCN1-wash (Table 2). The grafting of biomolecules was performed by soaking washed glasses in a solution of polyphenols (1 mg/ml for GA and TPH and 5 mg/ml for GPH) for 3 h at 37 °C [29,33]. At the end of the soaking period samples were gently washed two times in ultrapure water and let dry under a laminar flow cabinet (FASTER CYTOSAFE) in dark conditions. Functionalized samples were named SCNA + GA, SCNA + GPH, SCNA + TPH, SCN1 + GA, SCN1 + GPH and SCN1 + TPH (Table 2).

2.3. In situ reduction of silver nanoparticles

Functionalized biomaterials with the mentioned organic molecules were soaked 1 h at 37 °C in a 0.005 M $AgNO_3$ aqueous solution in order to obtain the in situ reduction of silver nanoparticles (Ag NPs) on the glass surface, exploiting the reducing action of previously grafted polyphenols. At the end of the soaking period, samples were gently washed in ultrapure water and let

dry under a laminar flow cabinet in dark conditions. Polyphenols grafted and silver modified samples were named SCNA + GA + Ag, SCNA + GPH + Ag, SCNA + TPH + Ag, SCN1 + GA + Ag, SCN1 + GPH + Ag and SCN1 + TPH + Ag (Table 2). Washed samples were subjected to the same treatment for comparison purposes and were named SCNA-wash + Ag and SCN1-wash + Ag (Table 2).

2.4. Physico-chemical characterization

Photometric measurements in the UV–vis spectral region (CARY 500 Varian spectrophotometer) were performed to quantify the amount of active polyphenols on the glass surface by means of the Folin&Ciocalteu method [34], as previously described by the authors [29,33]. A standard calibration curve was obtained with GA solutions of known concentration and used for GA quantification on the samples [30,28,29,33]. As far as natural polyphenols (a complex blend of various mono- and polyciclic type of phenol-based molecules) are concerned, their concentration was calculated in gallic acid equivalents units, employing to the same calibration curve [28,29].

Surface chemical composition and chemical state of elements were analyzed by means of X-ray Photoelectron Spectroscopy (XPS, PHI 5000 VERSAPROBE, PHYSICAL ELECTRONICS) in order to determine the presence of biomolecules after functionalization and silver nanoparticles after in situ reduction.

Field Emission Scanning Electron Microscopy (FESEM-EDS SUPRATM 40, Zeiss and Merlin Gemini Zeiss) was employed for the investigation of silver nanoparticles precipitation on samples surface. Samples were sputter coated with a thin Cr layer (<5 nm) before analyses.

2.5. Antibacterial activity evaluation

SCNA and SCN1 samples functionalized with polyphenols extracted from green tea leaves and the same after *in situ* reduction of Ag NPs were considered for the antibacterial tests, because they showed the best results in terms of functionalization among the molecules of natural origin. Just washed samples were also tested for control purposes.

2.5.1. Bacterial strains and growth conditions

The exponentially-growing biofilm pathogen *Staphylococcus aureus* (clinical isolate from the Hospital Maggiore of Novara) strain was used to evaluate the antibacterial activity of samples. Bacteria were cultivated on blood-agar plates (SintakS.r.l., Corsico, Milan, Italy) at 37 °C in aerobic conditions for 48 h until round single colonies were obtained. Plates were then stored at 4 °C until use.

2.5.2. Biofilm formation

Specimens were placed into the wells of a 12 multiwell plate (Nunc Delta, Nunclone). Then, 500 mL of fresh bacterial culture were prepared by inoculating about 4–5 single colonies into Luria Bertani broth (LB, Sigma-Aldrich, Milan, Italy); cultures were incubated at 37 °C in a Gallenkamp orbital shaker incubator at 200 rpm for 16 h. Exponentially-growing bacterial suspensions were then diluted in fresh LB medium at a final concentration of 1×10^7 cells mL^{-1} according to McFarland standard 1.0 [35]. 1 mL of the broth culture was collected and used to contaminate specimens; plate was incubated at 37 °C in rotation (90 rpm) for 90 min (adhesion phase). The supernatant containing planktonic cells was then removed, while biofilm former cells, attached to the specimens' surfaces, were rinsed with 1 mL of fresh LB medium (separation phase) [36,37]. Plate was incubated 24 h at 37 °C in a humid atmosphere to allow mature biofilm growth.

Table 3
Polyphenols amount on the surface of glass samples.

Glass	Polyphenol content – GA-equivalents [mg/ml] (mean ± stdev)		
	GA	GPH	TPH
SCNA	$0.0000 \pm 7.2832 \cdot 10^{-8}$	$0.0000 \pm 7.071 \cdot 10^{-6}$	$0.0000 \pm 7.071 \cdot 10^{-6}$
SCN1	0.0004 ± 0.0002	0.0000 ± 0.0005	0.0025 ± 0.0006

2.5.3. Bacterial cell viability

To assess the growth capacity of the bacterial after 24 h of direct contact to specimens' surface, compared to that of untreated controls, bacterial viability was evaluated by the validated quantitative colorimetric metabolic 2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenyl amino) carbonyl]-2H-tetrazolium hydroxide assay (XTT, Sigma). Briefly, 100 μL of XTT solution (3 mg mL-1 in acetone containing 0.1 M menadione) were added to each well and plates were incubated at 37 °C for 5 h in the dark; 100 μL were then collected from each well, centrifuged for 2 min at 1200 rpm to remove any debris, and the optical density (o.d.) was evaluated using a spectrophotometer (SpectraCount, IBM, NY, USA) at 490 nm [38]. Experiments were performed in triplicate.

