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In situ reduction of silver nanoparticles on bioactive glasses functionalized with polyphenols

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Abstract

The realization of surfaces with antibacterial properties decorated with silver nanoparticles through a green approach is a promising research challenge.

In this research work two different bioactive glasses have been surface functionalized with polyphenols (gallic acid and natural polyphenols extracted from red grape skins and green tea leaves) and silver nanoparticles deposited on their surface by in situ reduction from a silver nitrate solution. The presence of biomolecules and silver nanoparticles has been investigated by means of UV-Vis spectroscopy, X Ray Photoelectron Spectroscopy (XPS) and Field Emission Scanning Electron Microscopy (FESEM). Antibacterial activity of modified surfaces has been verified against *S. aureus*.

Keywords: bioactive glasses, polyphenols, silver nanoparticles, in situ reduction

1. Introduction

Silver is known from ancient times for its broad spectrum antibacterial activity and widely investigated as multi-purpose antibacterial agent. The raising diffusion of bacterial resistance to common antibiotics, recently defined as global threat [R], 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 [I1, I2] and numerous silver nanoparticles-loaded products come into the market in various application fields (e.g. personal care/cosmetics, textiles/shoes, electronics, household products, filtration/sanitization, medical) [II]. Despite the wide diffusion of nanosilver, its potential toxicity for health and environment is not completely known up to now [II]. The antibacterial mechanism of silver nanoparticles is not yet fully understood, however a multiple action has been proposed: interaction with the bacterial cell wall and its consequent damage, induction of oxidative stress by ROS (Reactive Oxygen Species) production and sustained release of silver ions [I3, I4]. Thanks to the high surface to volume ratio and the multiple mode of action silver nanoparticles present superior antibacterial activity compared to both 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) [I5, I6]. The main drawbacks of common synthesis routes for silver nanoparticles is the

employment of toxic chemicals, high temperature, pressure and energy, so new green and environmentally friendly strategies are needed. Various green synthesis approaches have been investigated such as the use of microorganisms (bacteria, fungi, yeasts, algae), polysaccharides and plants extracts [I6, I7, I8, I9]. 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 [I6]. 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.

Numerous experimental strategies have been reported in the scientific literature for the green synthesis of colloidal silver nanoparticles [I6-I19] but few works consider in situ reduction of silver nanoparticles on substrates [I20, I21, I22].

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 (model molecule) and with polyphenols from red grape skin and green tea leaves extracts. The effect of glass surface reactivity on the ability to graft biomolecules and subsequently to induce in situ reduction of silver ions has been investigated by means of UV-Visible, X Ray Photoelectron Spectroscopy (XPS) and Field Emission Scanning Electron Microscopy (FESEM). The antibacterial activity of Ag-NPs doped bioactive glasses has been evaluated against *S. aureus*.

This research work presents a promising strategy to obtain innovative smart biomaterials which combine the peculiar features of bioactive glasses (bioactivity, ion release), polyphenols (antioxidant, antibacterial, bone stimulating) and silver (antibacterial). In this route silver nanoparticles results embedded on the glass surface reducing risk concerns related to free metallic nanoparticles.

2. Materials and Methods

2.1 Glass synthesis

Two different bioactive glasses, designed in the authors laboratories, were considered: SCNA and SCN1. The molar composition and melting/annealing conditions are reported in Table 1. SCNA is a highly stable glass due to the presence of alumina among its constituents 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) as model molecule and polyphenols extracted from red grape skin (GPH) and green tea leaves (TPH) were considered for the surface functionalization.

Gallic acid was purchased from Sigma Aldrich (GA 97.5–102.5% titration, G7384, Sigma Aldrich) while natural polyphenols were extracted by conventional solvent extraction method, as previously described by the authors in [F2, F3].

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 [F2, F3, F1, F4, F5]. Washed samples will be named SCNA-wash and SCN1-wash (Table 2). The grafting of biomolecules was performed by soaking washed glasses in a solution of polyphenols (1mg/ml for GA and TPH and 5 mg/ml for GPH) for 3h at 37°C [F3, F6]. 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 will be 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 samples were soaked 1h at 37°C in a 0.005M $AgNO_3$ solution in order to obtain the in situ reduction of silver nanoparticles (Ag NPs) on the glass surface exploiting the reducing action of 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 will be 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 will be named SCNA-wash+Ag and SCN1-wash+Ag (Table 2).

