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Abstract

Ferrimagnetic bioactive glass ceramics are promising biomaterials in the field of bone substitution and cancer treatment for their ability to bond to bone (bioactive behavior) and to be heated by the application of an external magnetic field (hyperthermia). Surface functionalization of these materials with polyphenols is a challenging and innovative strategy in order to impart them additional functional and specific properties (e.g. antioxidant, anticancer and antibacterial). Gallic acid (GA) is a phenolic acid which can be considered a good model molecule for polyphenols due to its simple structure and representative properties. In the present paper GA has been grafted to a ferrimagnetic glass ceramic (SC-45), in bulk and powder forms, in view of its potential clinical applications (such as hyperthermic treatment of cancer combined with the anticancer action of GA). The grafting process has been optimized in order to preserve GA activity. The effectiveness of the functionalization procedure has been demonstrated by means of Scanning Electron Microscopy equipped with Energy dispersive Spectroscopy (SEM-EDS), X-ray Photoelectron Spectroscopy (XPS), Thermogravimetric analysis-gas evolved analysis (TGA-EGA) and Folin&Ciocalteu tests (F&C). Release tests have been performed in double distilled water at 37°C and 43°C to verify the stability of the material.

Keywords: Ferrimagnetic bioactive glass ceramics , gallic acid, surface functionalization, cancer treatment, hyperthermia

Highlights

Gallic acid (GA) was grafted to a ferrimagnetic glass ceramic (SC-45)

The grafting process was optimized in order to preserve GA redox activity

SEM-EDS, XPS, TGA-EGA and F&C tests confirmed GA presence on SC-45 surface.

Introduction

Bioactive glasses and glass-ceramics are particular biomaterials able to allow the growth of hydroxyapatite layer on their surface. After implantation, these materials react with physiological fluids by means of a series of ion exchange reactions that culminate with the precipitation of calcium phosphates and the crystallization of hydroxyapatite on the biomaterial surface [1, 2]. In the biological environment a series of

biological reactions (absorption of biological molecules, action of macrophages, attachment and differentiation of stem cells, osteoblasts proliferation, generation and crystallization of matrix) follow the inorganic ones and lead to new bone formation [1, 2]. The structure of these materials make possible to obtain many different compositions with peculiar and tailored properties. Varying the silica content it is possible to modulate the glass bioactivity from almost inert to highly bioactive and substituting SiO_2 with P_2O_5 it is possible to obtain fully resorbable structures [3]. The introduction of different modifying oxides allows the release of ions with specific stimulation ability for cells: Ca^{++} for osteoblast proliferation, differentiation, and mineralization, Mg^{++} for cellular adhesion and bone formation, Zn^{++}, Cu^{++}, Ag^{+} for an antibacterial action, to cite only some examples [4, 5]. The effect of different ions on the bone bonding and antibacterial properties of bioactive glasses have recently been reviewed [6, 7]. It has been observed that bioactive glasses and their dissolution products can stimulate bone regeneration by affecting some cellular functions at the genetic level [8].

Thanks to their ability to induce the formation of an hydroxyapatite layer and to stimulate the formation of new bone tissues bioactive glasses and glass-ceramics have been historically intended for orthopedic and dental applications. In the last years their ability to bond to soft tissues, to act as carriers for drugs and bioactive molecules, to exploit specific functional properties based on their composition and crystalline phases suggested the possibility of their application in different fields such as soft tissues regeneration, wound healing, cancer therapy and local delivery [9, 10]

The authors previously developed a ferrimagnetic glass-ceramic (SC-45) [11-13] characterized by magnetite crystals embedded in an amorphous bioactive matrix (analogous to Bioglass®). The presence of magnetic crystals make possible to heat the material by the application of an external magnetic field. This peculiar feature allows the application of this glass-ceramic for hyperthermia in cancer treatment. Moreover in a previous research work the grafting of chemotherapeutic drugs has been considered in order to couple hyperthermic therapy with pharmacological one [13]. More recently, powders of SC-45 glass-ceramic have been used as dispersed phase to develop a new composite PMMA-based bone cement with bioactive and ferrimagnetic properties [14].

Polyphenols are a class of natural molecules, present in many plants and vegetable-based foods and beverages. An increasing interest in their study can be registered in the last years mainly due to the potential health benefits associated with these substances, such as anti-oxidant, anti-carcinogenic, antibacterial, anti-inflammatory, cardio- and neuro-protective effects [15-18]. An inhibitory effect of various polyphenols (including gallic acid) on molecular mechanisms associated to chronic inflammation, tumor genesis, progression, invasion and metastasis, have been documented *in vitro* and *in vivo* [19-21]. Gallic acid (3,4,5-trihydroxybenzoic acid) is a simple and small molecule belonging to phenolic acids and it can be considered representative for this class of molecules. A pro-apoptotic effect of gallic acid has been

evidenced against several cancer cells lines, such as lung cancer cells [22], HeLa cervical cancer cells [23], prostate carcinoma DU145 cells [24] and oral cancer cells [25]. A preferential pro-apoptotic effect of gallic acid against cancer cells compared to healthy ones, has been observed in vitro [23, 25]. The ability of this molecule to inhibit the growth and progression of prostate cancer after oral administration has been observed in a mouse model [26].

Despite of a wide research on the effects of the pure molecule on *in vitro* and *in vivo* models, few attempts of its coupling with artificial carriers have been reported. Combination of gallic acid with chitosan [27-29], dendrimers [30], Mg/Al layered double hydroxide [31], magnetite and gold nanoparticles [32, 33] can be cited as examples.

In a previous paper [34] the authors reported the possibility to effectively graft gallic acid to bioactive surface of glasses with different bioactivity. In the present research work, for the first time, the authors report gallic acid grafting to a ferrimagnetic bioactive glass-ceramic. The presence of a crystalline phase (magnetite) and of iron ions affect both the application of the modified materials (specifically intended for cancer treatment) and also the physical-chemical characteristic of the surface and the consequent results of the functionalization process.

This paper aims to functionalize with gallic acid (GA) and characterize a bioactive and ferrimagnetic glass-ceramic in view of its potential clinical applications, as that of hyperthermic treatment of cancer combined with the anticancer action of GA. The surface grafting of GA to SC-45 glass ceramic (in bulk and powder forms) has been optimized. The modified surfaces have been characterized from the morphological and chemical points of view, by way of scanning electron microscopy, X-ray Photoelectron Spectroscopy, Folin&Ciocalteu test and Thermogravimetric analyses, in order to determine the presence and redox reactivity of GA. Release tests have also been performed in order to evaluate the eventual molecular release. The antioxidant/pro-oxidant activity and selective cytotoxic behavior against cancer cells will be investigated and reported in future papers.

