Surface functionalization of bioactive glasses with natural molecules of biological significance, part II: grafting of polyphenols extracted from grape skin

Surface functionalization of bioactive glasses with natural molecules of biological significance, part II: grafting of polyphenols extracted from grape skin

Xin Zhang[1], Sara Ferraris[1], Enrico Prenesti[2], Enrica Verné[1]

[1] Politecnico di Torino, Applied Science and Technology Department, C.so Duca degli Abruzzi 24, Torino 10129, Italy.

[2] Università degli Studi di Torino, Department of Chemistry, Via Pietro Giuria 7, Torino 10125, Italy.

Corresponding author:

Sara Ferraris

e-mail: sara.ferraris@polito.it

Phone: 0039-0110905768

Fax: 0039-0110904624

[1]**Abstract**

Polyphenols, as one of the most important family of phytochemicals protective substances from grape fruit, possess various biological activities and health-promoting benefits, for example: inhibition of some degenerative diseases, cardiovascular diseases and certain types of cancers, reduction of plasma oxidative stress and slowing aging. The combination of polyphenols and biomaterials may have good potential to reach good bioavailability and controlled release, as well as to give biological signaling properties to the biomaterial surfaces. In this research, conventional solvent extraction was developed for obtaining polyphenols from dry grape skins. The Folin&Ciocalteu method was used to determine the amount of total polyphenols in the extracts. Surface functionalization of two bioactive glasses (SCNA and CEL2) was performed by grafting the extracted polyphenols on their surfaces. The effectiveness of the functionalization was tested by UV spectroscopy, which analyzes the amount of polyphenols in the uptake solution (before and after functionalization) and on solid samples, and XPS, which analyzes the presence of phenols on the material surface.

Key words: surface functionalization, bioactive glasses, polyphenols, extraction

Introduction

Grapes as table food and winemaking sources had a long and abundant history, dated to the Ancient Greek and Roman civilizations. Due to the various nutrient elements in grape, such as vitamins, minerals, carbohydrates, edible fibers and phytochemicals, nowadays substantial scientific attention has been focused on the potential biomedical

[1] **Abbreviations**
GA Gallic Acid
PH phenols extracted from grape skins
CA Citric Acid

effect of grapes and grape products. Various biomedical research studies have been centered on the investigation of the role of nutrients from grape in several areas including cardiovascular health, cancer development and progression, Alzheimer's disease and other neurodegenerative disorders, aging and alterations in cognitive and motor function, antiviral activity, oral health, immune function, and diabetes [1-2]. Among the phytochemicals protective substances in grape, polyphenols play an important role according to their notable biomedical properties and health-promoting benefits [3]. The phenolic compounds mainly include simple phenols, phenolic acids (both benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins. In plants, phenolic compounds may act as phytoalexins, antifeedants, and attractants for pollinators, contributors to the plant pigmentation, antioxidants, and protective agents against UV light, among others [4]. In grape skin, the phenolic compounds usually contain proanthocyanidins, ellagic acid, mycicetin, quercetin, kaempferol, trans-resveratrol, etc. [5]. Pastrana-Bonilla's [6] research, have quantified the antioxidant activity of muscadine grape skin extracts as 12.8 μmol TE/g expressed according to the Trolox Equivalent Antioxidant Capacity (TEAC) assay. Falchi et al. [7] have found that the ischemic reperfusion injury was significantly inhibited in the rats isolated of heart after 30 days' consumption of the extracts from flesh and grapes skin which exhibited equal effect of cardioprotection. Hudson et al. [8] have reported that extracts from grape skin could induce prostate tumor cell lines apoptosis with high efficiency.

In order to obtain these useful components, different extraction methods of polyphenols have been developed. Liquid-liquid extraction is the main method used for grapes [9]. The extraction conditions of phenolic compounds are influenced by solvent, temperature, extraction time and the ratio of sample-to-solvent. The common solvents used for extraction includes ethanol, methanol, acetone or formic acid and water in different ratio. Besides, some improved methods have been developed Hong et al. [10] used microwave-assisted extraction technique to optimize the isolation of phenolic compounds from grape seeds. Ultrasound-assisted extraction has been employed to extract resveratrol from grapes [11]. In addition, supercritical fluid

extraction [12] and aqueous two-phase system extraction [13] have made advances in grape phenol extraction. Total phenolic content is typically determined by way of the Folin&Ciocalteu method using gallic acid as standard [14]. In order to improve the molecular stability and bioavailability of the molecules of biological interest, an interesting approach their grafting on a carrier. Polymeric matrices have been widely used as carrier for osteogenic growth factors [15, 16]. It is also possible to graft biomolecules on the surface of biomaterials for implants with different grafting strategies such as simple adsorption, covalent bonding or release from a degradable carrier [17, 18]. In this way the functionalized biomaterial surface acts as localized carrier for the biological principle and this represents a challenging strategy to send specific signals to cells and tissues via *in situ* delivery. Many research papers deal with the surface grafting of proteins, enzymes and drugs [15, 16, 19-21] on different kinds of biomaterials, although few are related to polyphenols and natural biomolecules [22, 23].

Among the different classes of biomaterials, bioactive glasses have been widely studied for orthopedic and dental applications, due to excellent biocompatibility and osteoinduction ability [24, 25]. A number of surface functionalization techniques made it possible to graft, on bioactive glass surfaces, various kind of biomolecules such as proteins, growth factors and enzymes [26, 27], however, few research have been focused on the combination of bioactive glass ceramics and natural bioactive phytochemicals. Some researches indicated phenol functionalization of carbon nanotubes to improve their mechanical and dynamic properties [22]. It has been observed that the incorporation of resveratrol (stilbenes from grapes, peanuts and berries) with porous poly-ε-caprolactone (PCL) surface can improve the alkaline phosphatase (ALP) activity of rat bone marrow stromal cells and can enhance mineralization of the cell-scaffold composites in vitro [23].

The aim of this research is to graft polyphenols, extracted from grape skin, to bioactive glasses in order to combine the bioactive properties of glass with the biological activities and health-promoting benefits of polyphenols. Compared with the commercial molecules, the utilization of nutraceuticals from natural sources offers the

opportunity to exploit the waste products of food industry for obtaining high added value products for healthcare applications. In this paper, attention is mainly paid to the extraction of polyphenols, the grafting conditions and the analysis after surface functionalization.

