Babeș – Bolyai" University, Faculty of Physics

Synthesise and characterisation of bioactive glasses loaded with Ascorbic Acid

Ph.D. Thesis

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Key words : Sol-gel, Xerogel, Aerogel, Ascorbic Acid(Vitamin C), Bioactivity, Bovine serum Albumin (BSA)

Contents

Introduction

The aim of this thesis is to synthesize and characterize new materials with possible future application in tissue engineering and regenerative medicine.

Regenerative medicine/tissue engineering is a rapidly growing multidisciplinary field involving scientists, engineers, and physicians to develop biological substitutes that can mimic tissues for diagnostic and research purposes and can replace (or help regenerate) diseased and injured tissues. The properties and applications of materials (synthetic and natural) that are used in tissue regeneration are commonly called biomaterials [1, 2]. Some of the most promising biomaterials for application in bone tissue engineering are bioceramics such as hydroxyapatite (HA), calcium phosphates, bioactive glasses and related composite materials. In case of silicate based bioactive glasses the reactions on the material surface induce the release and exchange of critical concentrations of soluble Si, Ca, P and Na ions, which can lead to favourable intracellular and extracellular responses promoting rapid bone formation [3, 4]. With respect to biomaterials used in bone tissue applications, it has been shown that the presence of oxygen-derived free radicals can lead to a gradual decrease in bone formation. The importance of introducing antioxidants in these systems to create a suitable substrate that favourites the growth of osteoblast and to diminish the activity of osteoclasts, shows a significant increase in the last years [5, 6, 7].

The work of this thesis is based on preparation and characterization of sol- gel derived Xerogel and Aerogel glasses loaded with Ascorbic Acid in different ways. The bioactive property of the materials is given by bioactive glass in the formula $56SiO_2 – 40CaO – 4P_2O_5$. For preparation, sol-gel method was chosen, because it permits a low temperature preparation and a better controllability of the bioactive glass structure and morphology.

This thesis beside this introduction which presents the motivation and objectives is divided in five chapters. The first chapter presents the literature aspects of biomaterials, incorporating background information about sol-gel method, xerogel and aerogel formation, focused on surface and protein interaction and on the importance of vitamin C in bone formation. The second chapter gives a description of the characterization techniques used for analysis of the materials in this research work and describe also the protocols for in-vitro studies. The third chapter gives a detailed description of the analyses of the pharmaceutically used Ascorbic Acid. The last two chapters present the experimental procedures both preparing and analysing the samples, and also the obtained results are discussed.

2. Experimental Devices

2.1. Differential Thermal Analyses (DTA) and Thermogravimetric analyses (TG)

The DTA and TGA experiments were carried out under air, using Shimadzu DTG-60H analyser in alumina crucibles. In order to investigate the thermal behaviour, the dried samples were heated from room temperature, with heating rate of 10 ºC/min, to 500^{o}C for vitamin C, to and to 1000 ^{o}C for the other samples.

2.2. X-ray Powder Diffraction (XRD)

The structure of the samples was analysed by X- ray diffraction with a Shimadzu XRD-6000 diffractometer using Cu Ka radiation (λ =1.5418 Å), with Ni-filter. The spectra were recorded between 10o and 80º in 2θ continuous scanning modes, with steps of 2º/ minute. The operation voltage and current were 40 kV and 30 mA, respectively. Phase identification was realised by comparing the experimental XRD patterns to standard inorganic crystal structure data (JCPDS).

2.3. Fourier-Transform Infra-Red spectroscopy

JASCO 6200 FTIR spectrometer was used to characterize the presence of specific functional groups in absorption configuration in the 4500 to 400 cm^{-1} spectral range. A small amount of each sample was mixed with potassium bromide (KBr) powder, pressed as a pellet, and analysed at a resolution of 4 cm-1 and a sample scan of 256.

2.4. Specific Surface area and porosity evaluation

The specific surface area and pore volume of the samples were determined with a Qsurf Surface analyser based on Brunauer–Emmett–Teller (BET) method using N2-adsorption– desorption

2.5. Scanning electron microscopy

Scanning electron microscopy (SEM) images were taken with a FEI Quanta 3D FEG 200/600 microscope. In order to amplify the secondary electrons signal, the samples were covered with gold.

