Surface functionalization of bioactive glasses with natural molecules of biological significance, part I: Gallic Acid as model molecule

Surface functionalization of bioactive glasses with natural molecules of biological significance, part I: Gallic Acid as model molecule

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Abstract

Gallic Acid (3,4,5-trihydroxybenzoic acid, GA) and its derivatives are a group of biomolecules (polyphenols) obtained from plants. They have effects which are potentially beneficial to heath, for example they are antioxidant, anticarcinogenic and antibacterial, as recently investigated in many fields such as medicine, food and plant sciences. The main drawbacks of these molecules are both low stability and bioavailability. In this research work the opportunity to graft GA to bioactive glasses is investigated, in order to deliver the undamaged biological molecule into the body, using the biomaterial surfaces as a localized carrier. GA was considered for functionalization since it is a good model molecule for polyphenols and presents several interesting biological activities, like antibacterial, antioxidant and anticarcinogenic properties. Two different silica based bioactive glasses (SCNA and CEL2), with different reactivity, were employed as substrates. UV photometry combined with the Folin&Ciocalteu reagent was adopted to test the concentration of GA in uptake solution after functionalization. This test verified how much GA consumption occurred with surface modification and it was also used on solid samples to test the presence of GA on functionalized glasses. XPS and SEM-EDS techniques were employed to characterize the modification of material surface properties and functional group composition before and after functionalization.

Key words: Surface functionalization, bioactive glasses, gallic acid

Introduction

Gallic Acid (3,4,5-trihydroxybenzoic acid, henceforth GA) and its derivatives are present in many plants and are common components of food and beverages of plant origin, such as grape and tea [1]. GA can be extracted from wood, fruits, wine, different alcoholic beverages and various medicinal plants often used in traditional Chinese medicine [2]. Singh *et al.* isolated various antioxidant phytochemicals from tomatoes, including GA [3]. In Owen's [4] research, carbon fiber was taken as the raw

material to extract several classes of polyphenolic substances with the organic solvent extraction, a number of techniques were employed for their analysis: liquid-chromatography electrospray-ionization mass spectrometry (LC-ESI), nano-electrospray-ionization mass spectrometry (ESI-MS) and gas-chromatography mass spectrometry (GC-MS); a total of 24 polyphenol compounds were identified and the amount of GA was 42 % of the total phenol mass.

Because of its good biological and pharmacological abilities, it has antioxidant, anti-allergic, anti-carcinogenic, anti-mutagenic and antibacterial properties, GA has been widely used in food additives and cosmetics. In addition, GA is employed as a source material for inks, paints and color developers [5]. Agarwal *et al.* reported that GA is a major active agent in grape seed extract and induces apoptosis activity in DU145 human prostate cancer cells [6]. GA can also scavenge free radicals of L1210 leukemia cells, which could cause an aberrance of normal tissue [7]. On the other hand some authors reported the pro-oxidant and cytotoxic effect of gallic acid in certain conditions [8, 9, 10]. Moreover it has been evidenced that GA coupling with gold nanoparticles can reduce its toxicity [11].

Nowadays, much attention has been paid to the combination of biomaterials and biological molecules. Surface functionalization of biomaterials with biomolecules is a promising strategy in order to target specific signals to cells and tissues directly from material surfaces.

Many research papers dealing with the surface grafting of proteins, enzymes and drugs can be listed [12-16] but few consider polyphenols and natural biomolecules. However, there is an increasing interest for this kind of substances within the scientific community. For instance, GA has been successfully employed as an intermediate functional molecule for further immobilization of vascular endothelial growth factor (VEGF) [17]. Furthermore, in addition to the study and use of the pure molecule, GA has also been combined with organic and inorganic carriers in order to take advantages of its antioxidant, antibacterial and anticancer properties.

As far as organic carriers are concerned, recently the coupling of GA with chitosan has been investigated by a number of research groups in order to exploit GA

antioxidant properties in the food industry and in pharmaceutical and medical applications [18-20].

Regarding inorganic carriers, GA has been intercalated in Mg/Al layered double hydroxide by co-precipitation [21] to obtain a gradual release of the antioxidant molecule in physiological media.

Finally, the coupling of GA with inorganic nanoparticles has been explored. In particular, magnetite nanoparticles coated with a GA layer have been synthesized for the controlled release of GA in cancer treatment [22]. Moreover, GA has been used to induce significant antibacterial properties and to act as stabilizing agent with gold nanoparticles [23].

Scientific studies on the surface functionalization of synthetic supports with biological molecules and drugs are mainly related to polymers, metals and ceramics and only a few papers have been published on the modification of bioactive glasses.

Bioactive glasses and glass–ceramics are very attractive materials, widely investigated for many years and mainly used in the field of bone reconstruction [24]. They are known for their peculiar surface reactivity when soaked in water or aqueous solutions, like simulated body fluids (SBF), TRIS buffer or physiological media [24-28]. When soaked in simulated or biological fluids, these glasses are prone to various surface modifications which stimulate the precipitation of a hydroxyapatite layer on them. This feature is related to their ability, in vivo, of promoting effective osteointegration [29].

As well-documented by the literature [30], the bioactivity mechanism starts with a rapid ion exchange at the glass surface, between the alkaline ions of the glass and the hydrogen ions of the solution. This causes the formation of silanols that, by further polycondensation, develop a silica gel layer on top of the glass. In this layer the adsorption of calcium and protonated forms of phosphate ions is promoted [27], leading to the crystallization of hydroxyapatite. Due to their hydroxylation properties, bioactive glasses can be successfully functionalized, without further surface activation treatments, if the fast condensation of silanols to silica gel is prevented and thus the presence of free -OH groups on their surface is preserved.

In this research, two bioactive glasses with different degree of bioactivity were chosen as model surfaces for grafting with GA. SCNA is a glass characterized by a simple composition and high stability while CEL2 is a highly bioactive glass [31]. These two biomaterials were previously developed and fully characterized by the authors; their different degree of surface reactivity in simulated body fluids allowed the optimization of their surface activation and the understanding of the role of exposed surface groups on grafting with several biomolecules, like carnosine, bone morphogenetic dipeptide and alkaline phosphatase [32, 33]. Bioactive glasses have been chosen as model surface because the combination of their peculiar properties, such as biocompatibility, bioactivity, and tailorable surface reactivity, with the biological properties of GA, represents a challenging approach to the development of implants with improved therapeutic performances. Moreover synthetic supports like glasses are intended to improve the stability and bioavailability of GA at the desired site.

Materials and methods

Samples preparation

Two bioactive glasses (SCNA and CEL2), with different degree of bioactivity, were considered as substrates for GA grafting. The glasses were produced by traditional melt and quenching techniques, as described in several previous papers [31-33]. Composition and melting conditions for the both materials are reported in table 1.
All materials were used both in the bulk and in the powder form.