2.6. Statistical analysis

All tests were performed in triplicate. The Kruskal – Wallis analysis followed by Conover test as *post-hoc* were used to determine significance that was set at $p < 0.05$

3. Results and discussion

3.1. Photometric determination of the polyphenol content on the glass surface

The results of the Folin&Ciocalteu test on glass samples are reported in Table 3.It was impossible to detect a significant amount of polyphenols (GA, GPH and TPH) on SCNA surface by this technique, while a certain amount of GA and TPH was registered on SCN1 one. These results are in accordance with previous ones obtained by the authors on the same glass [30,28] and can be attributed to the low reactivity of SCNA and the moderate surface area of glass slices. The increase in reactivity obtained with SCN1 composition allows a more effective grafting of biomolecules, except for GPH.

3.2. XPS analyses

Atomic percentages of elements from survey spectra are reported in Table 4.

No significant differences in the surface amount of Si and Ca can be highlighted between SCNA and SCN-1, in accordance to their theoretical bulk composition. The low amount of sodium recorded on SCN-1 and its absence on SCNA (compared to the theoretical bulk composition) can be ascribed to both ion release in the washing solution and also to a sort of hiding due to carbon presence at the surface. XPS is sensitive to the outermost surface layer (few nanometers) and a significant amount of carbon has been registered also on washed samples due to hydrocarbon absorption from the atmosphere, as widely documented in the literature for reactive surfaces [39–42]. EDS analyses (not reported), that interest a higher penetration depth (around 1 μm) detect a sodium amount closer to the theoretical one. This phenomenon makes XPS survey analyses not completely suitable and exhaustive for the characterization of material reactivity and functionalization. High resolution

Table 4
Atomic percentages of elements from XPS survey spectra.

SCNA								
Element	wash	wash + Ag	GA	GA + Ag	GPH	GPH + Ag	TPH	TPH + Ag
O	56.8	55.1	59.6	55.6	57.0	49.9	53.6	54.6
C	21.7	18.0	15.1	15.2	20.6	26.3	22.3	20.5
Si	15.4	20.1	22.9	23.4	21.3	19.3	19.6	19.2
Ca	3.5		0.8			0.3	1.3	0.5
Al	2.6	2.6	1.6		1.2		2.7	
N							0.4	
Ag		4.2		5.9		4.0		5.2
Cl						0.2	0.1	
SCN1								
O	53.4	48.7	53.0	49.3	52.6	50.7	50.0	50.9
C	25.3	29.3	27.1	29.5	30.0	27.4	31.2	26.6
Si	17.1	16.8	17.9	16.4	14.3	16.4	17.5	16.4
Ca	2.8	2.1	1.1	0.2	1.9	0.3	1.0	1.1
Na	1.3	1.0			1.0	0.3		0.9
N		1.0	0.9	0.5	0.2		0.1	0.1
Ag		1.1		4.1		5.0	0.2	3.9

spectra of the oxygen region (not reported) has been recorded and confirmed hydroxyls exposition on both glasses after washing.

No significant trends can be registered for carbon and oxygen as characteristic elements of grafted biomolecules. The detailed analysis of carbon region is necessary in order to investigate the effectiveness of the surface functionalization procedure, as discussed in the following.

On the other hand, a significant amount of silver can be detected on Ag-treated samples, confirming the surface ability to induce the reduction of Ag(I) and the subsequent uptake. A higher silver content can be observed on polyphenol grafted SCN1, while no significant differences between functionalized and washed samples can be noted on SCNA samples. The nature of deposited silver (metallic/ionic) was investigated by the detailed analysis of Ag region in the following. A small amount of silver has been detected on SCN1 + TPH, the amount can be considered negligible because it falls in the instrumental error (0.1–0.2% at.) and can be attributed to contamination.

The detailed analysis of carbon region for washed and functionalized samples is reported in Fig. 1.

On the washed samples (Fig. 1a and e) two main contributions, at about 284.8 eV and 289 eV, can be detected and attributed to C—C/C—H from hydrocarbon contaminants and carbonates respectively [39–42]. Surface contaminations by atmospheric hydrocarbons are always present onto reactive surfaces [39–41], as already observed, together with carbonates, on bioactive glasses by the authors [28–30,43]. A small signal at about 287 eV can be also detected on SCN1-wash; C—O bonds can correspond to this energy, but it can also come from contaminants on this sample.

The signal of carbonates disappears after surface functionalization, as previously observed by the authors [28–30]. A significant signal at around 286 eV appears on all the polyphenols grafted glasses (Fig. 1b, c, d, f–h) and can be attributed to C—O bonds in polyphenols molecules [44–46]. This signal was previously observed on polyphenols grafted bioactive glasses by the authors [28–30]. A moderate contribution in the 288 eV around can be observed on SCN1 samples after polyphenols grafting. It can be attributed to the C=O bonds [44–46] in carboxylic groups of polyphenols or in hydroxyl groups of polyphenols oxidized to quinones, as previously observed by the authors [28–30]. The appearance of the C=O signal only on the most reactive glass can be associated with a higher ion release in the functionalization medium with consequent higher alkalinization and polyphenol oxidation during grafting for this material.

The signal at 284.8 eV persists after the functionalization but its relative intensity is lower than on the washed surfaces, especially for SCNA. It must be underlined that the C—C/C—H signal on the washed sample can be attributed to surface hydrocarbon contaminants from the atmosphere. In absence of surface functionalization, and for a stable glass for which carbonatation is low, it is the most significant signal in the carbon region. After functionalization the signal related to the characteristic functional groups of the biomolecules become more relevant compared to this contribution and its relative intensity decreases.

The detailed analysis of the silver region for Ag-treated samples is reported in Fig. 2. For all the samples, the main contribution is given by the signals at about 368.2 eV and 374.3 eV, attributable to metallic silver (Ag^0) [47,48]. A second contribution at lower binding energies (about 367.7 eV and 373.1 eV) can be observed, except for SCN1-wash + Ag sample, and attributed to silver oxides (AgO, Ag_2O) [47,48]. This contribution is almost negligible for SCNA samples, while it becomes significant for SCN1 + GA + Ag, SCN1 + GPH + Ag and SCN1 + TPH + Ag.