2.4 Physico-chemical characterization

Photometric measurements in the UV-visible spectral region (CARY 500 Varian) were performed in order to quantify the amount of active polyphenols on the glass surface by means of the Folin&Ciocalteu method [FC] as previously described by the authors [F3, F6]. A standard calibration curve was obtained with GA solutions of known concentration and used for GA quantification on the samples [F1, F2, F3, F6]. As far as natural polyphenols are concerned the concentration was calculated in gallic acid equivalents according to the same calibration curve [F2, F3].

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 (< 5nm) before analyses.

2.5 Antibacterial tests

SCNA and SCN1 samples functionalized with polyphenols extracted from green tea leaves and the same after in situ reduction of silver nanoparticles 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 (Sintak S.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 and planktonic bacterial cells

Specimens were placed into the wells of a 12 multiwell (Nunc Delta, Nunclone). 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 $1x10^7$ cells mL^{-1} according to McFarland standard 1.0. One mL of the broth culture was collected and used to contaminate specimens; plate was incubated at 37°C in rotation (90 rpm) for 90 minutes (adhesion phase). The supernatant containing planktonic cells was then removed, while biofilm cells, attached to the specimens' surfaces, were rinsed with 1 mL of fresh LB medium (separation phase). Plate was incubated 24 h at 37°C in a humid atmosphere to allow mature biofilm growth.

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 (3mg 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. Experiments were performed in triplicate.

2.6 Statistical analysis

All tests have been performed in triplicate. One-way ANOVA, with a significance level $p<0.05$, has been applied for statistical analysis of the data.

3. Results and discussion

3.1 Photometric determination of the phenol content on the glass surface

The results of the Folin&Ciocalteu test on glass samples are reported in Table 3.

It was not possible 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 [F1, F2] 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 allow a more effective grafting of biomolecules, except for GPH.

3.2 XPS analyses

Atomic percentages of elements from survey spectra are reported in Table4.

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 its precipitation. 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) will be investigated by the detailed analysis of Ag region in the following.

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

On the washed samples (Figure 1a and 1e) 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 [C1-C4]. Surface contaminations by atmospheric hydrocarbons are always present onto reactive surfaces [C1-C3] and have been already observed, together with carbonates, on bioactive glasses by the authors [F1-F3, C5]. A small signal at about 287 eV can be also detected on SCN1-wash, C-O bonds can correspond to this energy, but can come from contaminants on this sample.

The signal of carbonates disappears after surface functionalization, as previously observed by the authors [F1-F3]. A significant signal at about 286.8 eV appears on all the polyphenols grafted glasses (Figure 1b, 1c, 1d, 1f, 1g and 1h) and can be attributed to C-O bonds in polyphenols molecules [P1, P2, P3]. This signal has been previously observed on polyphenols grafted bioactive glasses by the authors [F1-F3]. A moderate contribution in the 288 eV around can be observed on SCN1 samples after polyphenols grafting. It can be attributed to C=O bonds [P1-P3] in carboxylic groups of polyphenols or in polyphenols oxidized to quinones, as previously observed by the authors [F1-F3]. The appearance of 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 detailed analysis of silver region for Ag-treated samples is reported in Figure 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) [Ag1, Ag2]. 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) [Ag1, Ag2]. 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 [F3].

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 Figure 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 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 (Figure 4).

A moderate reaction layer can be observed on SCNA samples after the various modification processes as an irregular texture (Figure 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 (Figure 1). 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 tests

Washed, TPH grafted and TPH grafted and Ag treated samples were considered for the antibacterial tests in order to evaluate the effect of both natural polyphenols and silver nanoparticles on bacterial viability.

The results of antibacterial tests are reported in Figure 5.

A first moderate (11-13%), but statistically significant ($p<0.05$), reduction in bacterial viability has been induced by tea polyphenols grafting. An increase in the antibacterial activity of the samples (35-36% reduction in bacterial viability) has been obtained after the in situ reduction of silver nanoparticles.

No significant differences have been observed between the two glasses.

Conclusion

Gallic acid and polyphenols extracted from red grape skin and green tea leaves have been successfully grafted on two bioactive glasses with different reactivity. The ability to graft biomolecules increases with the glass surface reactivity. Grafted biomolecules, but in a certain measure also surface hydroxyl groups, make possible the reduction of silver ions to silver nanoparticles on the glass surface. Bioactive glasses functionalized with tea polyphenols and, more evidently, after in situ reduction of silver nanoparticles present an antibacterial activity against *S. aureus*.