The bulk materials were obtained by pouring the melted glass on a brass plate. The obtained bars were annealed in a furnace (annealing condition are reported in table 1) in order to release the residual stresses and cut with an automatic cutter (Struers Accutom 5). Glass slices were then polished with SiC abrasive papers (120-4000 grit) in order to obtain plane and homogeneous surfaces.

For the preparation of powders, the melted glasses were poured in water in order to obtain a frit. The frit was ball milled and sieved up to a grain size low than 20 μm.

Surface activation

In order to graft GA on the material surface it is necessary to have free –OH groups suitable for the functionalization.

As far as silica-based glasses and glass-ceramics are concerned, it is possible to effectively expose reactive hydroxyls by means of simple water washings and this process was optimized by the authors in previous works [32, 33].

The activation step includes a 5 min. washing treatment in acetone in an ultrasonic bath, in order to remove surface contaminants, and 3 subsequent water washing steps (5 min each) in an ultrasonic bath for –OH groups exposition [32]. At the end of the surface activation, samples were dried in air at room temperature. The surface activated samples will be named glass-washed.

GA grafting

A stock solution of 1.0 mg/ml of GA in double distilled water was prepared by dissolving GA (GA 97.5-102.5% titration, G7384, Sigma Aldrich) in double distilled water for about 2 h under magnetic stirring. For each sample 5 ml of 1.0 mg /ml GA solution was employed. Activated samples (both bulk and powder ones) were soaked in GA solution for 24 h at 37° C. In order to prevent the light irradiation of GA (which is a light-sensitive molecule) all the holders were covered with aluminum foils.

The effect of pH on the glass and molecular behavior was evaluated by citric acid addition to acidify the GA uptake solution. In fact the reactivity of bioactive glasses will induce a basification of the functionalization medium, as discussed in the results and discussion section. 0.5 M citric acid (CA, Citric Acid Monohydrate, ACS reagent 99.0-102.0%, Sigma Aldrich) in double distilled water was added drop wise to the GA uptake solution till the pH was 3.0. A part of samples was functionalized with CA-modified GA solutions.

After 24 h, the GA and GA-CA solutions from each bottle were moved to clean bottles for UV analyses., The samples (powders and bulks) were washed twice by double distilled water, dried in an incubator for 12 h at 37°C and stored in the dark. GA-grafted samples are labeled as *SCNA+GA* and *CEL2+GA*, respectively, while the ones functionalized with gallic acid and citric acid addition as *SCNA+GA+CA* and *CEL2+GA+CA*.

Functionalization solutions are indicated as *GA* (gallic acid uptake solution), *GA SCNA bulk/powder* (gallic acid uptake solution after SCNA bulk/powder samples), *GA CEL2 bulk/powder* (gallic acid uptake solution after CEL2 bulk/powder samples), *GA+CA SCNA bulk/powder* (citric acid added gallic acid uptake solution after SCNA bulk/powder samples), *GA+CA CEL2 bulk/powder* (citric acid added gallic acid uptake solution after CEL2 bulk/powder samples)

3 samples for each type were considered both for UV and pH measurements and results reported as mean±standard deviation.

UV-Visible analyses

GA solutions were analyzed by means of UV-Vis Spectroscopy (CARY 500 VARIAN) before and after samples soaking, in order to estimate the amount of molecule grafted onto the material surface.

The total amount of phenols in the solution was determined by the Folin&Ciocalteau method [34]. In brief, 2 ml of the solution to be tested was mixed with 6 ml of water and 0,5 ml of Folin&Ciocalteau reagent (Folin&Ciocalteau phenol reagent 2 M with respect to acid, 47641, Sigma Aldrich) were added. Mixtures were gently mixed and after 3 min. 1,5 ml of 20% (p/V) Na_2CO_3 solution were added. After 2 h reaction, UV measurements were carried out. Preliminary test were also performed putting the Folin&Ciocalteau reactive mixture directly on functionalized samples in order to determine the surface grafted gallic acid.

A standard calibration curve was prepared. Six solutions, with defined GA concentrations (0.0025, 0.005, 0.01, 0.02, 0.03 and 0.04 mg/ml), were prepared and tested with the Folin&Ciocalteau method described before.

XPS analyses

X-Ray Photoelectron Spectroscopy (XPS, PHI 5000 VERSA PROBE, PHYSICAL ELECTRONICS) was employed in order to verify the presence of GA on samples surface after the functionalization. Bulk samples were considered for this test. Both activated and activated and GA-grafted materials were analyzed.

The chemical composition of the outermost surface layer was determined by means of survey analyses while the chemical state of the most significant elements (C and O) was investigated by high resolution spectra in the proper binding energies ranges. Binding energies were corrected considering the standard shift respect to the C1s at 284.6 eV.

SEM observations

In order to study the reactivity and consequent surface modifications of bioactive

glasses during the functionalization process, washed and GA-grafted samples were observed by scanning electron microscopy (SEM) equipped with energy dispersive X-ray Spectroscopy (EDS), (SEM-EDS SEM-FEI, Quanta Inspect 200, EDS-EDAX PV 9900). Samples were sputter coated with a thin Cr-layer (to make them conductive) before observation.

Results and discussion

Macroscopic observation and pH measurements

Figure 1 shows the SCNA and CEL2 bulk samples before and after soaking into the uptake solutions, as well as the solutions themselves and their pH values (mean±standard deviation). It is evident that the surface of CEL2 samples changes from colorless to brown after GA uptake. This change of color is almost absent for SCNA. The same feature is observable for the uptake solutions. The solution color change is reduced when citric acid is added in the uptake solution.

It must be underlined that in the present setup, the two glasses were soaked in the GA solutions, which are characterized by a fairly acid pH value (3.22±0.30), and that they are not buffered. In aqueous solutions the amorphous silica network of bioactive glasses can be attacked by water (weakly dissociated) by the breaking of oxygen bridges and the forming of -OH. Moreover the ion exchange between Na^+ from the glass network and H_3O^+ from the solution promotes the formation of free -OH on the glass surface. In an acid environment the ion exchange is enhanced [32].

It is also well known that during soaking of bioactive glasses in aqueous solutions, the pH can change to more alkaline values [30], even in buffered simulated body fluids, if a static model of soaking is used. This happens because the ion exchange between Na^+ and H_3O^+ causes a depletion of H_3O^+ in the solution. In the present setup, the solutions were not buffered, and a strong change to alkaline values of pH can be observed, especially in the case of the most reactive glass (CEL2) and for powder

samples, for both glasses. In fact pH values of 3.43 for bulk SCNA, 7.99 for SCNA powders, 7.34 for bulk CEL2 and 8.68 for CEL powders have been recorded for the gallic acid solutions after samples soaking. A basic environment promotes a further hydroxylation of silica-based glasses through a direct disruptive action on the silica network catalyzed by the OH^- anions in the solution [32]. For bioactive glasses characterized by high reactivity (like CEL2) this effect is much stronger than for almost inert glasses. Moreover the high surface area of powder samples can favor the ion exchange process. A basic environment can also induce the oxidation of catechol groups on GA to generate quinone groups [17], the result is the typical brownish color that was noticed both on the glass surfaces and in the uptake solutions.