Materials and methods

Extraction of phenol compounds from grape skin

Fresh red grapes (Barbera) were provided by a small-scale producer in the north of Italy (vineyard situated in Vaglio Serra, Asti, Piedmont, Italy). The fresh grapes were divided into four parts: flesh, skin, seed and stem (in this case only grape skin was employed). Fresh red grape skin was dried in oven at 60°C and then grinded into small pieces.

Conventional solvent extraction was performed in a water-ethanol mixture (20:80, volume ratio) with the solid-liquid ratio 1:20 [28]. The extraction temperature was of 60° C and the extraction time of 60 min in a thermostatic bath. The extraction solution was separated from the grape skin through filtering and put into an oven, at 60° C, until the ethanol was completely evaporated. The extract was finally freeze dried and weighted. Phenols extracted from grape skins will be indicated as PH from now on.

Determination of total phenol content

Total phenol content of the extracts was determined by the Folin&Ciocalteu method [14]. In brief, 2 ml of the test solution were introduced in the test bottle and add 6 ml double distilled water, followed by adding 0.5 ml Folin&Ciocalteu reagent (Folin&Ciocalteau phenol reagent 2 M with respect to acid, 47641, Sigma Aldrich). After 3-5 minutes mixed, 1.5 ml Na_2CO_3 (20%) was added. The final solution was tested after 2 h reaction by UV absorption at 760 nm (CARY 500 VARIAN). 3 different samples were prepared and tested for each type of functionalization.

Gallic acid (GA 97.5-102.5% titration, G7384, Sigma Aldrich) was used as standard

for phenols quantification. 6 gallic acid solutions with increasing concentration (0.0025, 0.005, 0.01, 0.02, 0.03 and 0.04 mg/ml)were prepared and tested with the Folin&Ciocalteau method in order to obtain the calibration standard curve. The curve was used to calculate the total phenol content of the extracts.

Materials preparation

Two kinds of bioactive glasses were considered in this research as substrates for surface functionalization: SCNA (57% SiO_2, 34% CaO, 6% Na_2O, 3% Al_2O_3 mol%), which is characterized by a simple composition and a relative low bioactivity, and CEL2 (45% SiO_2, 3% P_2O_5, 26% CaO, 7% MgO, 15% Na_2O, 4% K_2O mol %) which presents a more complex composition and an higher bioactivity.

These two glasses were previously characterized in terms of characteristic temperatures, degree of bioactivity and surface reactivity after different washing treatments for surface functionalization with biomolecules [26, 27]. Both glasses were prepared by traditional melt and quenching techniques as described in [27, 29] and considered both in the bulk and powder forms. Reagents were melted in a platinum crucible and poured on a brass plate to obtain bars, or in water to obtain a frit. Glass bars were annealed in a furnace and then cut and polished in order to obtain homogeneous slices. Glass frits were milled and sieved up to obtain powders with a grain size lower than 20 μm.

Hydroxyl exposure

The first step of functionalization consisted in a cleaning and surface activation treatment. A procedure optimized in previous works on the surface modification of these glasses was employed [26, 27]. In brief, samples were washed in an ultrasonic bath once in acetone for 5 minutes and three times in water for 5 minutes each time. After these processes, samples were dried in air at room temperature.

Polyphenol grafting

100 mg of phenol extracts were dissolved in 20 ml double distilled water and mixed for about 2 h under magnetic stirring. Samples were soaked in phenol (PH) solution for 24 h at 37°C. For each sample 5 ml of 5.0 mg/ml phenol solution was employed.

The addition of citric acid (CA) in the PH uptake solution was performed (for bulk samples functionalization) in order to evaluate the effect of pH on the glass and molecular behavior. In fact, the ion release of bioactive glasses in the functionalization medium causes an increase of pH to basic values, as discussed in the results and discussion section and in previous papers [29, 30]. 0.5 M citric acid (CA, Citric Acid Monohydrate, ACS reagent 99.0-102.0%, Sigma Aldrich) was added drop wise to the PH uptake solution up to pH 3.0. A part of samples was functionalized with CA-modified PH solutions.

Solution pH was measured before and after samples soaking on 3 different samples and indicated as mean±standard deviation.

In order to prevent the light irradiation of phenol, all the holders were covered with aluminum foils. After 24 h grafting, the phenol solution from each sample was removed to clean bottles for UV analysis, while the powders and bulks glasses were washed twice by double distilled water and dried in incubator for 12 h at 37°C and stored in the dark.

The SCNA, CEL2 grafted with phenols will be named as *SCNA+PH* and *CEL2+PH*, while the ones functionalized with citric acid addition *SCNA+PH+CA* and *CEL2+PH+CA*.

PH solutions were analyzed by means of UV-Vis Spectroscopy with the Folin&Ciocalteau method (as described in the determination of total phenol content paragraph) before and after samples soaking, in order to estimate the amount of molecule grafted onto the material surface. The same technique was applied also to solid samples in order to determine the amount and activity of grafted phenols.

XPS analyses

In order to verify the presence of grape phenols (PH) on samples surface after the

functionalization, X-Ray Photoelectron Spectroscopy (XPS, PHI 5000 VERSA PROBE, PHYSICAL ELECTRONICS) was employed; survey analyses were performed for obtaining the chemical composition of the outermost surface layer, while high resolution spectra of the most significant elements (C and O) were collected for the determination of their chemical state. Bulk samples were considered for this test. Both activated and activated and PH-grafted materials were analyzed.

Binding energies were corrected considering the standard shift respect to the C1s at 284.6 eV.

SEM observations

Scanning Electron Microscopy (SEM) observations and Energy Dispersive Spettroscopy analyses (EDS) were performed (SEM-EDS SEM-FEI, Quanta Inspect 200, EDS-EDAX PV 9900) in order to study the reactivity and consequent surface modifications of bioactive glasses during the functionalization process. Washed and PH-grafted bulk samples were considered for this test. Samples were sputter coated with a thin Cr-layer (to make them conductive) before observation.