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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

Table 2: Samples names and treatments

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+GA grafting+in situ reduction Ag NPs	GA molecules/Ag NPs
SCNA+GPH+Ag	Acetone and water washing+GPH grafting+in situ reduction Ag NPs	GPH molecules/Ag NPs
SCNA+TPH+Ag	Acetone and water washing+TPH grafting+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+GA grafting+in situ reduction Ag NPs	GA molecules/Ag NPs
SCN1+GPH+Ag	Acetone and water washing+GPH grafting+in situ reduction Ag NPs	GPH molecules/Ag NPs
SCN1+TPH+Ag	Acetone and water washing+TPH grafting+in situ reduction Ag NPs	TPH molecules/Ag NPs

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

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								
Element	**wash**	**wash+Ag**	**GA**	**GA+Ag**	**GPH**	**GPH+Ag**	**TPH**	**TPH+Ag**
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

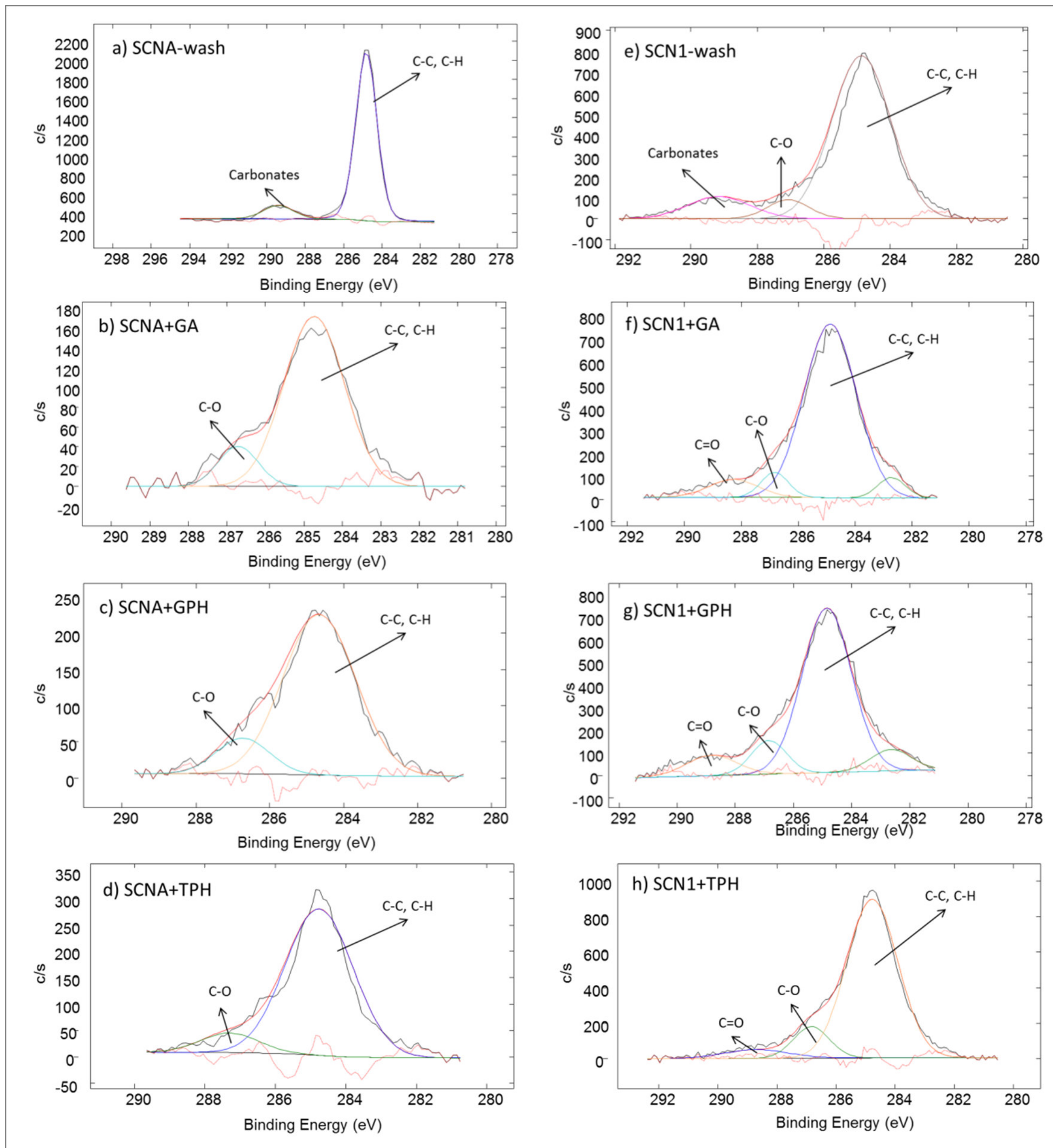

Figure 1: XPS high resolution spectra of carbon region

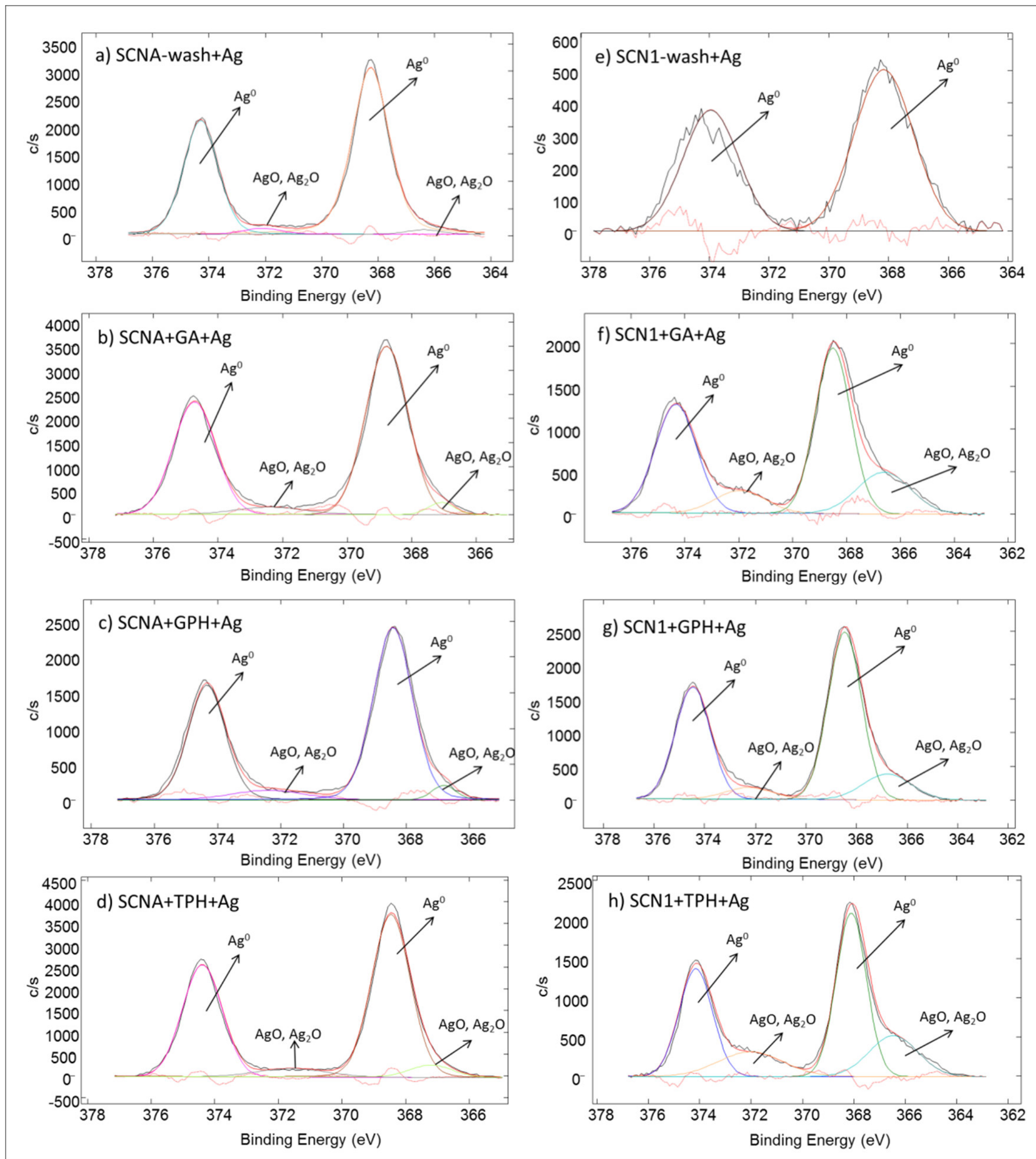

Figure 2: XPS high resolution spectra of silver region

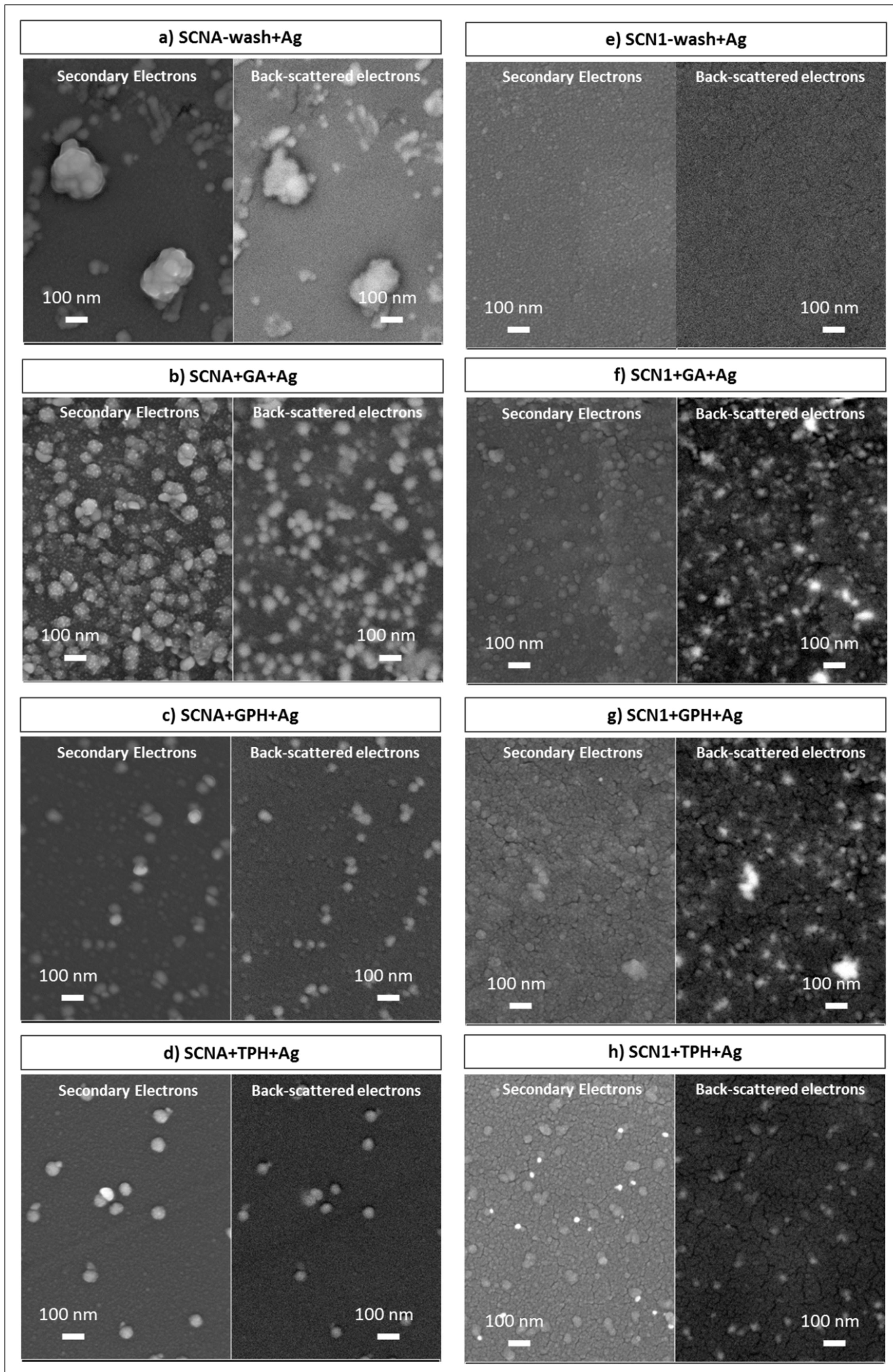

Figure 3: FESEM images (secondary and back-scattered electrons) of samples (200000x magnification)