2.6. Fluorescence spectroscopy

The Fluorescence spectra of BSA in reaction with Ascorbic Acid were performed on Jasco spectrofluorimeter FP-6300. The excitation wavelength was 295 nm and fluorescence spectra were recorded from 250 to 450 nm.

3. Characterization of Xerogel and Aerogel samples loaded with Ascorbic Acid

In order to evaluate the influence of ascorbate on the silicate glasses and conversely, there were prepared via sol-gel method: simple silica and a calco-phosphate silicate matrices, the ascorbic acid being incorporated in their compositions during the sol phase. The thermal behaviours of the samples were studied with DTA /TGA, the structure was analysed using XRD and FTIR. It was observed that, at the beginning the resulting solutions after addition of ascorbic acid to SiO_2 and SiCaP soles are colourless and transparent, but over time their colour is changed to pale yellowish. As has been reported in the literature [13], the density of the yellow coloration gradually increases over time and the colour becomes completely dark yellow as a result of oxidation. The results obtained on SiO_2 and silicates samples loaded with ascorbic acid during the sol phase of synthesis indicate also that the ascorbic acid has no negative influence on the gel formation. To improve the stability of the samples after addition of AA, and in order to prevent the drastically decrease of the specific surface area of the samples after AA addition, another loading method was applied by means of immersing dried and thermally treated gel powders in AA solution. According to the obtained results the best sample for other investigation and for vitamin C loading seems to be the thermally treated sample. The in vitro results confirm the fact (mentioned also in the literature) that vitamin C induces a more pronounced protein absorption on the examined samples surface and in the same time does not inhibit the bioactive layer formation.

One of the propose of this study was to incorporate ascorbic acid under argon atmosphere, in xerogel samples, which pH was adjusted at three different values during the sol synthesis (2, 4.5 and 7.5), and in aerogel sample dried supercritical and thermally treated at high temperature, and to characterise the structural, morphological and textural changes induced by the addition of Ascorbic Acid. The pH = 4.5 was chosen because this is the approximate pH of Vitamin C, pH 7.5 was used in order to compare xerogel and aerogel samples with same gelation time and, and pH=2 was used to see the difference between the XttAA with pH=2 were argon was not used and this one using argon for AA loading. Special attention was also focused on determining how Ascorbic Acid influence the bioactivity of the investigated samples and to investigate the protein binding capability of the Ascorbic Acid containing bioactive xerogel and aerogel samples.

3.1. Materials and Sample preparation

a) Synthesise of Xerogels with different pH

Sol – gel synthesized bioactive glasses were prepared, with a chemical composition of 56 mole % SiO2, 40 mole % CaO and 4 mole % of P2O5. The glass composition used in this study was chosen based on a previous report that demonstrated its bioactivity [8].

The precursor for SiO_2 was *tetraethoxysilane* $SiC_8H_{20}O_4$ (*TEOS*), for CaO was *calcium nitrate tetrahydrate* $Ca(NO_3)_2*4H_2O$ and for P_2O_5 was *triethyl phosphate* $C_6H_{15}O_4P$ (TEP).

The corresponding amount of *tetraethoxysilane was mixed with ethanol* and *distilled water (*H2O: TEOS: EtOH - 2:1:0.5 molar ratio*),* and stirred for one hour at room temperature on a magnetic stirrer. The precursor for CaO was dissolved also in distilled water at saturation point. The compounds were mixed together at room temperature together with TEP. Nitric acid (HNO_3 - 0.01M) was used in the synthesis, to catalyse TEOS and TEP hydrolysis. Each reactant was added in 1hour interval under continuous stirring.