Basification is reduced by citric acid addition (as reported in figure 3 for bulk samples), but it is not completely overcame. It must be underlined that CA addition allow to keep the pH of the uptake solution below 7.00 and that the literature reports a favorable pH for the transition of GA structure from phenol group to quinone ones at 7.40 [17]. The strong color change of the CEL2 uptake solution is significantly reduced by CA addition (figure 1).

UV-Vis measurements: GA standard curve and uptake solutions

The standard calibration curve, obtained by the UV measurements of standard GA solutions (in the range 0.0025 – 0.04 mg/ml of GA in double distilled water) after the reaction with Folin&Ciocalteu reagent, is reported in figure 2. Absorbance measurements were repeated at least three times in order to estimate the uncertainty, reported in the graph of figure 2 as error bar.

GA standard solutions with 6 different concentrations were tested by UV absorption technique. The standard calibration curve, obtained plotting the absorbance recorded at 760 nm vs the GA concentration [mg/ml], shows a very good linear relationship and a good value of correlation coefficient ($R^2 = 0.9997$).

Each GA solution content, before and after functionalization, was evaluated by

reaction with the Folin&Ciocalteau reagent (as previously described) and tested by UV spectrophotometry in order to determine the consumption of GA during grafting. The amount of GA was determined on the basis of the standard calibration curve. The bar diagram in Fig. 3 (GA functionalization series) shows the concentration of original GA and GA after soaking of SCNA bulk and powder samples as well as CEL2 bulk and powder samples. All the concentrations after functionalization are lower than original GA ones. Compared with SCNA, it is notable that CEL2 consumed more GA than SCNA on both bulk and powder samples (the values are 0.41026 and 0.07457 mg/ml respectively). Due to the higher surface reactivity of CEL2, more –OH groups can be exposed. Moreover, during functionalization the high bioactivity of CEL2 can lead to the condensation of –OH groups and to the formation of a silica gel layer. This layer can adsorb and retain an additional amount of GA. Since the powder samples have larger specific surface area than the bulk glasses, they can bind more GA molecules; in fact the reduction in the amount of GA in the functionalization medium is remarkably higher, for both glasses in the powder form. On the other hand it must be noticed that the strongly basic pH recorded after CEL2 (bulk and powder) and SCNA (powder) samples can lead to the change in the molecular structure of gallic acid and a possible different behavior with the Folin&Ciocalteau's reagent. The comparison between bare and CA added GA solution after glass functionalization (figure 3, CA addition series) indicate an apparent reduction of the GA uptake after CA introduction. It can be supposed that the significant reduction in the GA content after functionalization without CA addition is attributable in part to an effective uptake and in part to a change in the GA structure due to the basic environment. At the same time, the consumption of GA observable also after CA addition suggests the success of grafting to some extent.

Moreover preliminary test of the Folin&Ciocalteu assay on solid samples evidenced the presence of a notable amount of gallic acid on the surface, proving effectiveness of grafting and the activity of the grafted molecule. The amount is significantly higher on CEL2 than on SCNA, confirming the results obtained on the solutions.

XPS analysis

XPS analyses were performed in order to determine the presence of GA on glass surface after functionalization.

The atomic percentages of the elements, detected by means of the survey analysis, are reported in table 2.

It can be observed that, although a washing treatment was performed, a certain amount of unavoidable carbon contaminants still remain on the surfaces of SCNA and CEL2, as widely documented in the literature for XPS analyses of reactive materials [33, 35-38]. After GA grafting, both on SCNA and CEL2 surfaces, a certain increase in surface carbon and oxygen contents can be detected. The addition of citric acid in the functionalization medium induces a reduction in the surface carbon content and a more evident increase in the oxygen one.

Surface chemical composition also suggests a significant decrease in sodium and calcium content for both glasses, demonstrating an ion-exchange between bioactive glass surface and the grafting solution. After GA grafting, the silicon content is unchanged for SCNA, while it is higher for GA-grafted CEL2 if compared with the as washed CEL2. The increase in the surface Si content after functionalization increases for CEL2 and becomes evident also for SCNA when citric acid is added.

These observations suggest certain dissolution of the glass matrix in the functionalization medium added with citric acid, that lead to a higher SiO_2 exposition and higher removal of adventitious carbon.

The detailed analyses of carbon and oxygen regions were performed in order to identify the chemical groups present on the surfaces at the different steps of the modification processes.

The detailed analyses of carbon region on SCNA and CEL2 (figures 4 and 5) after washing (figure 4a and 5a) detect a notable signal at about 284.8 eV , it can be attributed to unavoidable hydrocarbon contaminations on reactive surfaces [33, 35-38]. A second contribution at about 289.4-289.6 eV can be assigned to carbonates [39] and

results of higher intensity on CEL2 samples.

After GA grafting, different contributions can be detected in the carbon region for both glasses (figures 4b and 5b). In particular, the carbonate signal disappears and, together with the carbon contaminants peak at 284.8 eV other signals can be observed: one at about 286.3 - 286.5 eV and the second one at 287.6. - 288.2 eV. They can be attributed to C-O and C=O bonds [40-43] respectively and they have been already detected on GA-functionalized surfaces [41]. The C=O signal is reduced when citric acid is added in the functionalization medium. This result confirms CA ability to limit the oxidation of GA to quinone.

The detailed analyses of oxygen region of washed samples (figures 6a and 7a) underline the presence of the typical signals for oxides (530.8 – 531.7 eV), silica (531.6-532.3 eV) and hydroxyls groups (533.2 – 533.5 eV) as already reported for surface activated glasses [32,33]. After GA grafting, the oxygen region is significantly different for the two materials, confirming their different surface reactivity. On SCNA sample (figure 6b) a notable increase in the -OH signal can be documented as further confirmation of GA presence. On the other hand CEL2 spectrum (figure 7b), together with the increase in the hydroxyls signal, a significant peak in the 531.35 eV around can be documented. This last signal can be ascribed to oxides but also to C=O groups [44]. Samples functionalized with CA added gallic solutions underline a significant reduction in this contribution (fig 7c), these data confirm a lower tendency to transformation of GA to quinone. These results are in accordance to the ones obtained in the carbon region.

SEM observations

The SEM observation of the glass surface, before and after GA grafting, clearly evidences the difference in the substrate reactivity.

Washed SCNA samples (Figure 8, first row) present a completely uniform surface with polishing lines slightly visible. After GA grafting, different surface morphologies can be observed: some areas (GA-1, second row in fig.8) present a quite cracked

surface with the typical morphology of the silica gel layer, while some other ones (GA-2, third row in fig. 8) are uniform and analogous to the untreated surface.

EDS analyses (table in figure 11) show more pronounced C enrichment and Na and Ca depletion for the GA-1 area if compared to the GA-2 one. It seems that the surface reactivity of the glass is not uniform; this phenomenon can be ascribed to the slower reaction kinetics of this material.