Materials and methods

SC-45 bioactive and ferrimagnetic glass-ceramic preparation

The bioactive and ferrimagnetic glass ceramic (SC-45), with the composition (wt %) 24.7 SiO_2, 13.5 Na_2O, 13.5 CaO, 3.3 P_2O_5, 14 FeO, and 31 Fe_2O_3, was prepared by traditional melt and quenching technique, as reported in [11-13]. In brief, commercial reagents (Na_2CO_3, $CaCO_3$, SiO_2, $Ca_3(PO_4)_2$, $FeSO_4*7H_2O$ and Fe_2O_3, >99.0%, Sigma Aldrich) were melted in a platinum crucible for 30 min at 1550°C and then poured on a brass plate. The heating rate applied in order to reach 1550°C was 10°C/min. A part of the obtained glass

ceramic was annealed 12h at 600°C and then cut and polished for obtaining bulk samples (SC-45 bulk). Another part was ball milled and sieved up in order to obtain powders with a grain size below 20 μm (SC-45 pow).

Surface activation and functionalization

In order to expose surface reactive hydroxyl groups (-OH) a water based pretreatment, previously optimized [13] for this specific glass-ceramic was applied. Each SC-45 sample, both bulk (slices 0.38±0.07 g in weight and 98±3 mm^2 of exposed area) or powders (0.10 g, 1.8 m^2/g) were soaked in 10 ml of double distilled water at 37°C for 1 week. At the end of the soaking period, the samples were let dry under a laminar flow cabinet (FASTER CYTOSAFE) in order to avoid surface contamination.

Various experimental conditions were considered for GA grafting, in order to find the optimal conditions able to preserve the biomolecule activity and to obtain an effective interaction with the glass-ceramic:

- GA grafting for 24h at 37°C (GA 24h@37°C),
- GA grafting for 24h at 37°C in citric acid-sodium citrate buffer (GA+buf 24h@37°C),
- GA grafting for 3h at 37°C (GA 3h@37°C),
- GA grafting for 3h at 37°C with citric acid addition (GA+CA 3h@37°C),
- GA grafting for 24h at 37°C with citric acid addition (GA+CA 24h@37°C).

For all the experiments a 1 mg/ml solution of gallic acid (GA 97.5-102.5% titration, G7384, Sigma Aldrich) was employed, as described in [34,35]. Except for the citric acid-sodium citrate buffer case (GA+buf 24h@37°C), GA was dissolved in double distilled water. In the (GA+buf 24h@37°C) case GA was dissolved in sodium citrate buffer at pH 3.0. Citric acid-sodium citrate buffer was prepared mixing 82 ml of 0.1 M citric acid and 18 ml of 0.1 M sodium citrate, as described in [36]. The addition of citric acid in experimental setups (GA+CA 3h@37°C) and (GA+CA 24h@37°C) was performed drop by drop in the aqueous solution of GA up to pH 3.0.

The employment of citric acid-sodium citrate buffer and the addition of citric acid were considered in order to avoid an excessive pH increase of GA solutions during functionalization and avoid its degradation [37,38]. In fact, the glass ceramic can induce a significant increase in the solution pH, due to ionic exchange, during the functionalization period [13].

At the end of the functionalization period, samples (both bulks and powders) were gently washed two times with double distilled water and let dry under a laminar flow cabinet.

All the containers for samples functionalization and storage were covered with aluminum foils in order to avoid light induced degradation of GA.

The pH of the functionalization solutions was measured at different steps of the treatment and their colors registered in order to evaluate possible alterations of GA.

Surface characterizations

The surface morphology and semi-quantitative chemical composition of bulk samples were investigated by Scanning Electron Microscopy equipped with Energy Dispersive Spectroscopy (SEM-EDS, SEM-FEI, Quanta Inspect 200, EDS-EDAX PV 9900), after sputter coating of the samples with Cr.

The chemical composition of the outermost surface layer was investigated by means of X-ray Photoelectron Spectroscopy (XPS, PHI 5000 VERSAPROBE, PHYSICAL ELECTRONICS) on bulk samples. Both survey spectra and high resolution spectra of carbon and oxygen regions were acquired in order to evaluate GA presence on functionalized samples.

Folin & Ciocalteu quantification of GA in solutions and on samples surface

The Folin & Ciocalteu (F&C) method [39] was employed for the quantification of GA both in the functionalization solutions and on sample surfaces. The method, widely employed for the total phenols quantification, determines the GA content by monitoring the reaction between GA, in the sample, and phosphotungstic/phophomolybdic acids, contained in the F&C reagent [34,39]. The principle of the test is based on the reduction ability of GA and the test signal is a consequence of a redox reaction of which GA is the limiting factor. As far as functionalization solutions are concerned, 2 ml of the test solution were mixed with 6 ml of double distilled water, 0.5 ml of F&C reagent (Folin & Ciocalteu phenol reagent 2 M with respect to acid, 47641, Sigma Aldrich) and 1.5 ml of Na_2CO_3 (20% w/V) and allowed to react for 2h before photometric measurements.

In order to detect the GA on SC-45 surface a modified F&C test was developed. Functionalized samples (bulk or powders) were immersed in 8 ml of double distilled water and added with 0.5 ml of F&C reagent and 1.5 ml of Na_2CO_3 (20% w/V) and allowed to react for 2h. Before photometric tests, solid samples were removed and the solution analyzed.

The amount of GA was determined in accordance to a standard calibration curve obtained as fully described in [34].

Gallic acid solutions have been tested by means of the Folin&Ciocalteu method before and after 37°C storage in the functionalization conditions in order to verify the maintenance of GA activity. The results confirm that GA is able to preserve its activity after 37°C incubation.

GA release

GA release from functionalized samples (SC-45+GA(3h@37°C)) was investigated in double distilled water. SC-45 powder samples (0.1 g) were soaked in double distilled water (10 ml) at 37°C or 43°C for 1 d or 7 d. The two temperature values were considered in order to simulate physiologic and hyperthermic conditions. At each experimental time, both the release solution and sample surfaces were analyzed by the F&C method.

Thermogravimetric analysis-gas evolved analysis (TGA-EGA)

Thermogravimetry (TGA) was performed using a PerkinElmer (Waltham, MA, USA) instrument connected with a time resolved FTIR to analyze the weight loss of SC-45 (both SC-45 pretreated and SC-45+GA(3h@37°C)) and the composition of the moieties simultaneously released from the heated samples. For the TGA analysis, the ultramicrobalance Pyris 1 from PerkinElmer, sensitivity 0.1 μg, was employed. The samples were heated at the rate of 20 °C/min under N_2 atmosphere (35 cm^3 min^{-1}). After the heating ramp, the samples underwent an isothermal treatment at 800 °C under O_2 atmosphere (35 cm^3 min^{-1}) for 15 minutes to convert any carbonaceous species to CO_2. For each TGA experiment a relatively large sample amount (ca. 15 mg) was employed to optimize the released amount of gases. Fourier transform infrared (FTIR) analysis of the gas was carried out with a Spectrum 100 (PerkinElmer) spectrometer equipped with a 10 cm thermostatized gas cell over the wavenumber region 600−4000 cm^{-1}. Time/temperature-resolved infrared profiles of the evolved gases were obtained by integrating the region of interest for each molecular species investigated.

Results

pH data and macro observations

pH values of GA solutions before and after SC-45 functionalization in different conditions are reported in Table 1. A significant increase in the pH can be observed for the functionalization of GA 24h@37°C powder samples. An attempt to limit the pH increase was made using (i) a citrate buffer, (ii) the addition of citric acid or (iii) the reduction of the grafting time (3h). GA solution is colorless but it turns to a dark color after the incubation with SC-45 samples.