The final solution was divided in three parts; each part was adjusted at different pH value 2, 4.5 and 7.5. The solutions pH was adjusted to 4.5 and 7.5 using ammonia NH_3. For adjusting the pH = 2 nitric acid was used.

b) Aerogel synthesise

Based on previous studies [9] and taking in consideration the fact that the drying procedure of the samples influence the ascorbic acid uptake and stability, aerogel type sample also were chosen for AA loading. The silicate aerogels in this study were prepared at Aerogel Lab, Department of Inorganic and Analytical Chemistry University of Debrecen, and have the same molar composition as the xerogel one described in previous section (56%SiO_2, 40%CaO, 4%P_2O_5). The chemical composition and precursors used in preparation are the same as in case of xerogel samples. The synthesis of silicate aerogels can be divided into 3 general steps:

1. Gel preparation
2. Aging of the gel and solvent change
3. Supercritical drying of the gel

After drying, the aerogel samples (figure 3.1.) were removed from the reactor and for better stability the supercritical dried samples were thermally threated at 1050 ^oC.

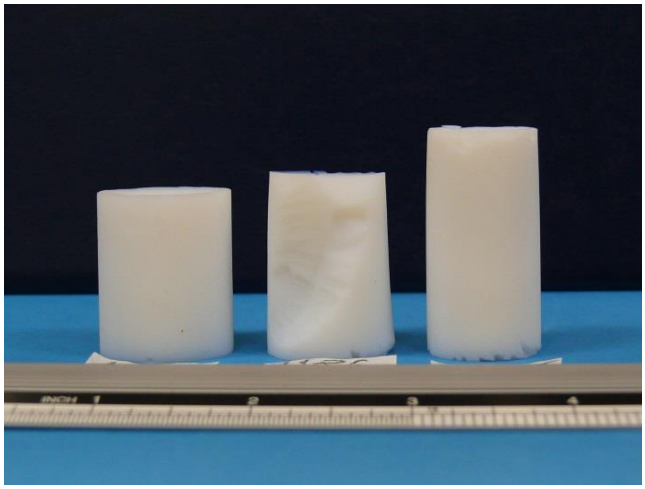


Figure 3.1.*aerogel samples after supercritical drying*

3.2. Incorporation of ascorbic acid

The incorporation of ascorbic acid in thermally treated, powder xerogel and aerogel samples was made under argon atmosphere to prevent ascorbic acid decomposition into dehydroascorbic acid in contact with air and water. In literature this technique, used to prevent the compounds from reacting with components of air (water or oxygen), is called air-free technique, and this method works best with argon, because this gas is heavier than oxygen.

The pharmaceutical grade ascorbic acid was dissolved in deoxygenated distillate water under argon atmosphere and the samples were also washed through with argon gas. The samples were kept under argon using Schlenk line, in ascorbic acid solution for 2 day after that were dried under vacuum at room temperature (Figure 3.2A and B)

(a) pH=2 *(b) pH=4.5* *(c) pH=7.5*

Figure 3.2A *the different pH samples immersed in ascorbic acid solution under argon atmosphere*

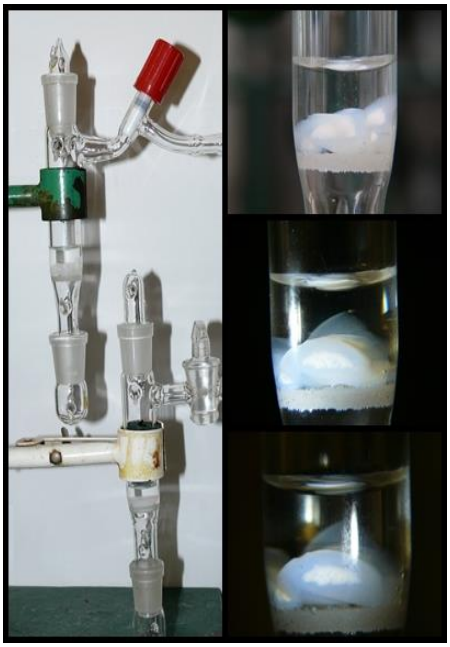


Figure 3.2B *Aerogel samples immersed in Ascorbic Acid solution under argon atmosphere.*

3.3. Results and Discussion

3.3.1. Differential thermal analyses and Thermogravimetric analyses

DTA and TGA were used to analyse the thermal behaviour of the xerogel and aerogel samples, to analyse the thermal decomposition of AA and also to see the presence of vitamin after loading.