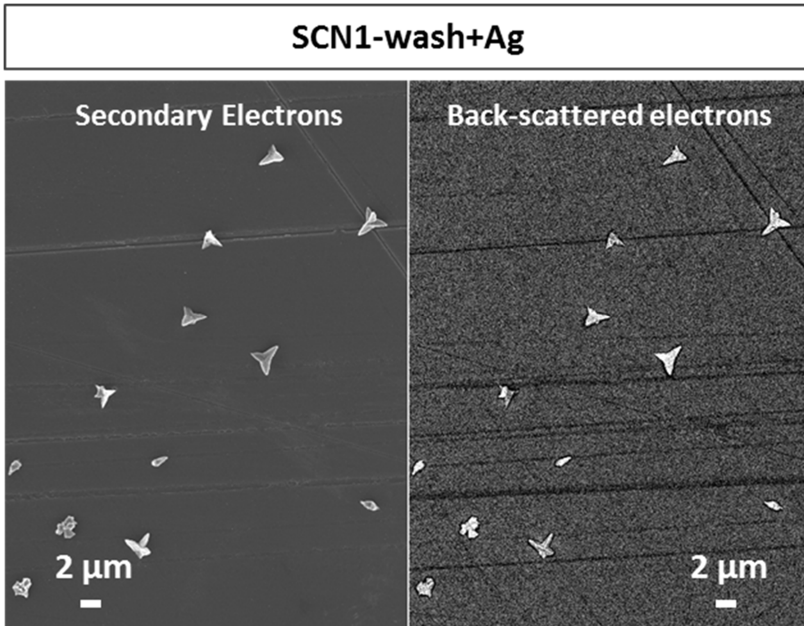

Figure 4: FESEM image (secondary and back-scattered electrons) of SCN1-wash+Ag (5000x magnification)

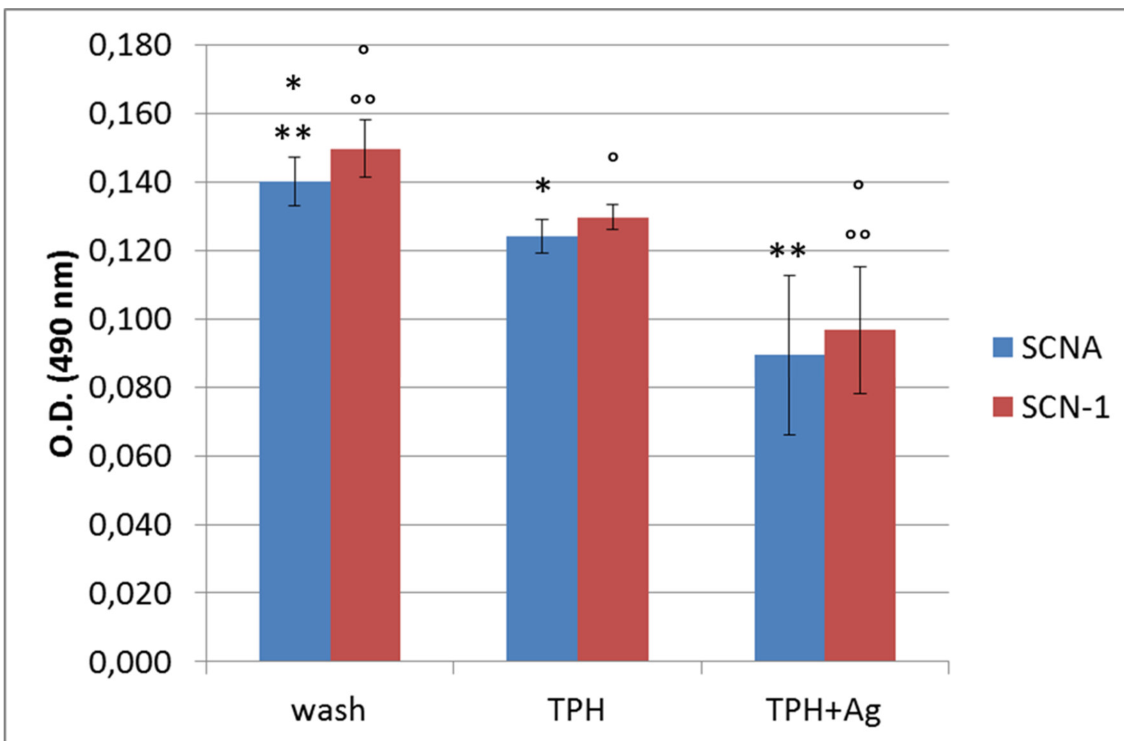

Figure 5: Bacterial viability on the tested samples

Graphical abstract