SC-45 samples are themselves brown-violet in color, due to the presence of magnetite crystals embedded in the amorphous matrix. No significant changes in their aspect can be evidenced after functionalization, except for the appearance of white precipitates on samples functionalized in citrate buffer. These samples resulted also extremely damaged and brittle after the functionalization.

SEM-EDS observations

Figure 1 reports SEM images and EDS analyses of SC-45 bulk samples after different surface treatments.

Magnetite crystals embedded in a glassy matrix can be clearly observed on the pretreated (1 week in water at 37°C) samples (Figure 1, SC-45 pretreated). EDS analysis individuates the main constituents of the glass ceramic; few carbon contaminations (always present on the surface of reactive materials [40-44]) are also detected. After GA grafting (24h@37°C) a thick reaction layer can be observed on the bulk SC-45 surface (Figure 1, SC-45+GA(24h@37°C)). EDS analysis reveals a significant carbon enrichment for this surface. An evident dissolution of the glass matrix can be observed after functionalization in citrate buffer (Figure 1, SC-45+GA+buf (24h@37°C)). Moreover numerous precipitates have been detected on these samples (images omitted for brevity). EDS and FTIR analyses (data not reported for brevity) on these precipitates suggest that they are constituted of calcium citrate. EDS analyses detect iron, oxygen and silicon on the surface free from precipitates, with a moderate amount of carbon.

XPS analyses

Table 2 reports the atomic percentages (obtained by XPS analyses) of elements detected on SC-45 bulk samples before and after GA grafting 24@37°C.

Figure 2 shows the high resolution spectra of oxygen region for SC-45 bulk samples before and after GA grafting 24@37°C

A significant increase in carbon and a notable reduction of iron, calcium and phosphorous can be detected on SC-45 surface after functionalization (Table 2)

Three main contributions at 530.58 eV, 531.58 eV and 533.69 eV can be detected in the high resolution spectrum of the oxygen region for the pretreated SC-45 sample (Figure 2a).

After GA grafting the low energy signal disappears, the one at about 531 eV is reduced and the one at about 532-533 eV is significantly increased (Figure 2b)

Folin&Ciocalteu quantification of gallic acid

The amounts of GA in the uptake solutions, before and after functionalization of SC-45 bulk samples 24h@37°C, are reported in figure 3a. A moderate lowering in the GA content results in the unbuffered uptake solution (GA SC-45 bulk 24h@37°C) but not for the citrate buffered one (GA+buf SC-45 bulk 24h@37°C).

Looking at the samples surface a significant amount of GA can be detected on SC-45+GA bulk 24h@37°C and a lower one on SC-45+GA+buf bulk 24h@37°C (Figure 3b).

Considering the high pH increase observed for GA solutions after 24h grafting at 37°C for powder samples and the bad results obtained with citrate buffer, further investigations on powder samples were performed with a reduction of the functionalization time to 3h (SC-45+GA (3h@37°C)) and optional addition of a small amount of citric acid for both 24h and 3h grafting (SC-45+GA +CA (24h@37°C) and SC-45+GA +CA (3h@37°C) respectively). Results of the F&C test for uptake solutions and powder samples are reported in figure 4.

A moderate reduction in the GA content can be registered in the uptake solution for all the tested grafting conditions. As far as powder samples are concerned (Figure 4b) GA is present on all the functionalized samples but its amount is higher for the SC-45+GA (3h@37°C). Considering these results, this experimental setup was considered for the further tests on powder samples.

GA release in double distilled water

The amount of GA released in water after 1 d and 7 d at 37°C and 43°C is reported in figure 5a, the residual GA on SC-45 surface in figure 5b. Table 3 reports the pH values of release solutions.

A very small amount of GA can be detected in water after 1d of soaking of SC-45+GA (3h@37°C) powder samples, and only traces can be detected after 7 days (Figure 5a). The amount of GA is lower for the 43°C test rather than that at 37°C. The amount of GA detected on the sample surfaces after soaking in water at 37°C is almost unchanged after 1 d and moderately lowered after 7 d. A sharp evident lowering in the GA concentration at the surface can be registered after soaking at 43°C.

pH measurements (Table 3) evidence a significant alkalization of the solution that is more evident after soaking for 7 days and at 43°C.

Thermogravimetric analysis-gas evolved analysis (TGA-EGA)

The thermal behavior of SC-45+GA (3h@37°C) and SC 45 pretreated was investigated in the temperature range of 30-800 °C. Each sample (ca. 20 mg) was heated to 800 °C at the rate of 20 °C/min under N_2 atmosphere to investigate possible desorption or pyrolytic processes occurring at the surface. When the temperature of 800 °C was reached, the atmosphere was switched to oxygen to fully oxidize both organic and metal species (i.e. iron) occurring in the samples. The gas evolved during the TGA was continuously monitored by FTIR evidencing that CO_2 and H_2O were evolved from both the samples during the heating ramp.

Figure 6A reports the thermograms recorded. Even though both SC 45+GA (3h@37°C) and SC-45 pretreated exhibited an overall weight loss of ca. 1.4 % during the heating ramp under nitrogen atmosphere, the thermograms differed the one from the other for some features. The first process observed was evidenced by a minimum on the derivative curve (figure 6B) at ca. 100 °C which was assigned to the loss of physisorbed water as evidenced by the FTIR analysis (figure 6C). An analogue process occurred also with SC 45 pretreated, but in this case the amount of the physisorbed water released (1 %) was higher than that observed with SC 45 GA (0.4%).

In the temperature range between ca. 250 to 700 °C, SC 45+GA (3h@37°C) underwent a second weight loss (0.7 %) which was not characterized by any distinct minimum peak on the derivative curve. During this process the evolution of CO_2, evidenced by the FTIR analysis, is consistent with the decarboxylation of gallic acid bound at the surface of the glass -ceramic. In the same temperature range, the thermal behavior of SC-45 pretreated was significantly different and a weight loss, evidenced by a well-defined minimum, occurred at a rather high temperature (ca. 650 °C) simultaneously with CO_2 release. Both the temperature at which the process occurred and CO_2 detected are consistent with the thermal decomposition of carbonates.

When the atmosphere was switched to O_2 , both the samples exhibited a gain of weight which is consistent with the oxidation of FeO to Fe_2O_3. The weight increase exhibited by SC-45 + GA ((3h@37°C) (0.9%) was significantly higher than that observed with SC-45-pretreated (0.5%). This result strongly indicates that the amount of iron (II) present in SC-45 + GA ((3h@37°C) was higher than that present in SC-45 pretreated suggesting that the interaction of GA can cause the reduction of the iron (III) of the glass-ceramic to iron (II). Moreover the FTIR analysis evidenced that during this process CO_2 and H_2O were released from both the samples. This result is consistent with the combustion of carbon containing species, possibly residues of degradation of GA on SC-45+GA (3h@37°C), and carbonates or organic contaminants on SC-45 pretreated. The FTIR thermogram recorded highlighted that the amount of both H_2O and CO_2 was very much higher for SC 45+GA (3h@37°C) (figure 6C and 6 D). This process was assigned to the combustion of a high amount of

the carbon containing species present on this sample. Such species were very likely the residual products of the pyrolysis of GA under N_2.