The thermal decomposition (DTA/TG) of the ascorbic acid powder and the re-crystallised one is presented in Figure 3.3. The obtained result is in agreement with the literature data and point out a thermal stability until around 200 ^{o}C. The ascorbic acid starts to decompose around 197 ^{o}C with a sharp decomposition evidenced by an endothermic peak and the maximum rate of decomposition occurring approximately at 235 ^{o}C and with an approximately 35% of weight loss observed in the TG curve [9]. The results shows that after recrystallization the decomposition starts earlier, around 192^{o}C and the maximum rate of decomposition is also shifted to lower value (223 ^{o}C). The TG results show weight loss appropriated (34.63%) to those obtained on AA before recrystallization.

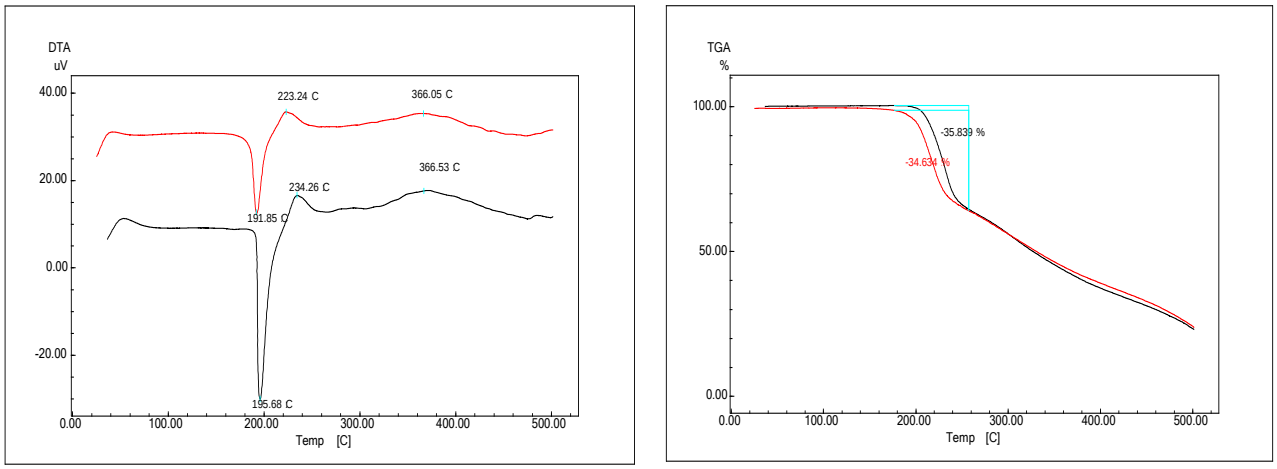
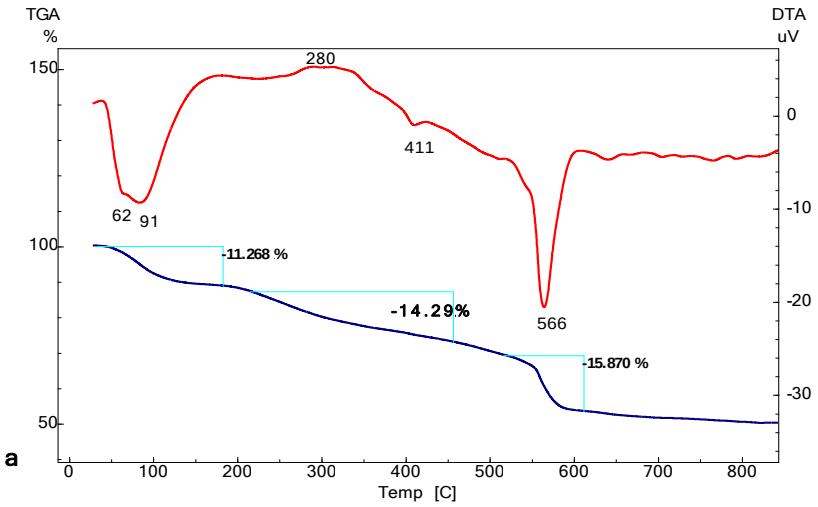


Figure 3.3 *DTA and TGA runs of pharmaceutically grade Ascorbic Acid (black) and recrystallized one (red)*

TGA/DTA curves of the three xerogel samples dried at 100 ^{o}C are presented in Figure 3.4. In each case endothermic peaks are observed in the temperature range of 50 - 125 ^{o}C, and are attributed to the loss of residual solvents such as ethanol (50-60 ºC) and water (90-95 ºC), events accompanied with an approximately 15% weight loss observed in the TG curve. The sample prepared at the pH value of 4.5 presents a third endodermic event in the mentioned temperature region (125 ºC), event attributed with water elimination that was absorbed from the atmosphere, this sample having higher hydrophilic property, fact also macroscopically visible.