Results and discussion

Macroscopic observations and pH measurements

Figure 1 shows the SCNA and CEL2 bulk samples and the uptake solutions before and after the functionalization process. As far as CEL 2 is concerned, it is evident that the surface of the glass changes from colorless to light brown after PH uptake. This change of color is almost absent for bulk SCNA and becomes more evident for SCNA powders and CEL2 (both bulk and powders) samples.

It must be underlined, as already said for the GA functionalization [29], that in the present setup, the two glasses were soaked in the phenol (PH) solutions, which are characterized by a fairly acid pH value (3.61±0.23, figure 1) and that they are not buffered. As described in the previous paper on GA functionalization [29] a significant ion release can be documented for bioactive glasses in aqueous media, and it is enhanced in an acidic environment [26]. The ion exchange between the glass and the solution induces a significant increase of the solution pH for static soaking models as reported in [30] and confirmed by experimental results (figure 1).

In the present work, 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 phenols after samples soaking are the following: 3.79 for bulk SCNA, 9.54 for SCNA powders, 8.53 for bulk CEL2 and 10.06 for CEL powders. These data confirm the one previously obtained with gallic acid [29].

A basic environment can favor both a further hydroxylation of the glass [26] and the oxidation of catechol groups of phenols to quinone groups [31], giving the typical brownish color that was noticed both on the glass surfaces and in the uptake solutions. The addition of citric acid in the uptake solution reduces the basification of the medium (as reported in figure 1 for bulk samples) and in particular keeps the pH value below 7.00. The change in the phenolic molecular structure has been reported at pH values of about 7.40 [31].

UV-Visible analyses

The total phenol content in the extract was determined by the Folin&Ciocalteau's method. The 5 mg/ml solution, prepared for glasses functionalization, was tested and compared with standard gallic acid solutions. Gallic acid standard solutions with 6 concentrations were prepared and tested by UV absorption photometry, to obtain a calibration standard curve for the quantification of phenols, as described in [29] and in materials and methods section. In order to determine the amount of phenols grafted to the bioactive glasses, the phenol content of the starting uptake solution was compared with the one after 24 h glass soaking.

Results are reported in figure 2 (PH functionalization series).

The first column (PH functionalization series) of the graph in figure 2 reports the phenol content in the extract solution used for grafting. The phenol concentration in the 5 mg/ml extracts solution results 0.083 mg/ml GA equivalent. As expected, this result suggest, a more complex composition of extracts if compared to a pure GA solution.

The other columns of the diagram in figure 2 (PH functionalization series) report the phenol content of solution after the reaction with bioactive glasses. It can be observed that all the concentrations after functionalization are lower than original phenol solution. Compared with SCNA, it is notable that CEL2 consumes more phenol on both bulk and powder samples (the values are 0.06078 and 0.02821 mg/ml respectively). Moreover, a higher consumption of biomolecules can be underlined for powder samples when compared to bulk ones. Both results are in accordance to the ones obtained with the same samples for gallic acid functionalization [29]. As far as the powders are concerned, the higher ability to graft biomolecules can be attributed to the higher surface area compared to bulk samples. Considering the difference between the two bioactive glasses, the higher ability of CEL2 in binding phenols can be explained with its higher reactivity. The formation of a porous silica gel layer during functionalization leads to the absorption of an additional amount of biomolecules. Moreover, the possible reduction of the phenol content due the

structural change of the molecules in basic environment must be considered, as previously done for GA functionalization [29]. Citric acid addition (figure 2, CA addition series) in the functionalization of bulk samples apparently reduce the uptake of phenols on the glasses surfaces (the reduction in GA-equivalent content is lower after the uptake). Since gallic acid and phenols are stable at acidic pH, as previously discussed in [29], it can be supposed that the strong basification induced by bioactive glasses (and especially by CEL2) soaking can lead to the partial change in the structure of the molecules and the impossibility to detect them by the Folin&Ciocalteau method. CA addition allows the maintenance of uptake solution pH below 7.00 reducing the structural oxidation of phenols. The uptake of a fraction of molecules with CA addition in the functionalization medium suggests a successful functionalization, as demonstrated by XPS analyses in the following paragraph.

Preliminary test were also performed putting the Folin&Ciocalteau reactive mixture directly on the functionalized samples in order to determine the surface grafted phenols. As already observed for GA-grafted bioactive glasses [29], the presence of a notable amount of phenols on the surface was evidenced, proving the success of functionalization. The amount is significantly higher on CEL2 than on SCNA, confirming the results obtained on the solutions and for GA grafting [29]. The Folin&Ciocalteau test on the sample surface is a measurement of the activity of phenols after grafting, in fact it determines the ability of grafted molecules to reduce Folin&Ciocalteau reagent. These measurements suggested that grape skin polyphenols are still active after the coupling with bioactive glasses.

XPS analysis

Table 1 reports the atomic percentages of elements on the surface of bioactive glasses before and after phenol functionalization, with and without citric acid addition.

The reduction in Ca and Na content after the grafting demonstrates an ion exchange between the glass and the solution due to the material reactivity. At the same time the

increase in the silicon content on CEL2 samples suggest a more evident reaction in the functionalization medium that can lead to the formation of a silica gel layer, as discussed in the SEM paragraph.

Although a good cleaning degree can be obtained by washing, a certain amount of unavoidable carbon contaminants still remain on the surfaces of SCNA and CEL2. These contaminations are always present on reactive surfaces, as widely documented in the literature [27, 32-35].

The total carbon and oxygen contents on the surface show a not completely clear trend, so, in order to discuss the presence of phenols on the glass surface, at the different steps of functionalization, the detailed analyses of oxygen and carbon regions are necessary.

The detailed analyses of carbon region for SCNA and CEL2 before and after PH grafting are reported in figures 3 and 4.

Two main contributions (at about 284.8 eV and 289.4-289.6 eV) can be observed in the detailed analyses of carbon region of SCNA and CEL2 after washings (figures 3a and 4a), as already reported in [29]. The first can be attributed to unavoidable hydrocarbon contaminations on reactive surfaces [32-35, 26], while the second one can be assigned to carbonates [36] and results higher on CEL2 samples, according to its higher reactivity.