Discussion

GA has been grafted to SC-45 ferrimagnetic glass ceramic (in bulk and powder forms) considering different experimental procedures aimed to optimize molecular immobilization and minimize its degradation.

Solution pH and color have been evaluated in order to monitor possible alterations of GA during functionalization.

GA presence and redox activity have been determined by means of SEM-EDS, XPS and F&C analyses.

A certain alkalization of the GA uptake solution has been observed after SC-45 soaking (Table 1). It is more evident for long soaking time and for powder samples.

The change in pH can be attributed to the ion release of SC-45 glass ceramic in the functionalization solutions [13] and a similar behavior has been already observed by the authors for the surface functionalization of bioactive glasses with GA [34] and natural polyphenols [45]. A higher ion release is associated with powder samples due to their higher surface area compared to bulk ones.

Bioactive glasses and glass-ceramics release various ions, involved in the bioactivity and cell stimulation processes, when soaked in aqueous media, ad discussed in the introduction. This phenomenon has been confirmed by means of EDS and XPS analyses for SC-45 glass-ceramic in the present paper. A moderate amount of Na, Ca and Fe can be released in solution from SC-45 samples. Ion release contributes to the changes in pH for the functionalization media and release test solution (especially because unbuffered release media have been considered in this work) and consequently can affect GA activity when non properly controlled. Moreover the presence of iron can strongly interact with gallic acid molecules, as discussed in the following (TGA-EGA experiments). The Fe-GA interaction can affect the antioxidant-pro-oxidant activity of the polyphenol. An in depth study of this phenomenon will be discussed in a future paper.

Together with the pH also the color of the solution changes after sample soaking.

Color change can be interpreted in term of the redox reactivity of GA, whose oxidized form (preferentially formed in alkaline environments and in the presence of light) tends to yellow-brown color (quinone form). Moreover, the mentioned color can further turns to black due to complex formation between gallate and Fe(III) ions [18].

SEM observations after pretreatment (1 week in water at 37°C) evidence magnetite crystals embedded in a glassy matrix (Figure 1). EDS analysis confirms the glass-ceramic composition with few carbon contaminations. The presence of carbon from atmospheric contaminants is often observed on the surface of reactive materials such as titanium and bioactive glasses [40-44]. A thick reaction layer appears after GA grafting (Figure 1, SC-45+GA (24h@37°C)) together with a considerable increase in the surface carbon content. The carbon content on SC-45+GA (24h@37°C) is three times higher than the one on SC-45 pretreated, this increase can be considered significant and can be attributed to GA grafting to surface -OH groups and to its retention in the reaction layer.

The glass matrix appears almost completely dissolved after functionalization in citrate buffer (Figure 1, SC-45+GA +buf (24h@37°C)). Numerous precipitates, identified as calcium citrate crystals by means of SEM-EDS and FTIR analyses (data omitted for brevity) have been detected on samples surface. Their presence can be due to the complexation between Ca ions from the dissolution of the glassy matrix and citrate ions from the buffer. In fact it can be supposed that sodium citrate buffer dissolve the glass matrix and induce the combination of calcium ion from the glass and citrate ions from the buffer itself. Magnetite crystals result exposed at the surface of the material due to the etching of the amorphous matrix (enhanced by the complexation ability of citrate ion towards calcium); EDS results show a prevalence of iron and oxygen with few carbon contaminants and silicon. A lower carbon content is a first signal of a less effective functionalization (as demonstrated by F&C and XPS analyses) due to the degradation of the glassy matrix (with consequent reduction of the exposed -OH groups) and the absence of the reaction layer.

XPS survey analyses confirm EDS observation, in fact a significant increase in the surface carbon content (as reported in Table 2 for SC-45+GA (24h@37°C) samples) can be interpreted as a first index of the GA grafting. The significant reduction in the iron, calcium and phosphorous contents can be attributed to ion release in the functionalization medium and also to the formation of a GA rich reaction layer.

The three contribution observed in the high resolution spectrum of the pretreated glass-ceramic (Figure 2a) can be attributed to oxides (530.58 eV), silica (531.58 eV) and hydroxyls groups (533.69 eV). These data confirm previous observations on pretreated SC-45 [13].

After GA grafting (Figure 2b) the signal of oxides is not visible, the silica one (531.04 eV) is significantly reduced while the -OH one (532.36 eV) is increased. These results are consistent with the hypothesis of surface grafting of GA molecules that hinder the glass substrate and expose hydroxyls groups.

The F&C test evidenced a moderate lowering of the GA content in the uptake solution of SC-45 bulk samples without buffer but not for the buffered ones (Figure 3a). The depletion of GA from the uptake solution can be attributed to its grafting on SC-45 surface.

Looking at the test results on solid samples (Figure 3b) a higher amount of GA can be detected on SC-45 samples functionalized without buffer compared to the buffered ones.

The F&C results on bulk samples confirm the SEM-EDS and XPS observations previously described. SC-45+GA samples are able to retain a significant amount of GA by grafting to -OH groups and retention in the reaction layer, while the SC-45+GA+buf ones are less effective, on the base of the phenomena previously hypothesized.

As far as powder samples are concerned a lower functionalization time (3h) or the addition of a small amount of citric acid (CA) have been considered in order to avoid an excessive pH increase without the damage induced on the glass-ceramic by the concentrate citrate buffer.

A certain lowering of the GA content has been registered for the uptake solution in all the tested conditions (Figure 4a). GA presence has been detected on all the functionalized powders but the amount is higher for the 3h@37°C ones (Figure 4b). These results suggest that the minimization of the functionalization time is sufficient in order to control the pH and obtain an effective grafting on powder samples.

Comparing the amount of GA on bulk and powder samples, it is evident that the latter is higher than the former. This behavior is due to the higher surface area exposed by powder samples. These results are in accordance with previous ones obtained on bioactive glasses functionalized with GA [34] and polyphenols from grape skin [45]. Surface roughness and topography affect the area exposed to the functionalization medium. In particular the exposition of a larger area for the glass matrix allows an higher availability of hydroxyls groups for GA grafting, improving the effectiveness of the functionalization.

Preliminary release tests in double distilled water (Figure 5a) evidenced a moderate amount of GA in in solution after 1d and only traces after 7d. These values are lower for the 43°C test. Looking at the sample surface (Figure 5b) the GA content is almost unchanged after 1d and moderately lowered after 7d for the 37°C test but significantly lowered for the 43°C test.

The apparent lower release detected after 7 days compared with the one after 1 day, as well as at 43°C compared with the one at 37°C, could be attributed to the high pH of the solutions (Table 3) that can alter the GA structure and make it undetectable by the F&C method. When the pH value did not rise above 8.7 (Table 3), any significant decrease in the amount of GA was detected. Higher values of pH, joined with the temperature increase, are able to damage the structure of GA. Release test in buffered solution (e.g. Simulated Body Fluid – SBF or Phosphate Buffered Saline – PBS) can offer a controlled pH (at about 7.4) and consequently a more controlled behavior for GA. On the other hand the interaction of the glass ceramic surface with SBF/PBS (due to material reactivity) and the possible interactions between these solutions and

GA molecule must be taken into consideration. These tests will be investigated in depth in further research works.