CEL2 washed samples present a polished surface with some pits and cracks (figure 9, first row). After GA grafting (figure 9, second row) the sample is completely covered by a cracked layer with the typical morphology of the silica gel. These observations support the ones obtained and discussed by XPS detailed analyses of oxygen region and confirm the presence of a uniform porous layer able to trap a higher amount of GA, if compared to SCNA. EDS analyses (table in figure 9) suggest a significant increase in the carbon content after the functionalization together with a reduction in the Na, Mg, Si, K, Ca percentages.

The reduction of Na, Mg, Si, K and Ca content after functionalization confirm an ionic exchange between the glass and the GA solution.

Conclusions

Two bioactive glasses, with different surface reactivity (SCNA and CEL2) were prepared as bulk and powder samples for functionalization with gallic acid (GA). After surface activation, hydroxyl groups were exposed on the material surface followed by GA grafting. Folin&Ciocalteau tests, on GA solutions and on solid functionalized samples, showed GA depletion, due to its uptake on glass surface during functionalization, and also GA grafting on samples surfaces. The amount of GA depleted from the solution, and grafted on the glasses, increased (for bulk samples) with the glass reactivity from SCNA to CEL2 and with the surface area from bulk to powders. XPS analyses of samples, before and after molecular anchoring, evidenced characteristic peaks for GA on functionalized glasses. Citric acid addition in the

functionalization medium made possible to preserve gallic acid structure during grafting. SEM observation of the surfaces confirmed the different reactivity of the two glasses, in fact a significant reaction layer was observed on CEL2 surfaces and a more irregular one on SCNA samples. Release tests are in progress in order to determine the ability of modified glasses to gradually release the grafted molecule. Moreover, in vitro tests have been planned for the evaluation of the biological properties of GA-grafted bioactive glasses.

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Figures and tables

Figure 1: Glass and uptake solution appearance and pH values (mean ± standard deviation) before and after functionalization