A significant change in the C spectrum can be observed after phenols grafting (figures 3b and 4b). At first, the disappearance of carbonate signal can be underlined on both glasses. At the same time, the appearance of two new contributions (at about 285.93-286.16 eV and 288.08-288.79 eV) was observed. They can be attributed to C-O and C=O respectively [37-39]. The same signals, with a higher intensity, were obtained on SCNA and CEL2 grafted with pure gallic acid [29]. As observed for GA-grafted glasses [29] CA addition in the functionalization medium induces a reduction in the C=O signal as indication of a lower oxidation of phenolic structures to quinones (figure 3c and 4c). This phenomenon is less evident for CEL2 samples; in fact also the solution pH is closer to values favorable for of oxidation of phenols to quinones (fig. 1).

Looking at the oxygen region of washed samples (figures 5a and 6a), the typical signals of oxides (530.8 – 531.7 eV), silica (531.6-532.3 eV) and hydroxyls groups (533.2 – 533.5 eV) can be observed, as already reported for surface activated glasses [13, 26-29].

After phenol coupling, silica signal is still present on both surfaces together with OH groups one. Hydroxyls peak is a first confirmation of the grafting of phenolic structures. The signal at about 531.6 eV can be attributed to both oxides and C=O groups [40]. Its noticeable presence after PH coupling can confirm the partial oxidation of phenols to quinones in basic environment. In fact citric acid addition (figure 5c and 6c) leads to the reduction of this peak in the oxygen region, as previously underlined also for the carbon region. These results are in accordance with the ones obtained for GA grafted bioactive glasses [29].

SEM observations

The surface of washed SCNA (figure 7 first row) appears homogeneous with no topographical features of interest, polishing lines are slightly visible. Any significant alteration in the morphology can be detected after the functionalization (figure 7, second row). The chemical analysis does not evidence significant variation.

CEL2 presents a completely different behavior (figure 8). After washing some pits and cracks are visible but a cracked surface layer, with the typical appearance of silica gel, can be observed after the functionalization. It has already been detected on GA-grafted CEL2 [29] and can be attributed to the glass reactivity in the functionalization medium. EDS analyses confirm the surface reactivity, the decrease in the Na, Ca and Mg content can be ascribed to the ionic exchange between the glass and the solution. Moreover, the significant increase in the carbon content can be due to the incorporation of organic molecules on the surface.

Considering UV, XPS and SEM analyses, it can be hypothesized that a part of the molecules could be covalently grafted to the glass surface and, in the case of the most reactive glass, a part could be adsorbed in the silica gel layer. An analogous

hypothesis has been supported by the authors for alkaline phosphatase grafting onto bioactive glasses [27, 41]. However, since in this paper we use a mixture of polyphenols extracted from natural sources (characterized by a wide set of molecular structures), that has not been speciated, this subject is of hard investigation. Further analyses will be performed in order to investigate more in depth this point.

Conclusions

Natural polyphenols were extracted from red grapes by water-ethanol extraction. The presence of phenols and their chemical activity in the extracts were verified by the Folin&Ciocalteau method.

The biomolecules obtained from the extraction were employed for the surface functionalization of two different bioactive glasses with the aim of obtaining smart biomaterials with both inorganic and biological properties.

UV absorption analysis, of phenol solutions before and after glass soaking and of solid samples after reaction with Folin&Ciocalteu reactive mixture, reveal that there is a significant level of capture of the biomolecules by the materials, and this increases with the glass reactivity and the surface area. Moreover, XPS investigations detected the signal of the characteristic groups of the biomolecules on the material surface after grafting. Citric acid addition in the functionalization medium made possible to preserve the phenolic structure avoiding oxidation.

This research work demonstrates that natural biomolecules can be coupled with synthetic inorganic materials. This result is extremely interesting in the surface functionalization field, since it makes it possible to prepare smart biomaterials able to combine the typical inorganic activity of bioactive glasses with specific biological properties of natural molecules (anti-oxidant, antibacterial, antiviral, anticancer and so on).

Release tests and in vitro biological analyses are planned. Moreover, analysis will be performed to determine the components speciation of the extracts.

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Figures and tables

Figure 1: Glasses and uptake solutions appearance before and after functionalization and pH measurements (mean±standard deviation).

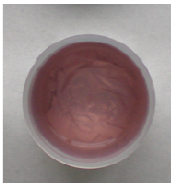
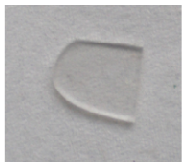
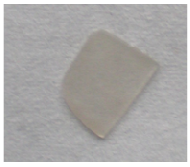
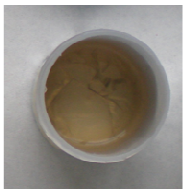
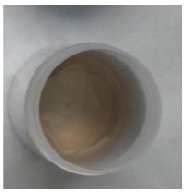
<table>
<tr><td>SCNA</td><td>SCNA+PH</td><td>SCNA+PH+CA</td><td rowspan="8">PH
 
pH=3.61±0.23</td></tr>
<tr><td>  </td><td>  </td><td>  </td></tr>
<tr><td></td><td>PH SCNA</td><td>PH+CA SCNA</td></tr>
<tr><td></td><td> 
pH=3.79±0.06</td><td> 
pH=3.12±0.12</td></tr>
<tr><td>CEL2</td><td>CEL2+PH</td><td>CEL2+PH+CA</td></tr>
<tr><td>  </td><td>  </td><td>  </td></tr>
<tr><td></td><td>PH CEL2</td><td>PH+CA CEL2</td></tr>
<tr><td></td><td> 
pH=8.53±0.03</td><td> 
pH=6.82±0.46</td></tr>
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Figure 2: UV analysis PH uptake solution before and after 1 day soaking of the different glass samples(PH functionalization series) and buffering effect of the citric acid addition on bulk samples (CA addition series)