TGA-EGA experiments (Figure 6) evidence a 1.4% weight loss for the SC-45+GA (3h@37°C) sample that can be attributed to two main processes.

The first one was evidenced by a minimum on the derivative curve (figure 6B) at ca. 100 °C and was assigned to the loss of physisorbed water as evidenced by the FTIR analysis (figure 6C). An analogue process occurred also with SC 45 pretreated, but in this case the amount of physisorbed water released was higher than that observed with SC-45+GA (3h@37°C). This result suggests a lower hydrophilicity of the bioglass upon functionalization with gallic acid.

A second phenomenon can be registered in the 250°-750°C range for SC 45+GA (3h@37°C) sample. It causes a weight loss of 0.7 %. The evolution of CO_2 evidenced by the FTIR analysis is consistent with the decarboxylation of gallic acid bound at the surface of the bioglass. A weight loss (evidenced by a minimum at about 650 °C) can be observed for SC-45 pretreated. During this process the evolution of CO_2, evidenced by the FTIR analysis (figure 6 D), was attributed to the decomposition of carbonates on the surface of SC-45 pretreated.

It is note of worthy that the same process was not observed with the SC-45+GA (3h@37°C) indicating that the treatment with gallic acid during the functionalization of SC-45 pretreated was able to dissolve the carbonate precipitated on the surface of the bioglass. This result confirmed what previously observed by some of the authors by means of XPS analyses on bioactive glasses functionalized with gallic acid and natural polyphenols [34, 45].

A weight gain can be observed for both samples when the atmosphere was switched to O_2. This process was assigned to the oxidation of iron (II) to iron (III) in the magnetite crystals according to the reaction:

$2FeO + ½\ O_2 \rightarrow Fe_2O_3$

SC-45+GA (3h@37°C) gained the 0.9 %, whereas SC 45 pretreated gained the 0.5 %. This result indicated that the amount of iron (II) in SC-45+GA (3h@37°C) is higher that contained in SC45 pretreated suggesting that the treatment of the bioactive glass with a reducing agent such as gallic acid caused the reduction of iron (III) in the magnetite crystals. The increased amount of iron (II) as a consequence of grafting GA at the surface could have a major role in the reactivity of such glass-ceramic towards biomolecules and influence its interaction with living cells and tissues after the implantation. This topic will be addressed in a future paper devoted to clarify the interaction of SC-45+GA (3h@37°C) with bio-molecules and assess its relevancy in biocompatibility/toxicity.

Moreover the FTIR analysis evidenced that during this process a high amount of CO_2 and H_2O were released from SC-45+GA (3h@37°C). This result is consistent with the combustion of organic species still present on the surface of SC-45+GA (3h@37°C) and derived from the degradation of gallic acid.

Conclusions

Gallic acid (GA) was grafted to a ferrimagnetic bioactive glass ceramic (SC-45). Grafting conditions have been optimized for both bulk and powder samples to obtain an effective immobilization and preserving GA activity. GA presence on SC-45 surfaces was demonstrated by means of SEM-EDS, XPS and TGA-EGA analyses and Folin&Ciocalteu test. A minimal GA release was observed in double distilled water at 37°C (but not at 43°C, within the sensitivity of the method) for SC-45+GA powder samples. GA was still present on SC-45 samples after 1 and 7 d in water at 37°C but significantly reduced after 1 and 7 d at 43°C.

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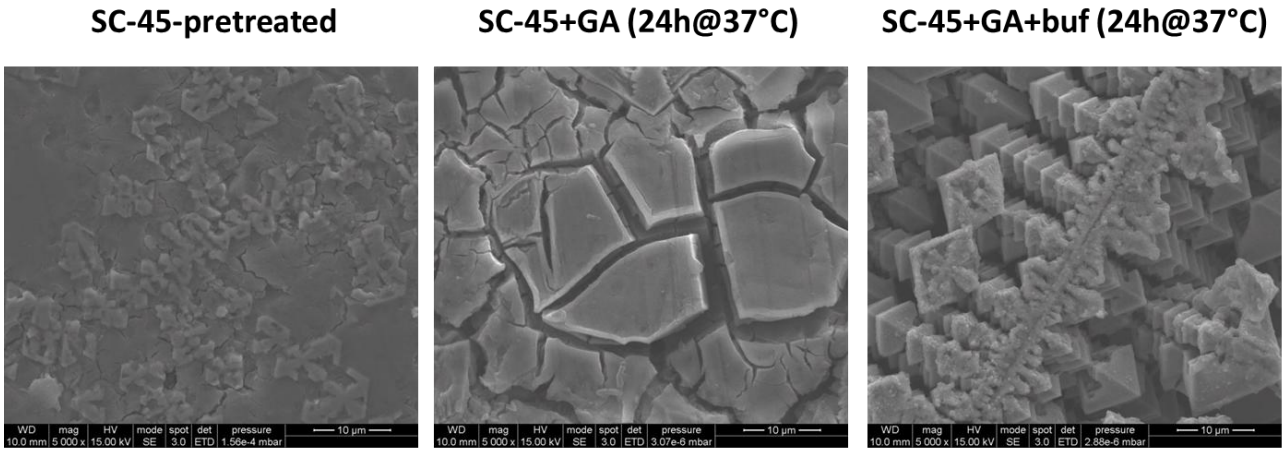
Figures and tables legend



Element	SC-45-pretreated	SC-45+GA (24h@37°C)	SC-45+GA+buf (24h@37°C)
C	10,80	31,42	13,28
O	53,79	43,78	43,20
Na	3,56	0,00	0,00
Si	8,17	9,45	6,81
P	0,99	0,35	0,00
Ca	4,76	1,62	0,56
Fe	17,92	13,38	36,15

Figure 1: SEM images and EDS analyses (%at) on a representative macro area (150x mag.) of SC-45 bulk samples after pretreatment (SC-45 pretreated), GA functionalization 24h at 37°C (SC-45+GA (24h@37°C)) and GA functionalization 24h at 37°C in citrate buffer (SC-45+GA+buf(24h@37°C)).