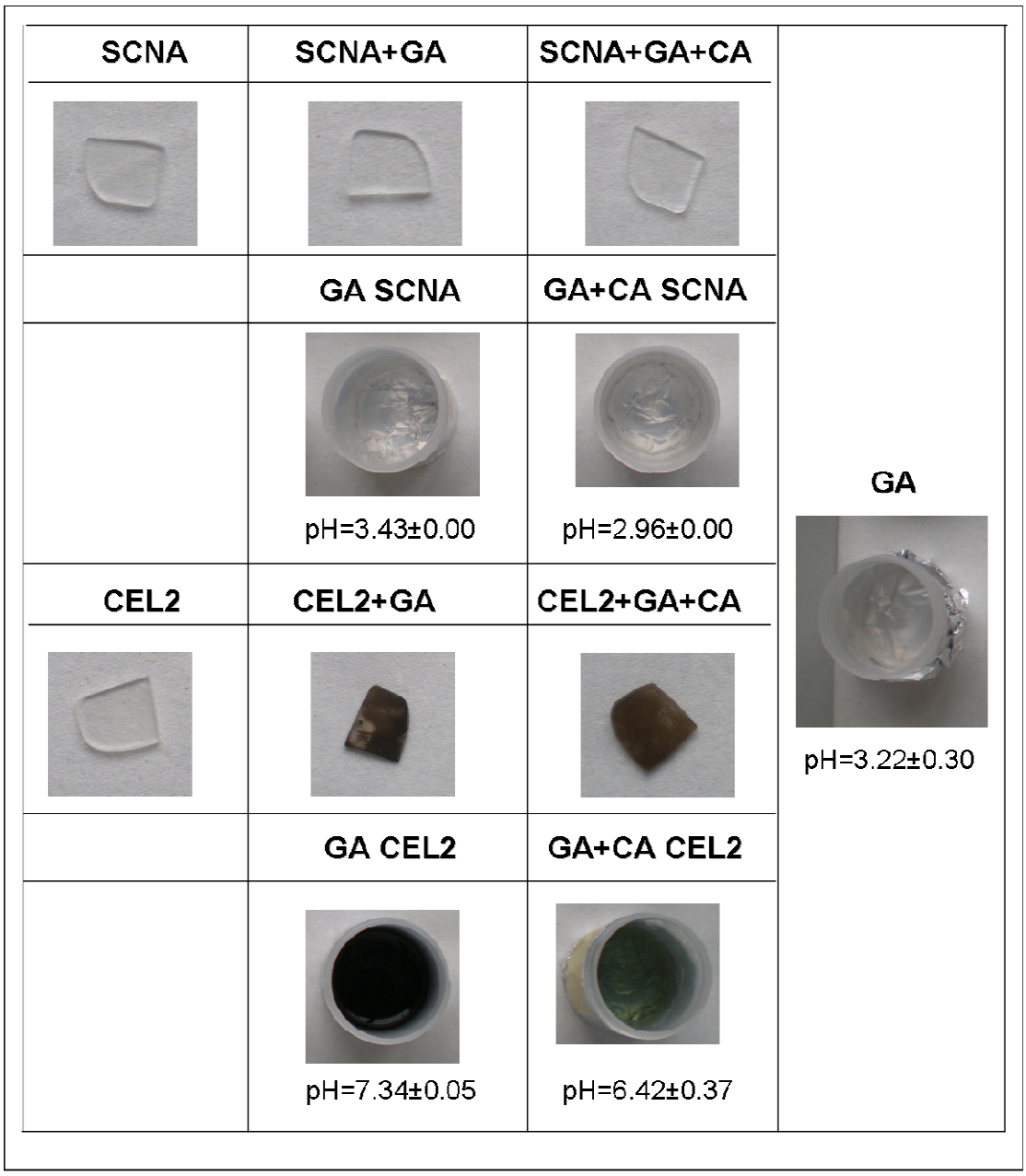


Figure 2: Standard calibration curve for GA

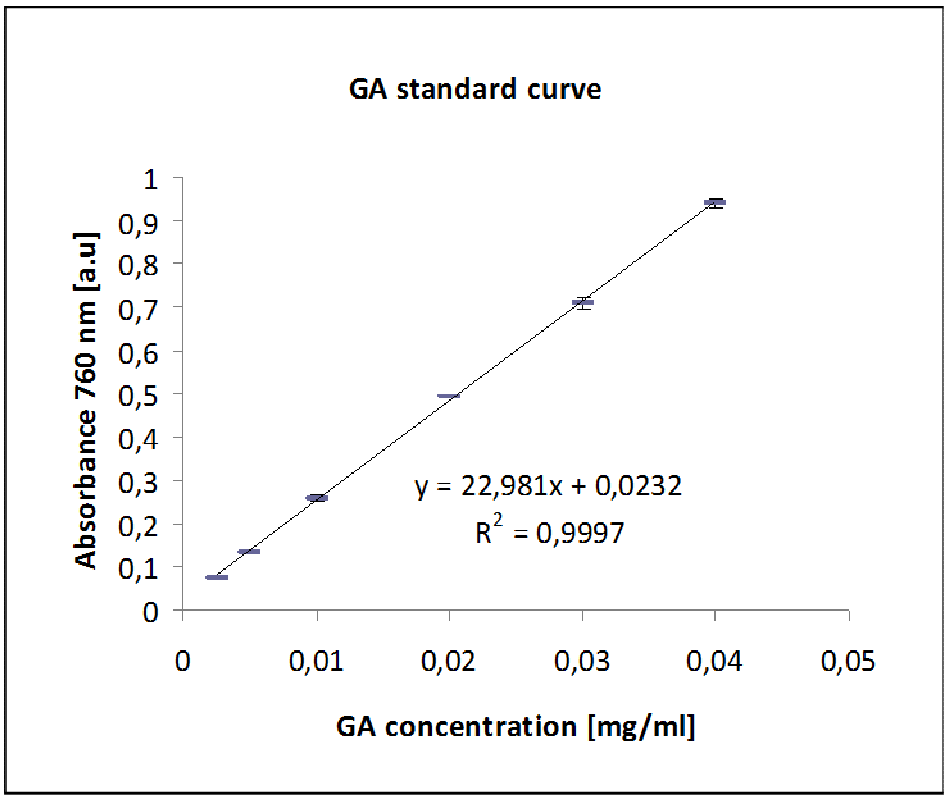


Figure 3: UV photometric results for GA uptake solution before and after 1 day soaking of the different glass samples, and effect of CA addition in the functionalization medium

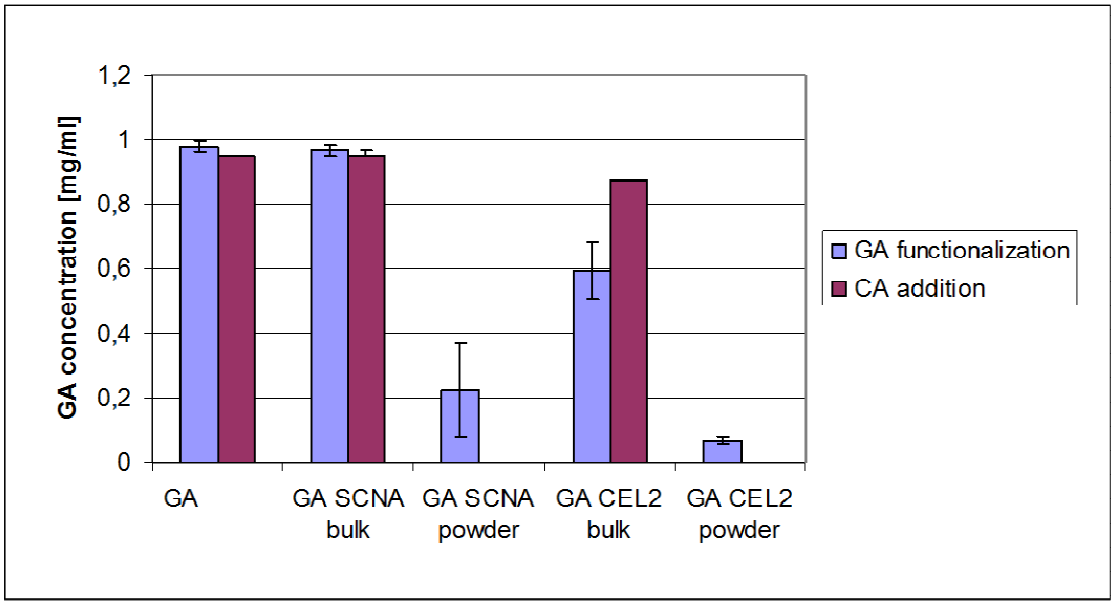


Figure 4: XPS detailed analysis of C region for SCNA washed (a), SCNA+GA (b), SCNA+GA+CA (c)

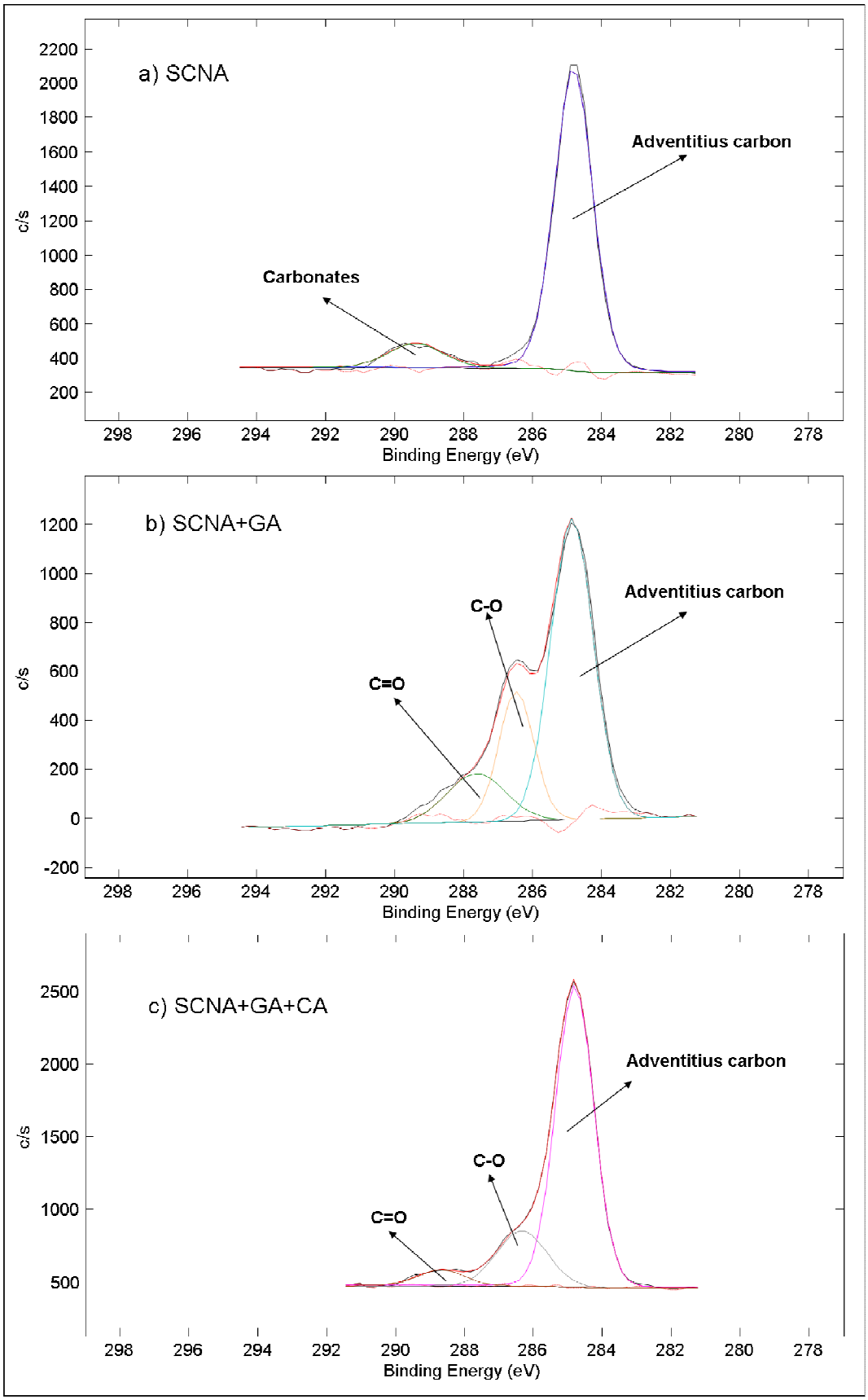


Figure 5: XPS detailed analysis of C region for CEL2 washed (a), CEL2+GA (b) and CEL2+GA+CA (c)