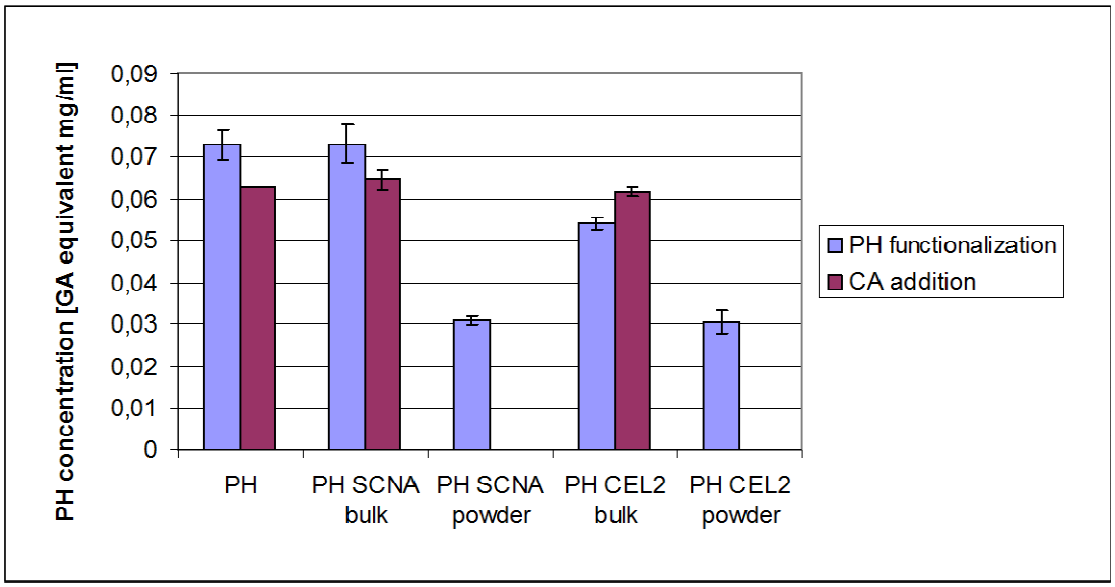


Figure 3: XPS detailed analysis of C region for SCNA (a), SCNA+PH (b) and SCNA+PH+CA (c)

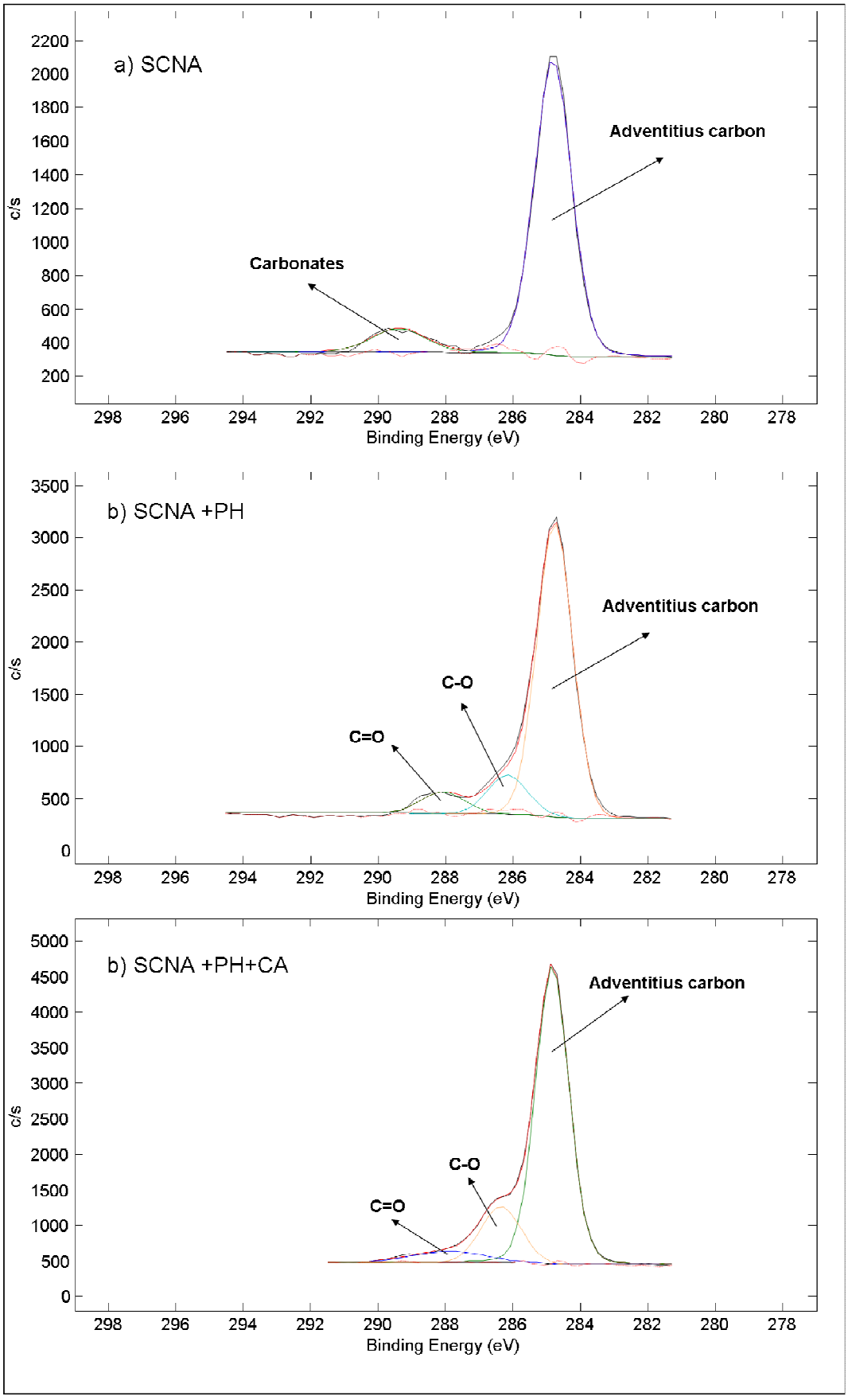


Figure 4: XPS detailed analysis of C region for CEL2 (a), CEL2+PH (b) and CEL2+PH+CA (c)