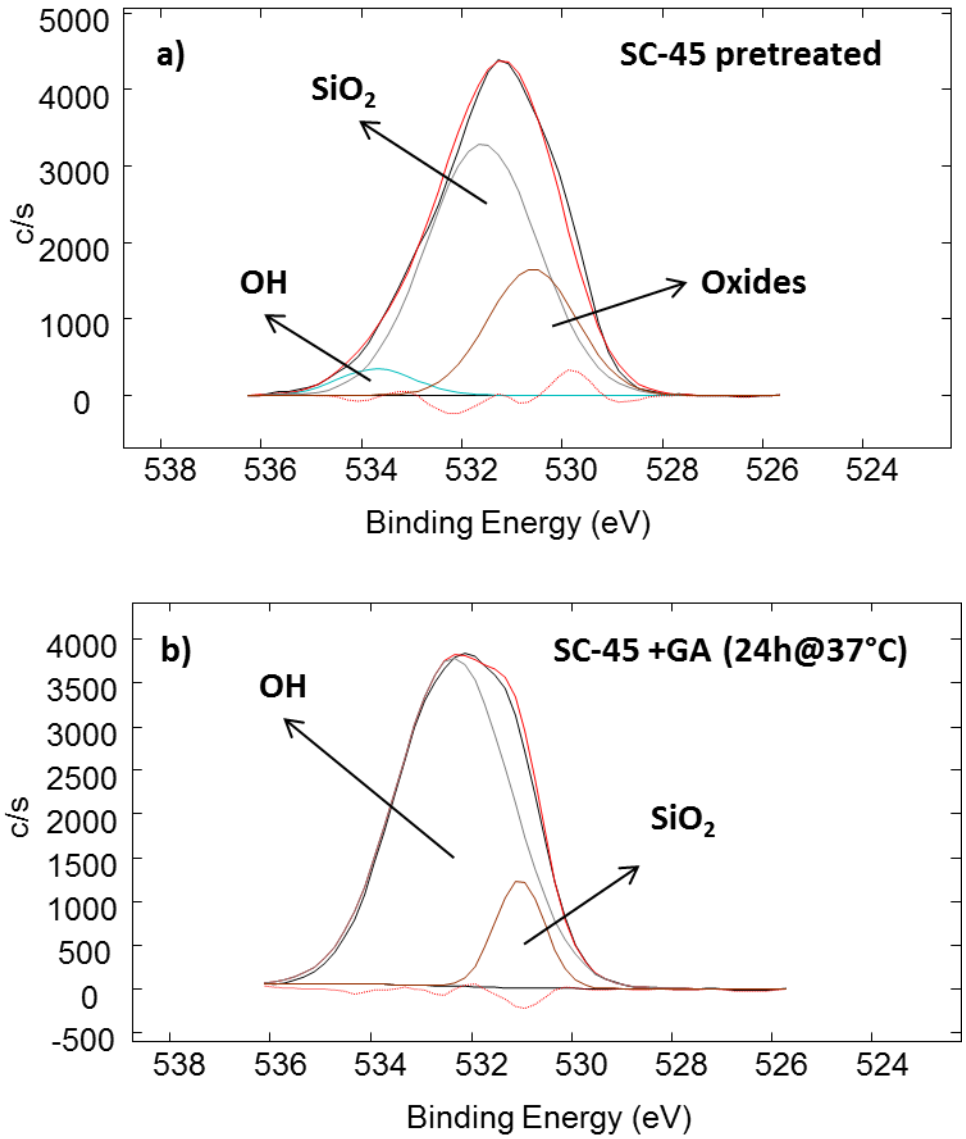


Figure 2: XPS high resolution spectra of oxygen region for SC-45 pretreated (a) and SC-45+GA (24h@37°C) (b) bulk samples.

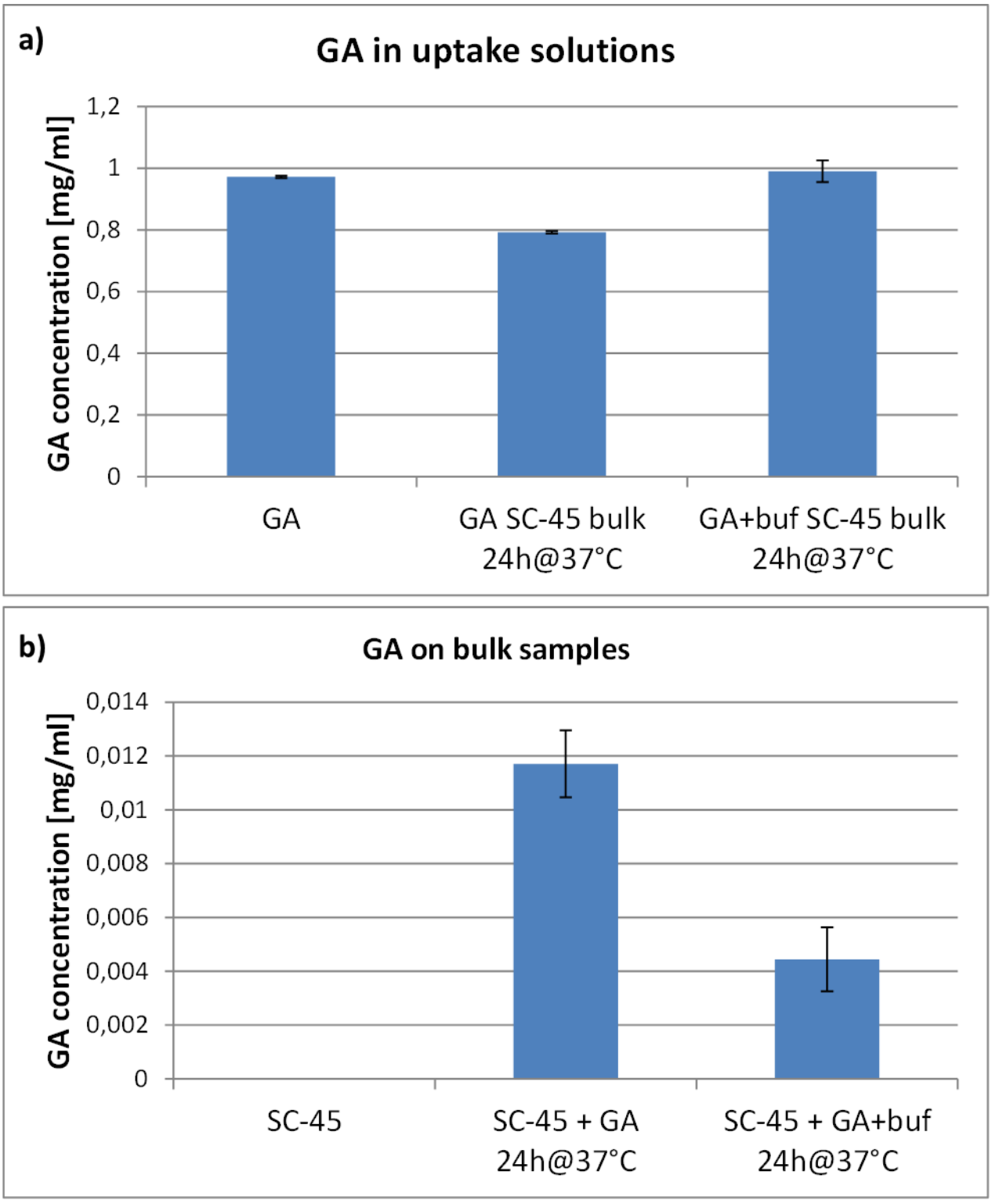


Figure 3: Folin & Ciocalteu determination of GA for surface functionalization of SC-45 bulk samples. a) GA in uptake solutions, b) GA on the surface of bulk samples.