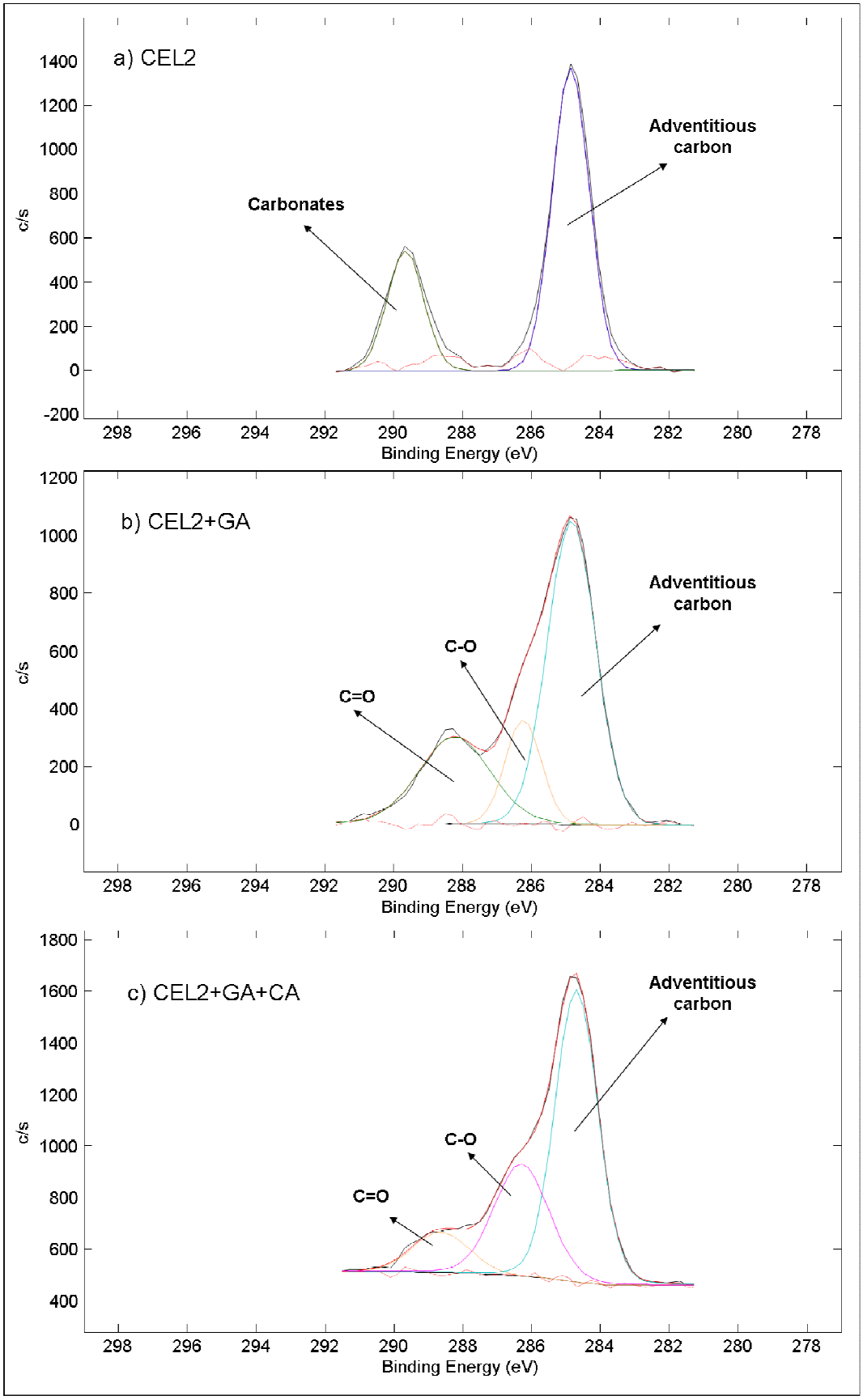


Figure 6: XPS detailed analysis of oxygen region for SCNA washed (a), SCNA+GA (b), SCNA+GA+CA (c)