The presence of metallic silver on the surfaces confirms their ability to reduce silver ions from the $AgNO_3$ solution of the treatment. This reducing ability can be ascribed to grafted polyphenols for functionalized surfaces. In the case of washed glasses a certain reducing ability can come from the surface exposed hydroxyl groups. This result is in accordance with the antioxidant ability of bioactive glasses previously observed by the authors [29].

The presence of silver oxides can be related to the absorption of silver ions in the surface reaction layer during the modification process. This phenomenon is more evident on functionalized SCN1 glass because of a higher reactivity and a more pronounced reaction layer, as confirmed by FESEM observations (paragraph 3.3).

3.3. *FESEM observations*

FESEM images have been recorded both in secondary electrons and in back-scattered ones modes in order to investigate surface morphology and discriminate the presence of silver nanoparticles. In fact Ag is heavier than the glass constituents and silver nanoparticles can be detected as bright spots on back-scattered electrons images.

FESEM images of samples (secondary electrons and back-scattered ones) are reported in Fig. 3.

Numerous particles, with a bright appearance in back-scattered images, can be observed on all the samples except of SCN1-wash + Ag. Particles form aggregates of at about 200 nm on SCNA-wash + Ag while particles with dimensions lower than 100 nm and small aggregates can be observed on SCNA + GA + Ag, SCNA + GPH + Ag and SCNA + TPH + Ag. Particles with even smaller

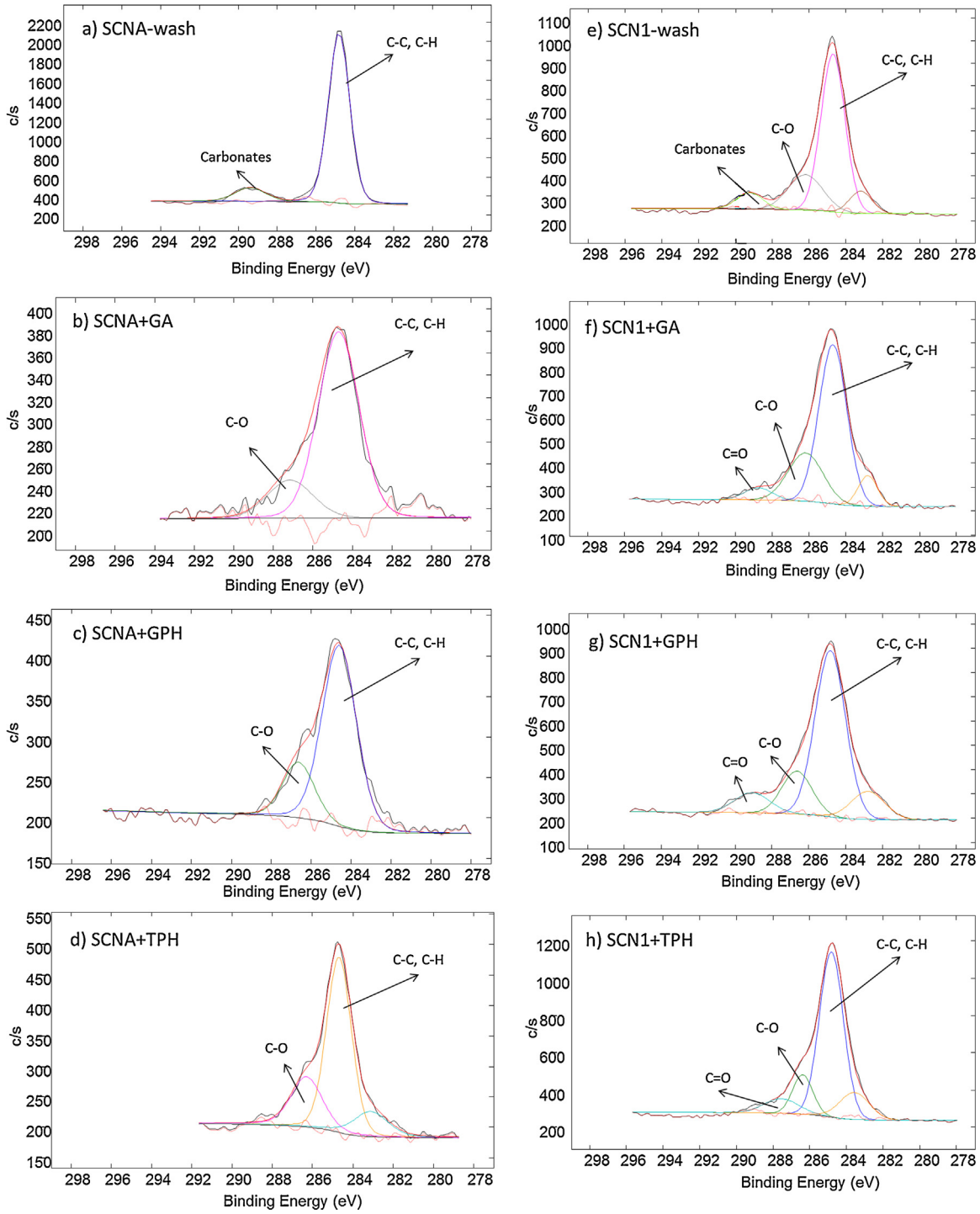


Fig. 1. XPS high resolution spectra of carbon region.

dimensions can be evidenced on SCN1 + GA + Ag, SCN1 + GPH + Ag and SCN1 + TPH + Ag. On the other hand only precipitates with bigger dimensions (few μm) and caltrop shape can be detected on SCN1-wash + Ag (Fig. 4).