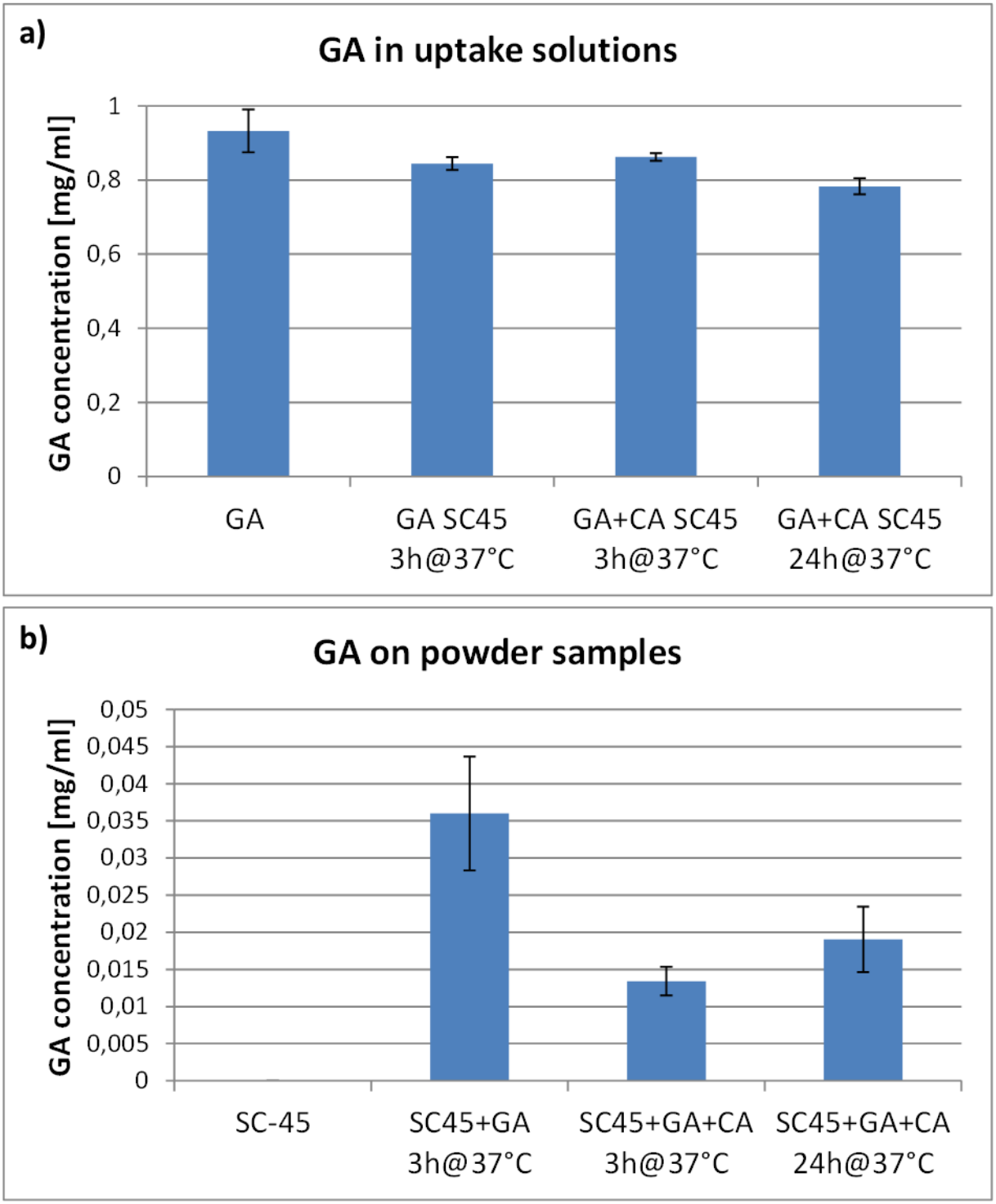
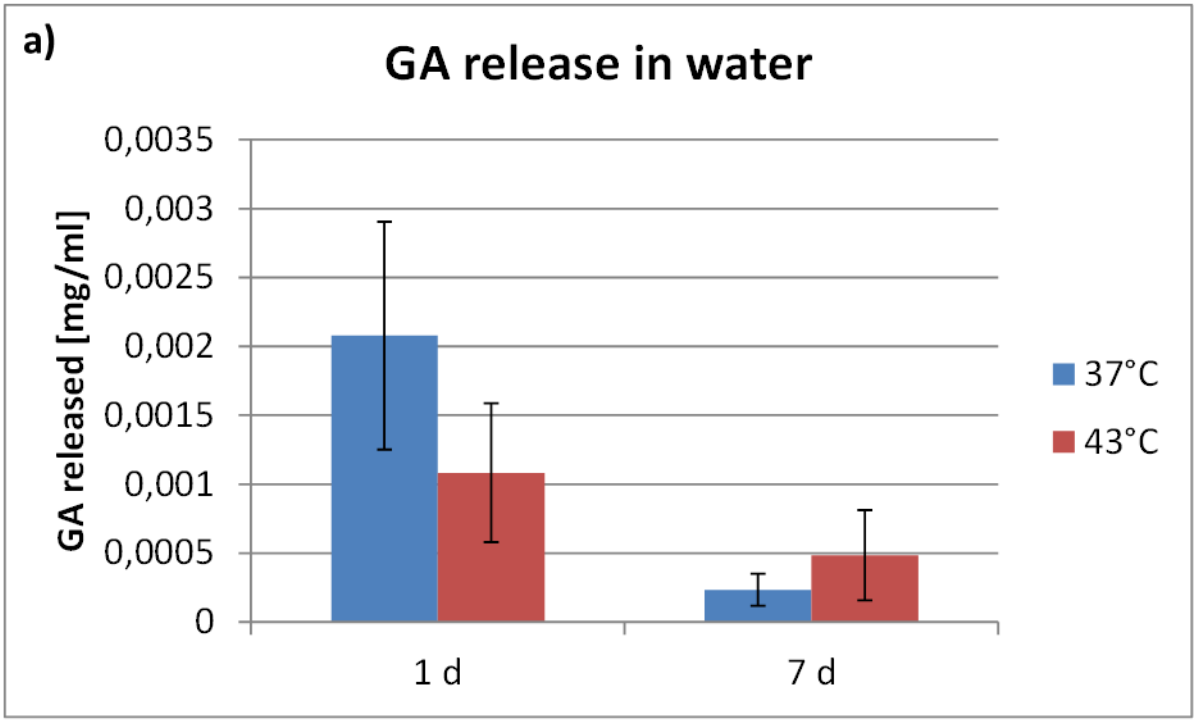


Figure 4: Folin & Ciocalteu determination of GA for surface functionalization of SC-45 powder samples. a) GA in uptake solutions, b) GA on the surface of powder samples.