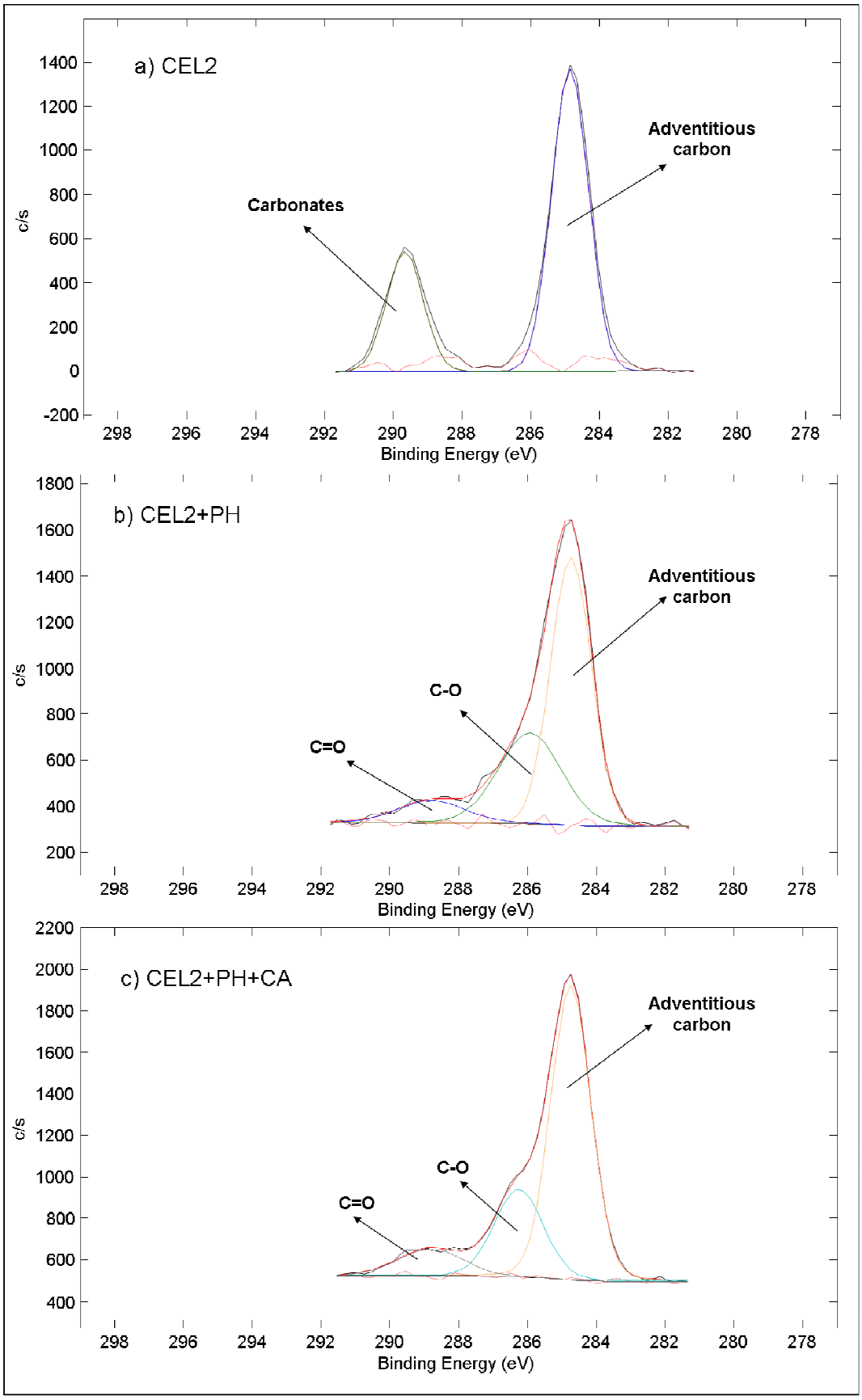


Figure 5: XPS detailed analysis of O region for SCNA (a), SCNA+PH (b) and SCNA+PH+CA (c)