A moderate reaction layer can be observed on SCNA samples after the various modification processes as an irregular texture (Fig. 3). On the other hand a significant reaction layer, more evident after grafting of biomolecules, can be observed on SCN1 samples, confirming the higher reactivity of this glass (Fig. 3). Considering both FESEM and XPS results it can be hypothesized that silver precipitates only as metallic nanoparticles on SCNA substrates and on SCN1-wash ones, while it is also adsorbed in ionic form in the reaction layer on SCN1 + GA + Ag, SCN1 + GPH + Ag and SCN1 + TPH + Ag.

EDS analyses confirm the presence of silver in all the observed precipitates.

3.4. Antibacterial activity

Since TPH grafted and TPH grafted/Ag treated samples showed the best results in terms of functionalization among the molecules

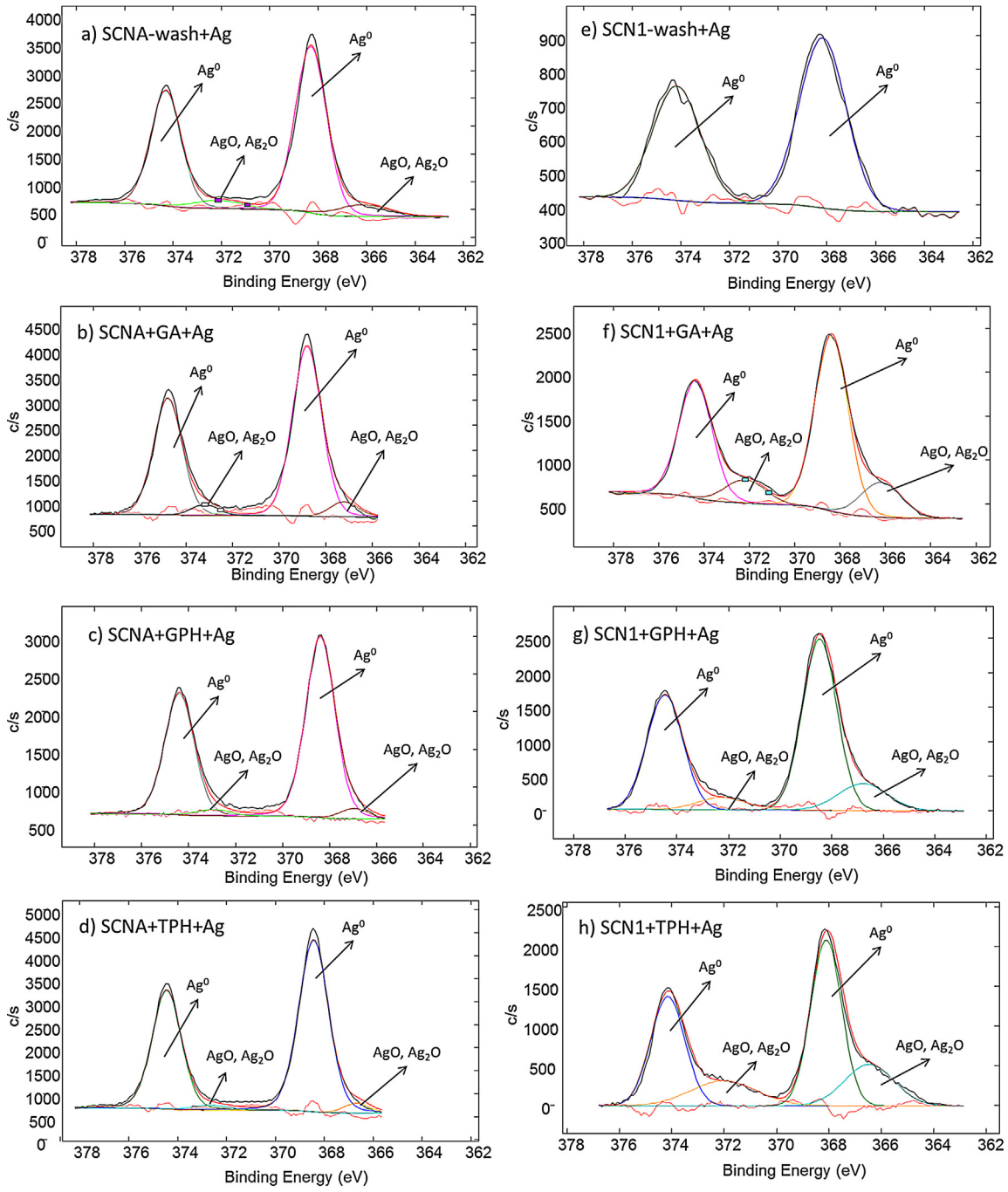


Fig. 2. XPS high resolution spectra of the silver region.

of natural origin, they were both considered for the antibacterial tests together with washed controls in order to evaluate the effect of both natural polyphenols and silver nanoparticles on bacterial viability. The main advantages in the use of natural extracts instead of synthetic chemicals are the possibility of recovery from natural sources, with economic and environmental benefits, and reduced toxicological concerns.

The results of antibacterial tests are reported in Fig. 5.

XTT analysis (Fig. 5a) revealed a statistical significant difference between washed control specimens (wash) and polyphenols + silver doped (TPH + Ag) ones for both SCNA ($p < 0.05$, indicated by *) and SCN-1 ($p < 0.05$, indicated by #). No significant differences were noticed between controls and polyphenols (TPH) grafted specimens ($p > 0.05$ for both SCNA and SCN-1 bioglasses).

Finally, bacteria viability after 24 h is reported in Fig. 5b in function of controls.