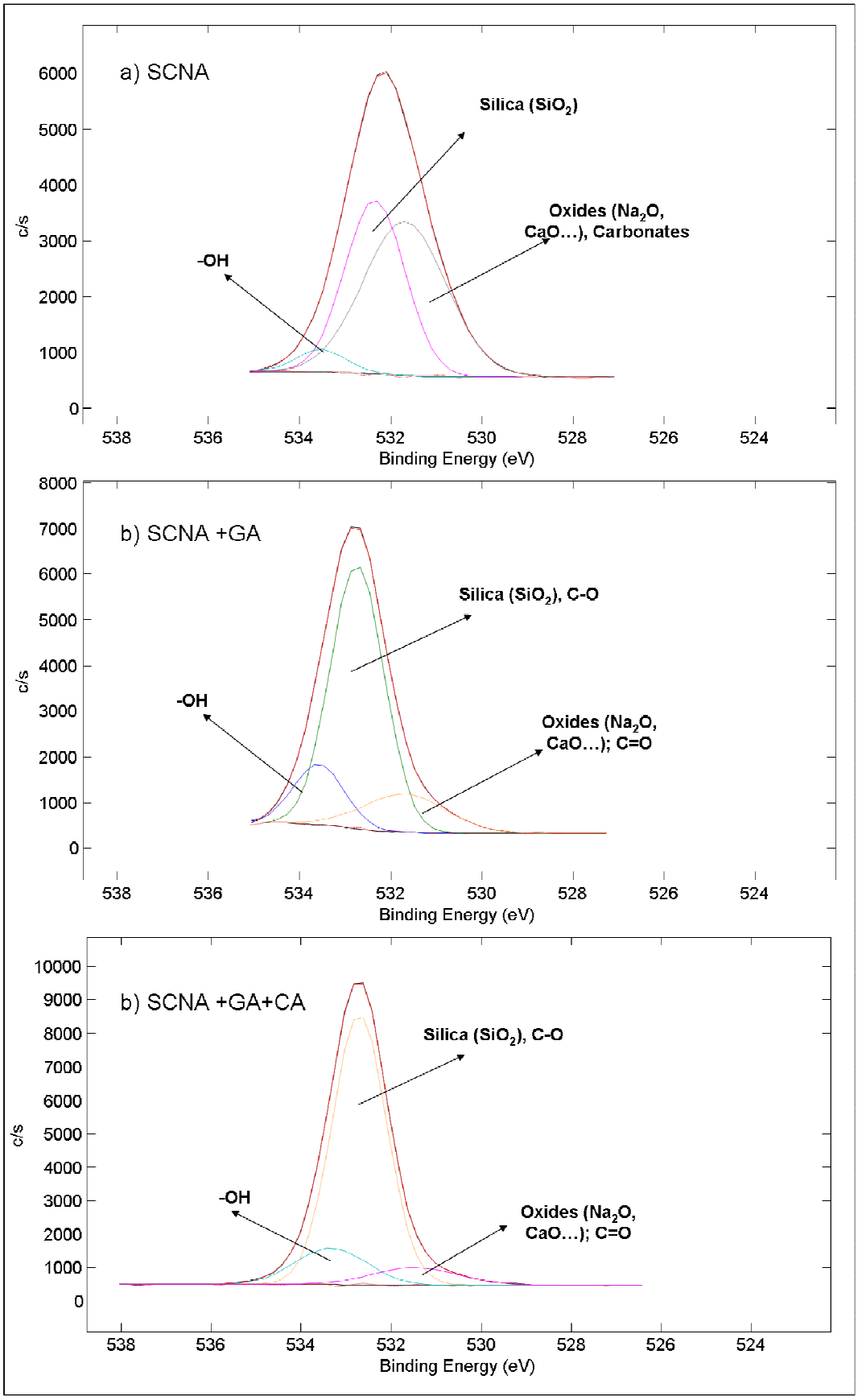


Figure 7: XPS detailed analysis of oxygen region for CEL2 washed (a), CEL2+GA (b) and CEL2+GA+CA (c)