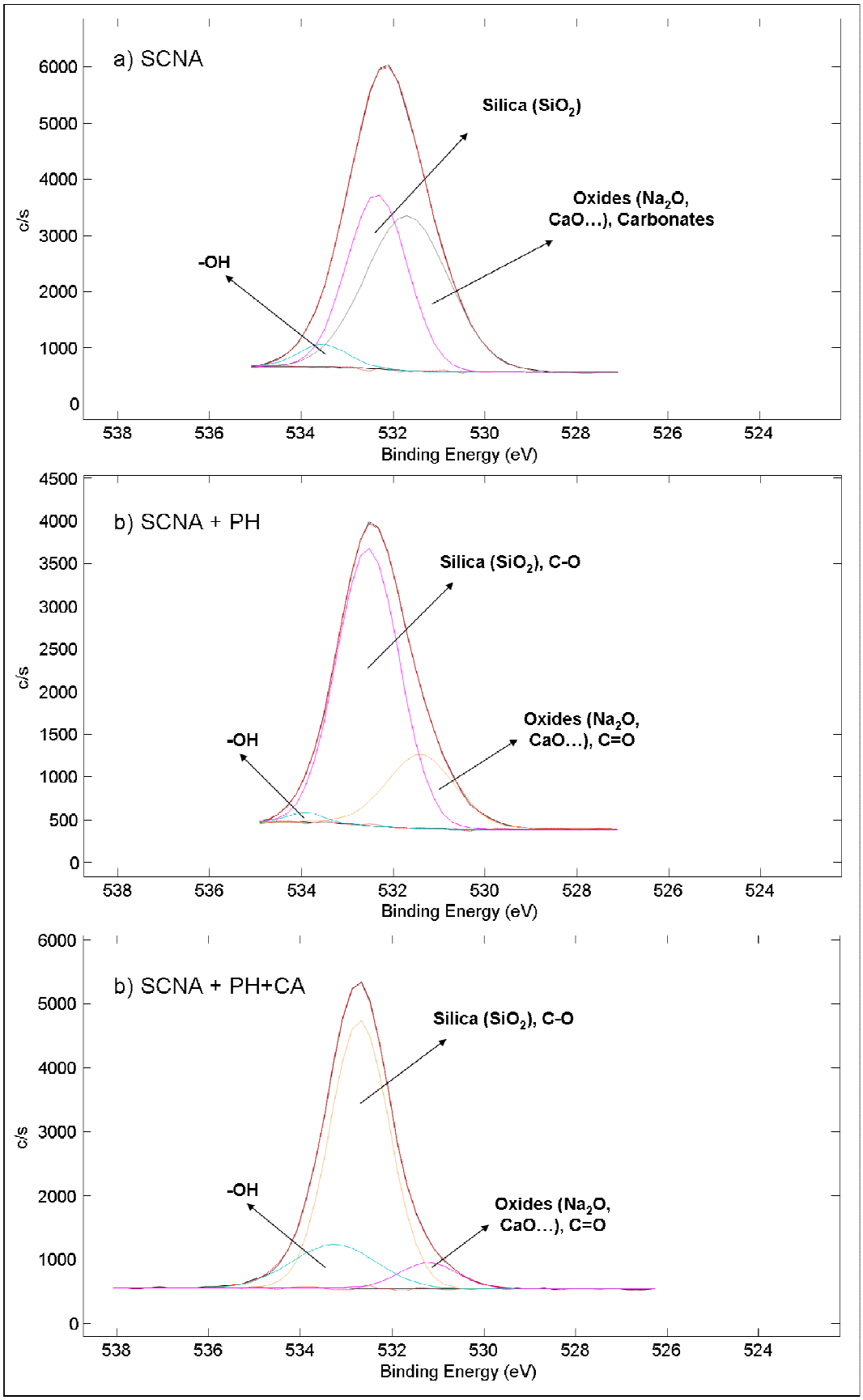


Figure 6: XPS detailed analysis of O region for CEL2 (a), CEL2+PH (b) and CEL2+PH+CA (c)