No significant differences have been observed between the behavior of the two glasses despite of the different reactivity and the different chemical state of silver on their surfaces (mainly metallic on SCNA and metallic and ionic on functionalized SCN-1).The antibacterial action of silver ions and nanoparticles is widely discussed in the literature and not completely understood yet [49–51] as well as the optimal effective therapeutic dosage [52]. As far as the surfaces prepared in the present research are concerned,

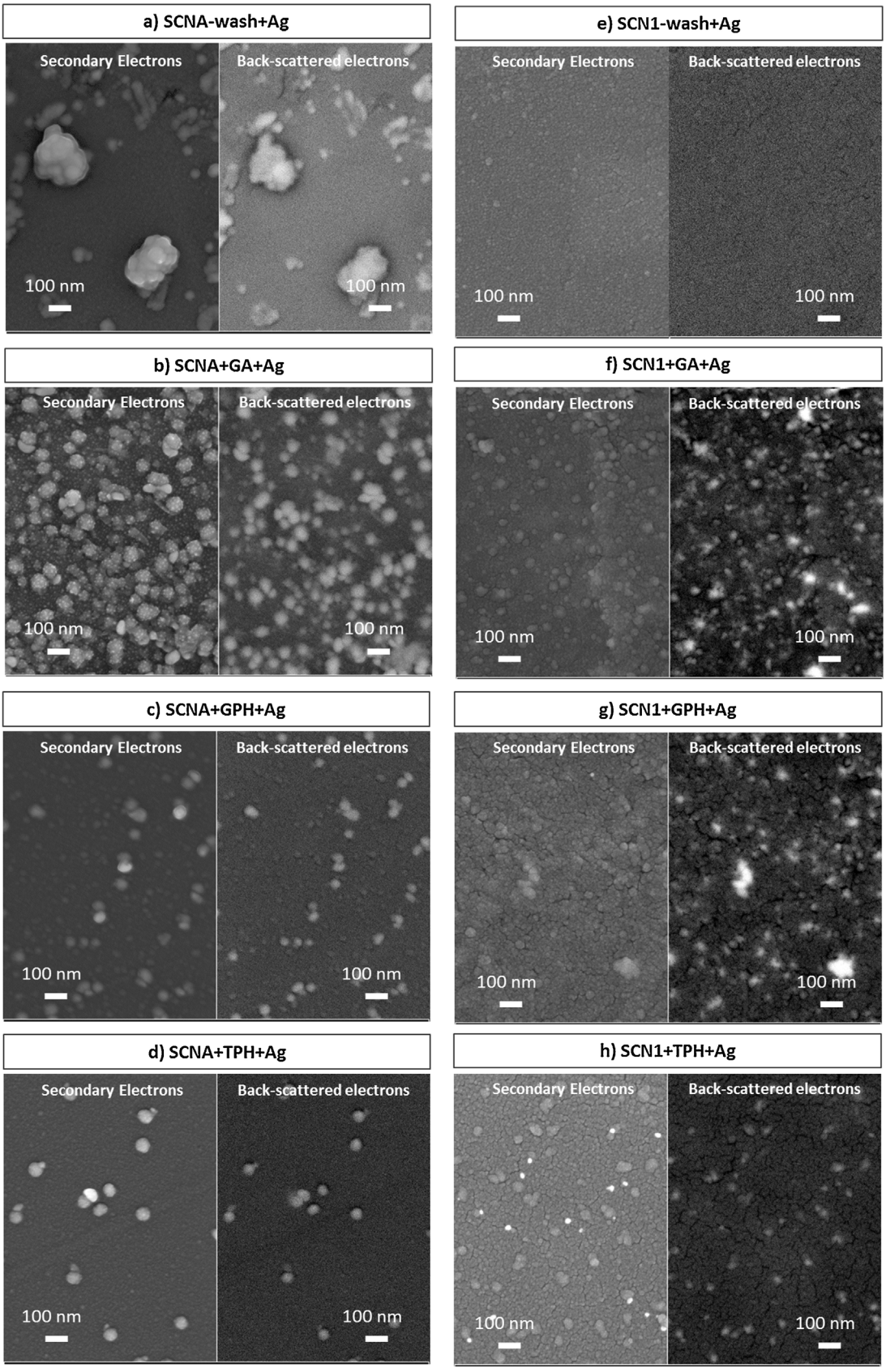


Fig. 3. FESEM images (secondary and back-scattered electrons) of samples (200,000x magnification).