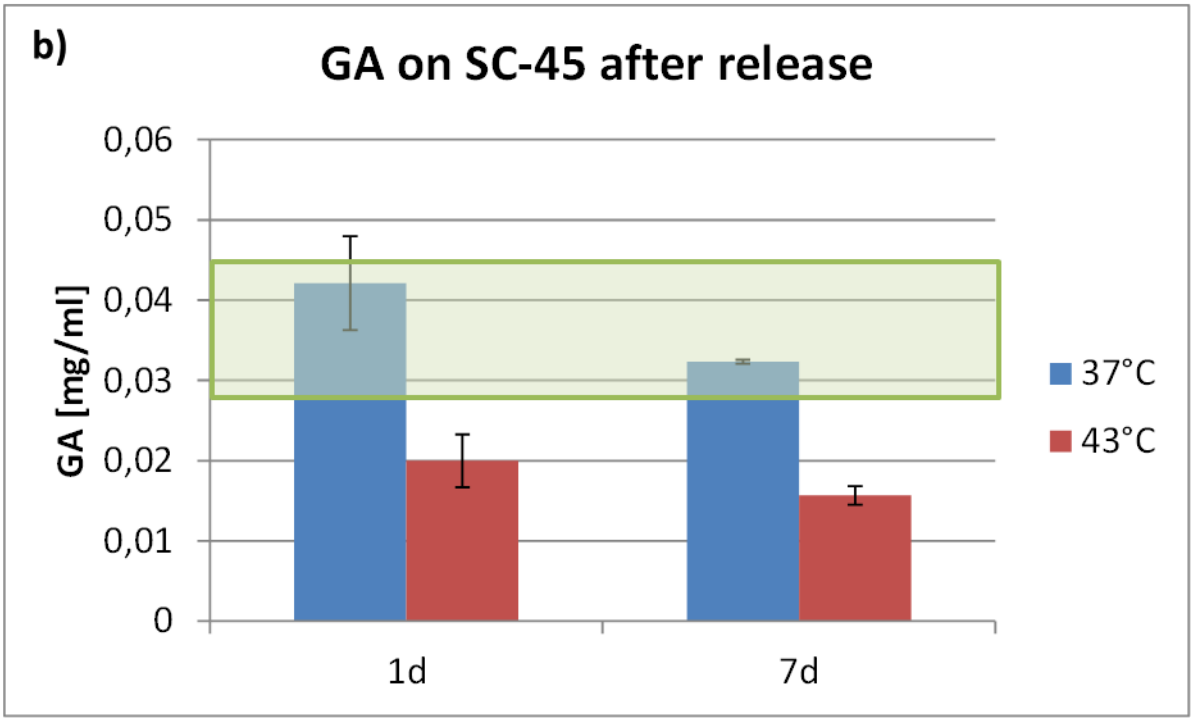


Figure 5: a) Amount of GA released in water after 1 d and 7 d soaking at 37°C and 43°C, b) amount of gallic acid on SC-45 surface after 1d and 7d soaking in water at 37°C and 43°C. The green box in b) reports the concentration range of GA on SC-45+GA(3h@37°C) before the release test.

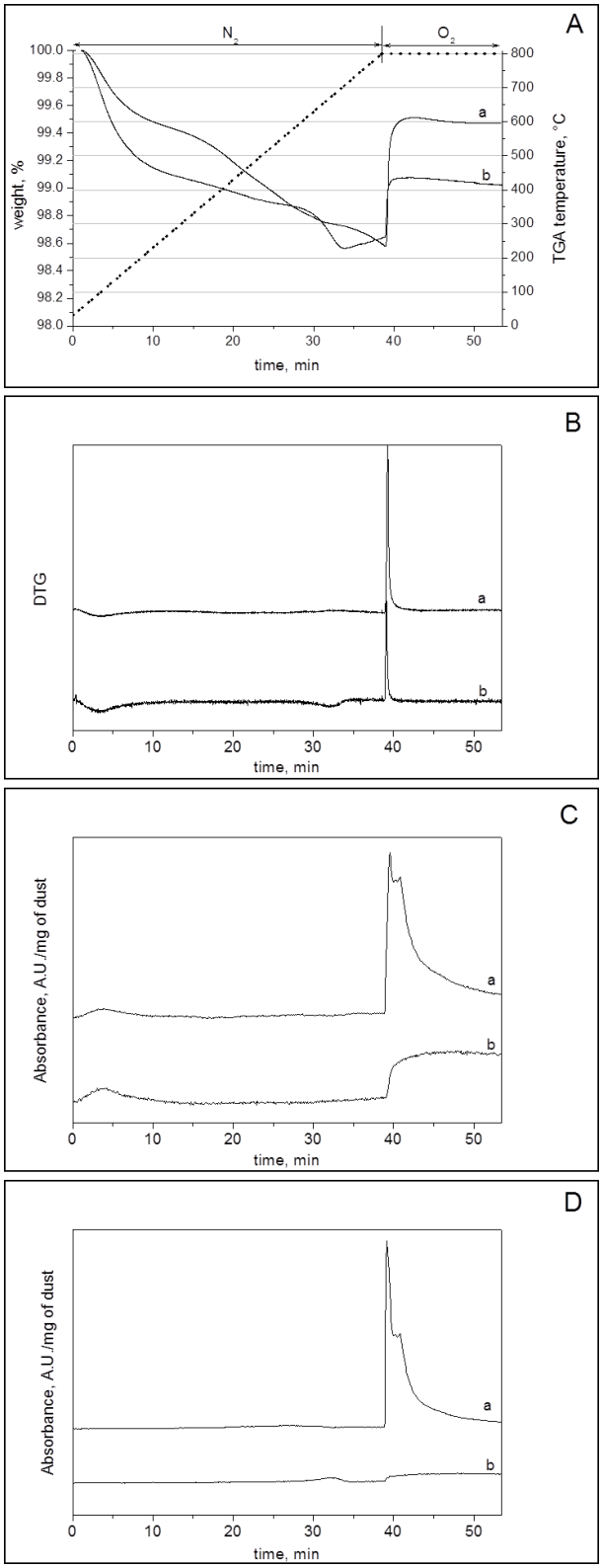


Figure 6. (A) Thermograms of SC 45+GA (3h@37°C) (a) and SC 45 pretreated (b) in the temperature range 30-800 °C. Dotted line: TGA temperature as a time function. (B) Derivative curves of the thermograms. (C) FTIR profiles of H_2O (D) FTIR profiles of CO_2 released during the thermal analysis.

Table 1: pH of GA solutions before and after SC-45 functionalization.

Substance/Material	**pH**
GA	3.18±0.02
GA-SC-45 bulk (24h@37°C)	4.03±0.07
GA+buf-SC-45 bulk (24h@37°C)	4.63±0.03
GA-SC-45 pow (*) (24h@37°C)	8.07±0.13
GA+buf-SC-45 pow (24h@37°C)	4.56±0.04
GA-SC-45 pow (3h@37°C)	6.48±0.53
GA+CA-SC-45 pow (3h@37°C)	4.73±0.25
GA+CA-SC-45 pow (24h@37°C)	6.43±0.40

Table 2: Atomic percentages of elements from XPS survey analyses

Element	**SC-45 pre-treated**	**SC-45+GA (24h@37°C)**
O	54.5	41.3
C	29.9	52.6
Fe	6.4	1.9
Ca	3.4	1.2
Si	2.4	2.9
P	1.9	-
N	1.6	-

Table 3: pH measurements in the release solutions.

	1 d	7 d
37°C soaking	8.71±0.07	8.81±0.14
43°C soaking	9.62±0.10	10.44±0.53