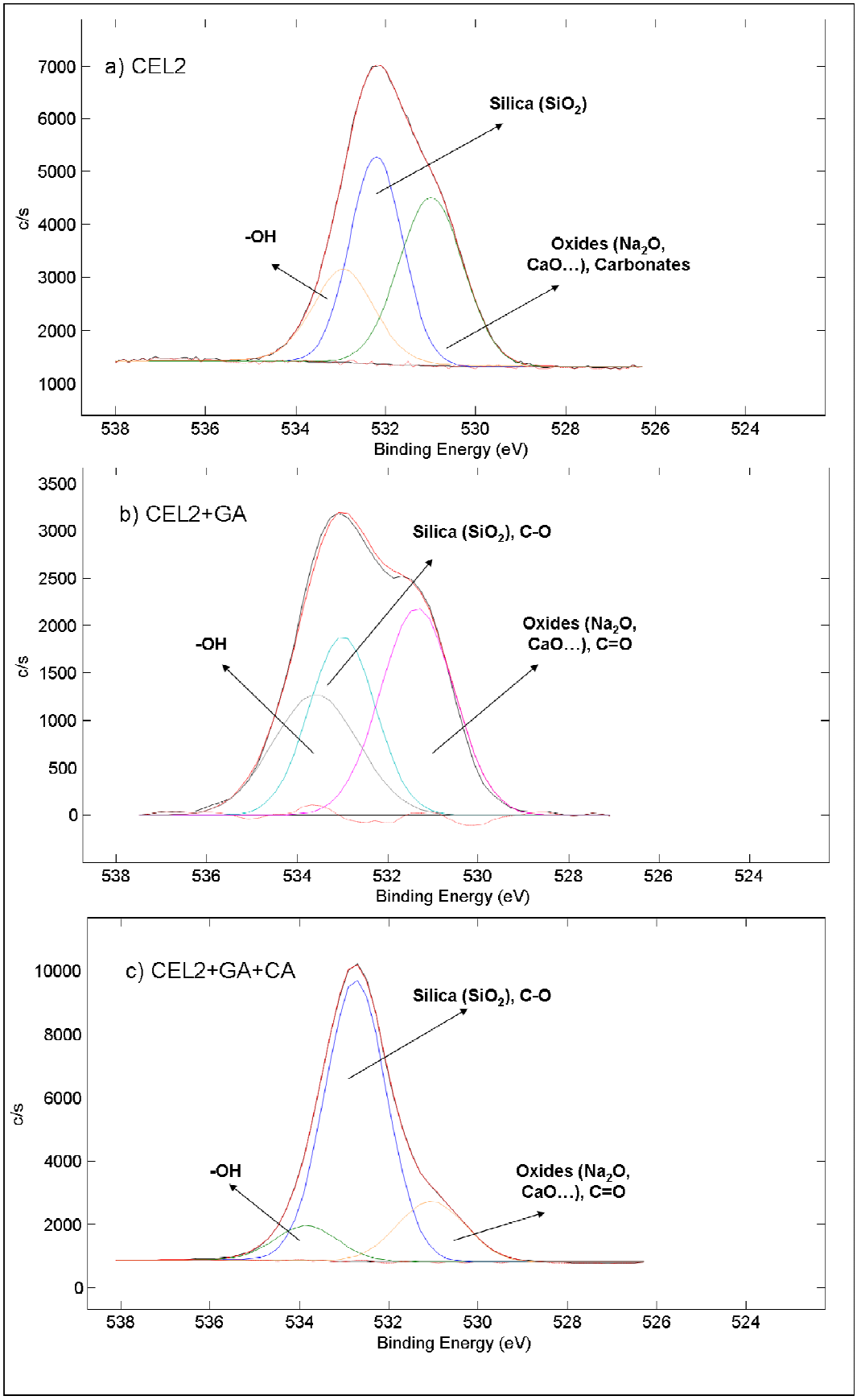
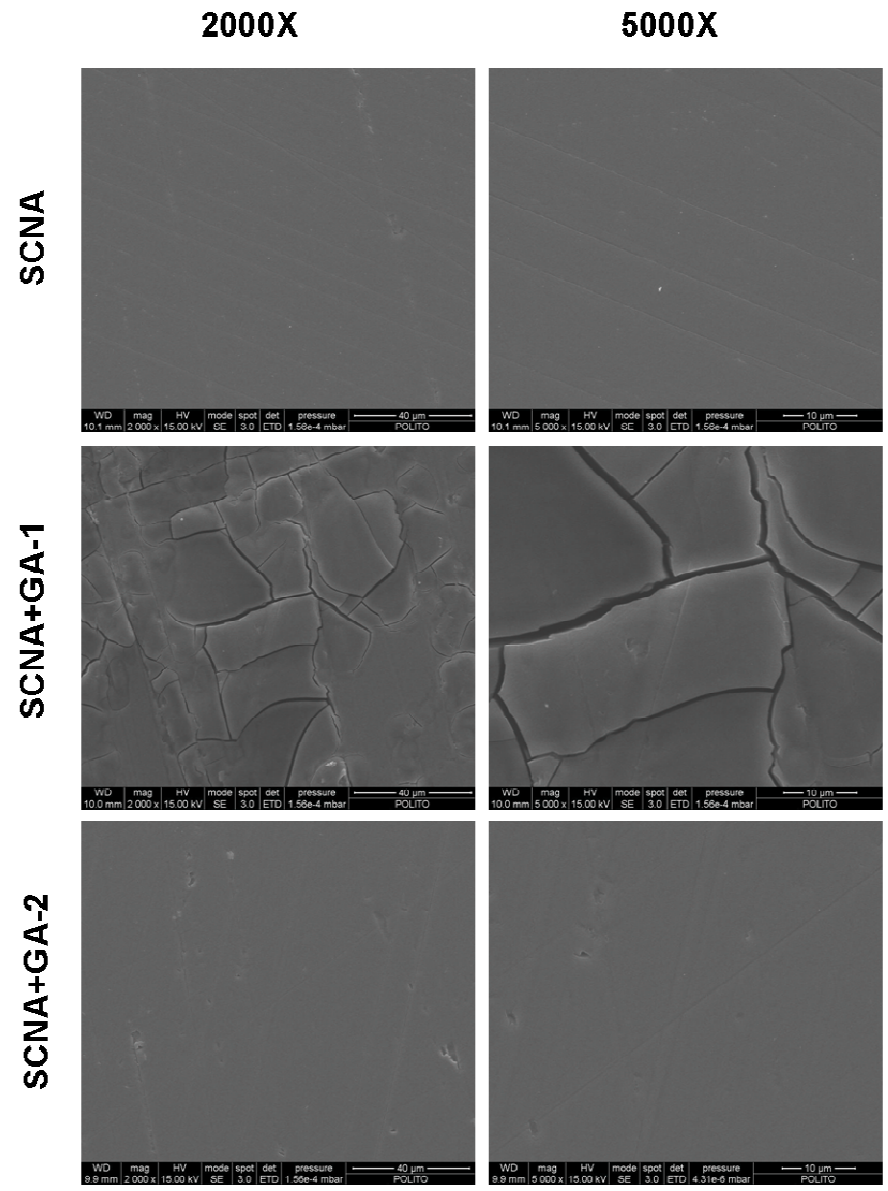
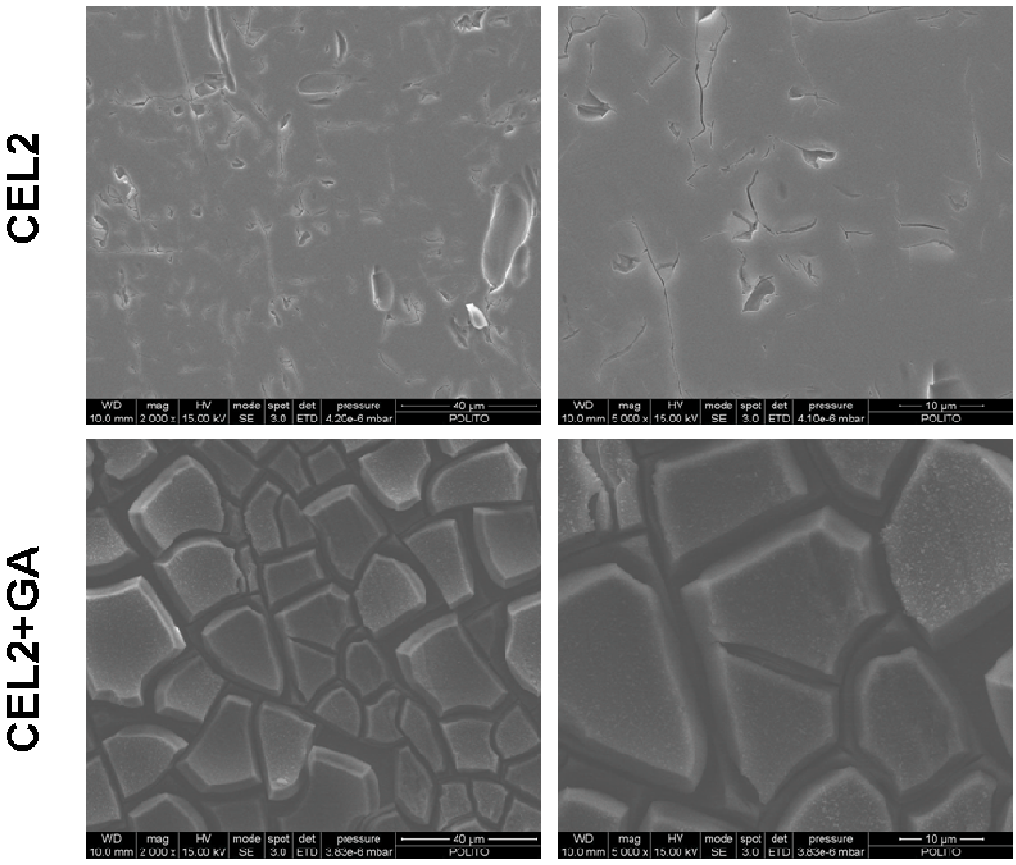


Figure 8: SEM images and EDS analyses of SCNA washed and GA-grafted



Element	SCNA	SCNA+GA-1	SCNA-2
C	7.95	8.77	6.96
O	52.38	58.74	54.04
Na	3.87	1.25	3.53
Al	2.45	2.53	2.35
Si	21.19	23.77	21.83
Ca	12.17	4.93	11.29

Figure 9: SEM images and EDS analyses of CEL washed and GA-grafted



Element	CEL2	CEL2+GA
C	8.57	38.63
O	49.63	44.03
Na	8.89	1.21
Mg	3.42	1.19
Si	15.84	5.18
P	2.26	2.88
K	2.54	0.44
Ca	8.85	6.43

Table 1: Glasses molar composition and melting/annealing conditions

	SCNA	CEL2
	Composition [Mol %]	
SiO_2	57	45
P_2O_5	-	3
CaO	34	26
MgO	-	7
Na_2O	6	15
K_2O	-	4
Al_2O_3	3	-
Melting	1h, 1550°C	1h, 1400°C
Annealing	10h, 600°C	12h, 500°C

Table 2: XPS atomic percentage of elements in the analyzed samples

	SCNA			CEL2		
Element [%at]	WASH	GA	GA+CA	WASH	GA	GA+CA
O	44,6	48,0	49.0	45,1	46,8	51.7
C	33,5	35,7	29.8	34,7	37,8	17.0
Si	14,9	14,2	19.9	5,7	8,9	16.4
Ca	4,8	1,1	0.2	6,8	5,0	2.3
Al	1,4	-	-	-	-	-
Na	0,8	-	-	4,3	0,5	0.7
P			-	1,8	0,9	0.2
Mg			-	1,3	<0,1	11.6
Other	-	1,1	1.1	<0,5	-	0.1