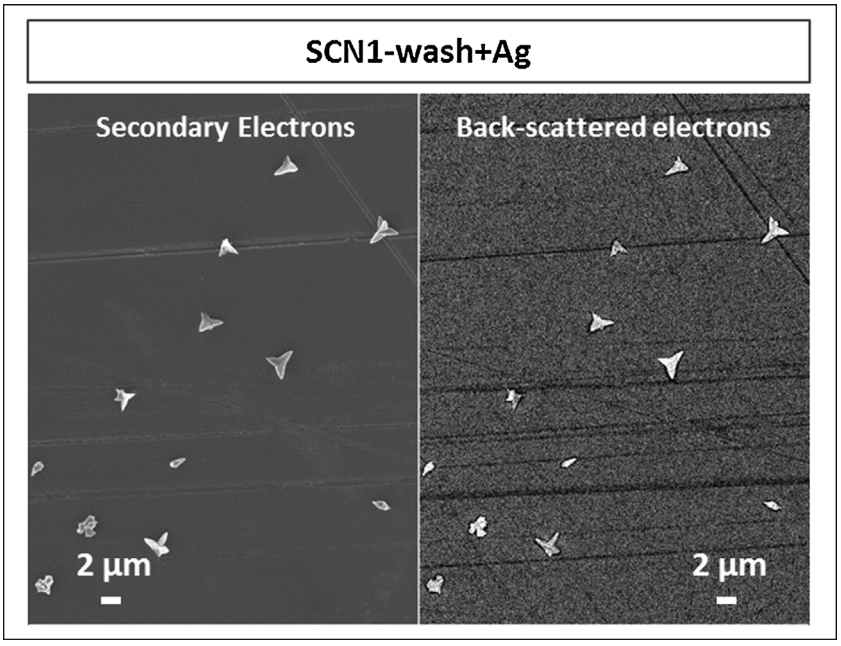
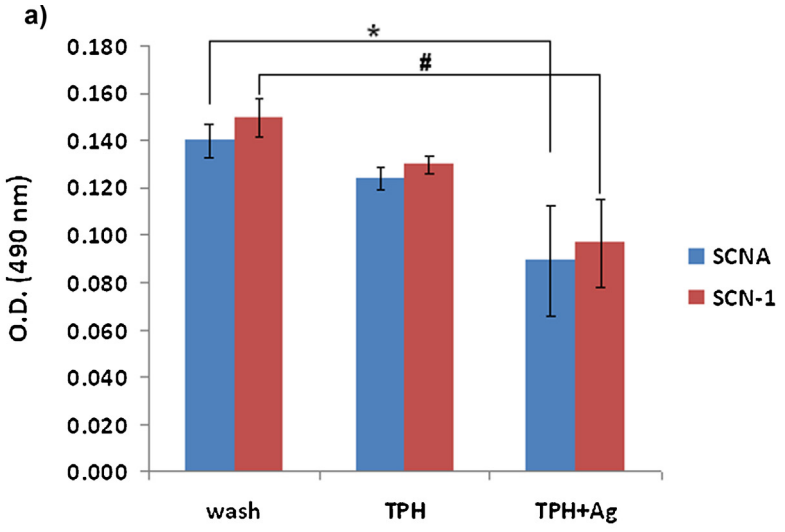


Fig. 4. FESEM image (secondary and back-scattered electrons) of SCN1-wash + Ag (5000x magnification).



b)

SCNA % Bacteria Viability (vs cnt)		
wash	TPH	TPH+Ag
(cnt)	88.5 +/- 0.5	63.8 +/- 2.3

SCN-1 % Bacteria Viability (vs cnt)		
wash	TPH	TPH+Ag
(cnt)	86.6 +/- 0.4	64.5 +/- 1.9

Fig. 5. *S. aureus* viability on the tested samples. XTT assay (a) showed significant differences between control (washed) and TPH + Ag specimens ($p < 0.05$, indicated by * and # for SCNA and SCN-1, respectively). In (b) the 24hs biofilm viability normalized towards controls are reported. Bars represent means and standard deviations.

the mechanism of the antibacterial action should be investigated in more detail together with the silver release and the biocompatibility, but this is out of the scope of the present preliminary study and will be discussed in a future research paper.

4. Conclusions

Gallic acid and natural polyphenols extracted from red grape skin and green tea leaves have been successfully grafted on two bioactive glasses with different surface reactivity. The ability to graft biomolecules increases with the glass surface reactivity. Polyphenolic biomolecules (as gallic acid or natural ones extracted by specific plant sources) grafted on the surface of bioactive glasses and, in a certain measure, also the surface hydroxyl groups of the glass, make it possible the reduction of the silver ions (coming from a silver nitrate aqueous solution) to metallic silver nanoparticles on the glass surface. The specific novelty of this paper consists in the green synthesis of colloidal silver nanoparticles directly operated

on the surface of each bioactive glass under examination previously grafted with natural organic reducing molecules (gallic acid or blends of polyphenols extracted from plants) also able to act as protective agents (antioxidant, antibacterial, vascular protective, bone stimulating activities). Bioactive glasses functionalized with tea polyphenols present a statistically significant antibacterial activity against *Staphylococcus aureus* after in situ reduction of silver nanoparticles. Finally, the strategy of preparation of the functionalized biomaterials developed opens the opportunity for a sustainable use of resources obtained from vegetal residues that would become wastes. The vegetable scraps of different machining operations can become valuable sources of biomolecules whose activities are useful in various sectors of impact to the health and to the economy.