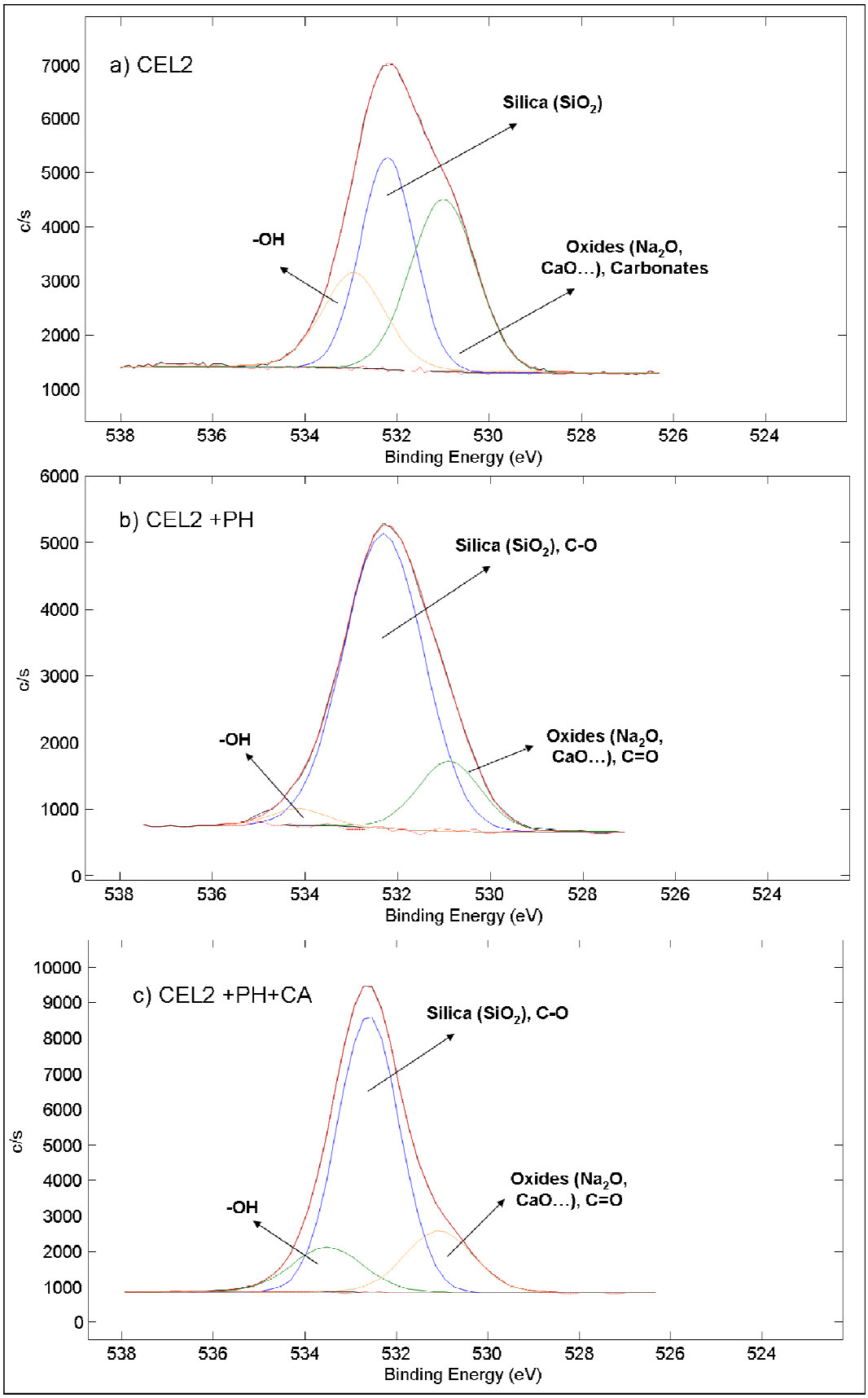
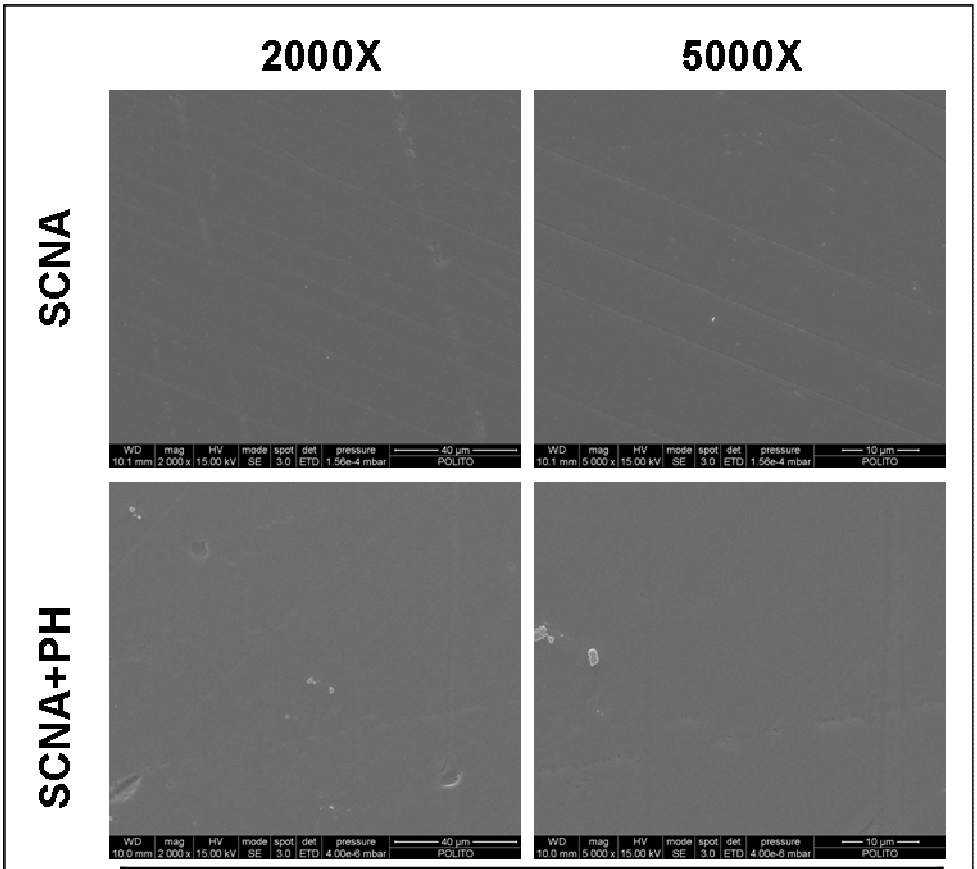
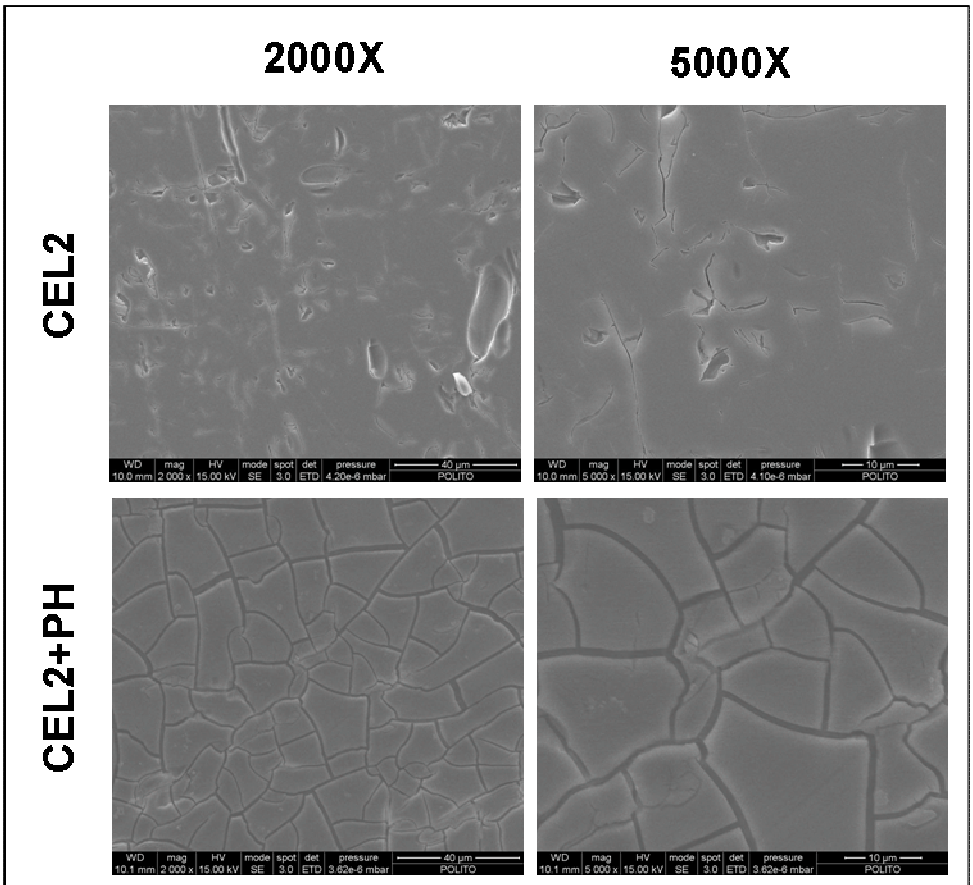


Figure 7: SEM images and EDS analyses of SCNA before and after phenols grafting



Element	SCNA	SCNA+PH
C	7.95	8.04
O	52.38	51.85
Na	3.87	3.69
Al	2.45	2.58
Si	21.19	20.95
Ca	12.17	12.90

Figure 8: SEM images and EDS analyses of CEL2 before and after phenols grafting



Element	CEL2	CEL2+PH
C	8.57	28.63
O	49.63	47.84
Na	8.89	1.91
Mg	3.42	2.44
Si	15.84	5.09
P	2.26	5.73
K	2.54	0.65
Ca	8.85	8.07

Table 1: XPS atomic percentages of elements in the analyzed samples

	SCNA			CEL2		
Element [%at]	**WASH**	**PH**	**PH+CA**	**WASH**	**PH**	**PH+CA**
O	44.6	32.6	31.9	45.1	49.0	49.1
C	33.5	52.0	57.3	34.7	28.6	20.7
Si	14.9	11.1	10.1	5.7	14.2	15.9
Ca	4.8	2.0	-	6.8	-	2.8
Al	1.4	-	-	-	-	-
Na	0.8	0.3	-	4.3	1.1	-
P	-	-	-	1.8	-	0.3
Mg	-	-	-	1.3	7.2	11.3
Other	-	<2.1	0.8	<1.8	<0.2	<0.1