References

[1] P. Stephens, Antibiotic Resistance Now 'global Threat', WHO Warns, 2014, http://www.bbc.com/news/health-27204988 (Accessed 20 April 2016).
[2] M. Rai, A. Yadav, A. Gade, Silver nanoparticles as a new generation of antimicrobials, Biotechnol. Adv. 27 (2009) 76–83.
[3] X. Chen, H.J. Schluesener, Nanosilver: a nanoproduct in medical application, Toxicol. Lett. 176 (2008) 1–12.
[4] S.W.P. Wijnhoven, W.J.G.M. Peijnenburg, C.A. Herberts, W.I. Hagens, A.G. Oomen, E.H.W. Heugens, B. Roszek, J. Bisschops, I. Gosens, D. van de Meent, S. Dekkers, W.H. De Jong, M. van Zijverden, A.J.A.M. Sips, R.E. Geertsma, Nano-silver – a review of available data and knowledge gaps in human and environmental risk assessment, Nanotoxicology 3 (2009) 109–138.
[5] J.T. Seil, T.J. Webster, Antimicrobial applications of nanotechnology: methods and literature, Int. J. Nanomed. 7 (2012) 2767–2781.
[6] M.J. Hajipour, K.M. Fromm, A.A. Ashkarran, D. Jimenez de Aberasturi, I. Ruiz de Larramendi, T. Rojo, V. Serpooshan, W.J. Parak, M. Mahmoudi, Antibacterial properties of nanoparticles, Trends Biotechnol. 30 (2012) 499–511.
[7] M. Ocwieja, Z. Adamczyk, M. Morga, K. Kubiak, Silver particle monolayers – formation stability, applications, Adv. Colloid Interface Sci. 222 (2015) 530–563.
[8] S. Ahmed, M. Ahmad, B.L. Swami, S. Ikram, A review on plants extract mediated synthesis of silver nanoparticles for antimicrobial applications: a green expertise, JARE 7 (2016) 17–28.
[9] P. Rauwel, S. Kuunal, S. Ferdov, E. Rauwel, A review on the green synthesis of silver nanoparticles and their morphologies studied via TEM, Adv. Mater. Sci. Eng. 2015 (2015), Article ID 682749.
[10] V.K. Sharma, R.A. Yngard, Y. Lin, Silver nanoparticles: green synthesis and their antimicrobial activities, Adv. Colloid Interface Sci. 145 (2009) 83–96.
[11] M. Ramya, S. Subapriya, Green synthesis of silver nanoparticles, Int. J. Med. Biol. Sci. 1 (2012) 54–61.
[12] G.A. Martinez-Castanon, N. Nino-Martinez, F. Martinez-Gutierrez, J.R. Martinez-Mendoza, F. Ruiz, Synthesis and antibacterial activity of silver nanoparticles with different sizes, J. Nanopart. Res. 10 (2008) 1343–1348.
[13] S. Kheybari, N. Samadi, S.V. Hosseini, A. Fazeli, M.R. Fazeli, Synthesis and antimicrobial effects of silver nanoparticles produced by chemical reduction method, DARU 18 (2010) 168–172.
[14] A.A. El-Kheshen, S.F.G. El-Rab, Effect of reducing and protecting agents on size of silver nanoparticles and their antibacterial activity, Der Pharma Chem. 4 (2012) 53–65.
[15] S. Panigrahi, S. Kundu, S.K. Ghosh, S. Nath, T. Pal, General method of synthesis for metal nanoparticles, J. Nanopart. Res. 6 (2004) 411–414.
[16] S.N. Barnaby, S.M. Yu, K.R. Fath, A. Tsiola, O. Khalpari, I.A. Banerjee, Ellagic acid promoted biomimetic synthesis of shape-controlled silver nanochains, Nanotechnology 22 (2011) 225605, 10 pp.
[17] J.A. Jacob, H.S. Mahal, N. Biswas, T. Mukherjee, S. Kapoor, Role of phenol derivatives in the formation of silver nanoparticles, Langmuir 24 (2008) 528–533.
[18] P. Dauthal, M. Mukhopadhyay, In-vitro free radical scavenging activity of biosynthesized gold and silver nanoparticles using Prunusarmeniaca (apricot) fruit extract, J. Nanopart. Res. 15 (2013) 1366, 11 pp.
[19] A. Panacek, L. Kvitek, R. Prucek, M. Kolar, R. Vecerova, N. Pizurova, V.K. Sharma, T. Nevecna, R. Zboril, Silver colloid nanoparticles: synthesis, characterization and their antibacterial activity, J. Phys. Chem. B 110 (2006) 16248–16253.
[20] K.M. Kumar, M. Sinha, B.K. Mandal, A.R. Ghosh, K.S. Kumar, P.S. Reddy, Green synthesis of silver nanoparticles using terminalia chebuls extract at room temperature and their antimicrobial studies, Spectrochim. Acta Part A 91 (2012) 228–233.
[21] Q. Sun, X. Cai, J. Li, M. Zheng, Z. Chen, C.-P. Yu, Green synthesis of silver nanoparticles using tea leaf extract and evaluation of their stability and antibacterial activity, Colloids Surf. A: Physiochem. Eng. Aspects 444 (2014) 226–231.
[22] R. Aladpoosh, M. Montazer, N. Samadi, In situ green synthesis of silver nanoparticles on cotton fabric using Sidlitzia Rosmarinus ashes, Cellulose 21 (2014) 3755–3766.
[23] Z. Liu, J. Yan, Y.-E. Miao, Y. Huang, T. Liu, Catalytic and antibacterial activities of green-synthesized silver nanoparticles on electrospunpolystirene nanofiber membranes using tea polyphenols, Compos. Part B 79 (2015) 217–223.
[24] Z. Wang, C. Xu, X. Li, Z. Liu, In situ green synthesis of Ag nanoparticles on tea polyphenols-modified graphene and their catalytic reduction activity on 4-nitrophenol, Colloids Surf. A 485 (2015) 102–110.
[25] W. Cao, L.L. Hench, Bioactive materials, Ceram. Int. 22 (1996) 493–507.
[26] S.M. Rabiee, N. Nazparvar, M. Azizian, D. Vashaee, L. Tayebi, Effect of ion substitution on properties of bioactive glasses: a review, Ceram. Int. 41 (2015) 7241–7251.
[27] H.M. Kim, F. Miyaji, T. Kokubo, C. Ohtsuki, T. Nakamura, Bioactivity of Na_2O-CaO-SiO_2 glasses, J. Am. Ceram. Soc. 78 (1995) 2405–2411.
[28] X. Zhang, S. Ferraris, E. Prenesti, E. Vernè, Surface functionalization of bioactive glasses with natural molecules of biological significance, part II: grafting of polyphenols extracted from grape skin, Appl. Surf. Sci. 287 (2013) 341–348.
[29] M. Cazzola, I. Corazzari, E. Prenesti, E. Bertone, E. Vernè, S. Ferraris, Bioactive glass coupling with natural polyphenols: surfacemodification, bioactivity and anti-oxidant ability, Appl. Surf. Sci. 367 (2016) 237–248.
[30] X. Zhang, S. Ferraris, E. Prenesti, E. Vernè, Surface functionalization of bioactive glasses with natural molecules of biological significance, part I: gallic acid as model molecule, Appl. Surf. Sci. 287 (2013) 329–340.
[31] E. Vernè, C. Vitale-Brovarone, E. Bui, C.L. Bianchi, A.R. Boccaccini, Surfacefunctionalization of bioactive glasses, J. Biomed. Mater. Res. 90A (2009) 981–992.
[32] E. Vernè, S. Ferraris, C. Vitale-Brovarone, S. Spriano, C.L. Bianchi, A. Naldoni, M. Morra, C. Cassinelli, Alkaline phosphatase grafting on bioactive glasses andglass–ceramics, Acta Biomater. 6 (2010) 229–240.
[33] S. Ferraris, X. Zhang, E. Prenesti, I. Corazzari, F. Turci, M. Tomatis, E. Vernè, Gallic acid grafting to a ferrimagnetic bioactive glass-ceramic, J. Non-Cryst. Solids 432 (2016) 167–175.
[34] V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic – phosphotungstic acid reagents, Am. J. Enol. Vitic. 16 (1965) 144–158.
[35] A. Cochis, L. Fracchia, M.G. Martinotti, L. Rimondini, Biosurfactants prevent in vitro Candida albicans biofilm formation on resins and silicon materials for prosthetic devices, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. 113 (2012) 755–761.
[36] A. Cochis, B. Azzimonti, C. Della Valle, R. Chiesa, C.R. Arciola, L. Rimondini, Biofilm formation on titanium implants counteracted by grafting gallium and silver ions, J. Biomed. Mater. Res. A 103 (2015) 1176–1187.
[37] A. Cochis, B. Azzimonti, C. Della Valle, E. De Giglio, N. Bloise, L. Visai, S. Cometa, L. Rimondini, R. Chiesa, The effect of silver or gallium doped titanium against the multidrug resistant Acinetobacter baumannii, Biomaterials 80 (2016) 80–95.
[38] B. Azzimonti, A. Cochis, M.E. Beyrouthy, M. Iriti, F. Uberti, R. Sorrentino, M.M. Landini, L. Rimondini, E.M. Varoni, Essential oil from berries of lebanese Juniperusexcelsa M. Bieb displays similar antibacterial activity to chlorhexidine but higher cytocompatibility with human oral primary cells, Molecules 20 (2015) 9344–9357.
[39] M. Textor, C. Sittig, V. Frauchiger, S. Tosetti, D.M. Brunette, Properties and bio-logical significance of natural oxide films on titanium and its alloys, in: M. Tengvall (Ed.), Titanium in Medicine, Springler-Verlag, Berlin, Heidelberg, New York, NY, 2001, pp. 171–230.
[40] M. Morra, C. Cassinelli, G. Buzzone, A. Carpi, G. DiSanti, R. Giardino, M. Fini, Surface chemistry effects of topographic modification of titanium dentalimplant surfaces: 1. Surface analysis, Int. J. Oral Maxillofac. Implants 18 (2003) 40–45.
[41] M.P. Ferraz, F.J. Monteiro, J.D. Santos, CaO–P2O5glass hydroxyapatite doublelayer plasma spray coating: in vitro bioactivity evaluation, J. Biomed. Mater. Res. 45 (1999) 376–383.
[42] X-Ray Photoelectron Spectroscopy Reference pages, C1s Carbonates: http://www.xpsfitting.com/2011/03/c-1s-carbonates.html, Copyright M.C. Biesinger (http://www.xpsfitting.com/) 2013 (8 May 2013, 11:14 a.m.).
[43] E. Vernè, S. Ferraris, C. Cassinelli, A.R. Boccaccini, Surface functionalization of bioglass® with alkaline phosphatase, Surf. Coat. Technol. 264 (2015) 132–139.
[44] A.V. Naumkin, A. Kraut-Vass, S.W. Gaarenstroom, C.J. Powell, NIST X-ray photo-electron spectroscopy database, in: NIST Standard Reference Database 20, Version 4.1, 2013, http://srdata.nist.gov/xps/selEnergyType.aspx (8 May 2013, 11:20 a.m.), 2012 copyright by the U.S. Secretary of Commerce on behalf of the UnitedStates of America. All rights reserved.
[45] J.F. Mowlder, W.F. Stickle, P.E. Sobol, K.D. Bomben, Handbook of X-ray photo-electron spectroscopy, in: A Reference Book of Standard Spectra for Identification and Interpretation of XPS Data, Physical Electronics, USA, 1995.
[46] G. Qiao, J. Su, M. He, Effect of (−) epigallocatechin gallate on electrochemicalbehavior and surface film composition of Co–Cr alloy used in dental restora-tions, Dent. Mater. J. 31 (2012) 564–574.
[47] M. Ferraris, S. Ferraris, M. Miola, S. Perero, C. Balagna, E. Verne, G. Gautier, C. Manfredotti, A. Battiato, E. Vittone, G. Speranza, I. Bogdanovic, Effect of thermal treatments on sputtered silver nanocluster/silica composite coatings on soda-lime glasses: ionicexchange and antibacterial activity, J. Nanopart. Res. 14 (2012) 1287.

[48] S. Ferraris, A. Venturello, M. Miola, A. Cochis, L. Rimondini, S. Spriano, Antibacterial and bioactive nanostructured titanium surfacesfor bone integration, Appl. Surf. Sci. 311 (2014) 279–291.
[49] B. Reidy, A. Haase, A. Luch, K.A. Dawson, I. Lynch, Mechanisms of silver nanoparticle release, transformation and toxicity: a critical review of current knowledge and recommendations for future studies and applications, Materials 6 (2013) 2295–2350.
[50] G.A. Sotiriou, S.E. Pratsinis, Antibacterial activity of nanosilver ions and particles, Environ. Sci. Technol. 44 (2010) 5649–5654.
[51] S. Ferraris, S. Spriano, Antibacterial titanium surfaces for medical implants, Mat. Sci. Eng. C 61 (2016) 965–978.
[52] J.L. Graves Jr., M. Tajkarimi, Q. Cunningham, A. Campbell, H. Nonga, S.H. Harrison, J.E. Barrick, Rapid evolution of silver nanoparticle resistance in Escherichia coli, Front. Genet. 6 (2015) 42.