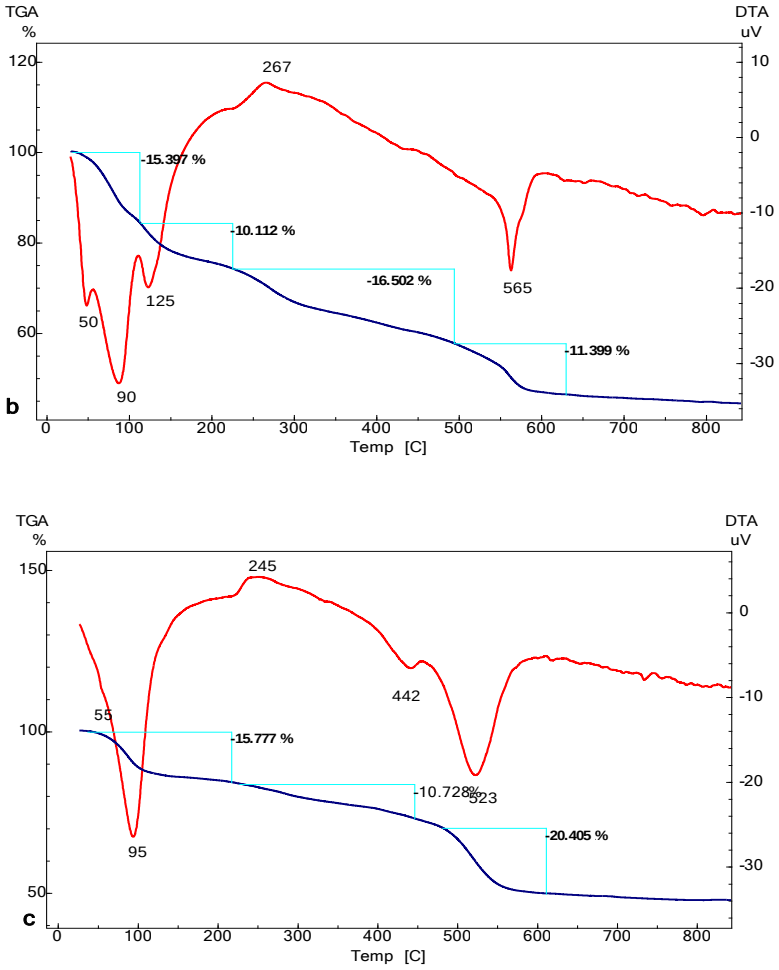


Figure 3.4 *DTA and TGA runs of sol-gel derived xerogel samples prepared at: pH= 2 (a), pH=4.5 (b), pH=7.5(c)*

Exothermic peaks are observed in 250 – 300 ^{o}C temperature range accompanied with a 15 – 20% weight loss in TG curve on each sample. This event is in agreement with the literature and can be associated with the decomposition of alkoxy groups remained from synthesis reagents. The events noticed between 400 ^{o}C and 570 ^{o}C, with a corresponding total weight loss between 25 – 30 %, are attributed to the loss of hydroxyls and nitrates (in the form of $CaNO_3 \cdot 4H_2O$ and HNO_3) used in the sol preparation [10] and the elimination of water captured in the pores. No significant weight loss can be observed above 700 ^{o}C, because this temperature is appropriate for a fully stabilization of the structures. The DTA and TGA results of the aerogel sample are presented in figure 3.5. As it was expected no significant event can be observed in the DTA curve, this means that major part of the residues from preparation are eliminated during the solvent change and supercritical CO_2 drying. The minor exothermic event at 430 ^{o}C is associated with the elimination of some residues captured in the pores.

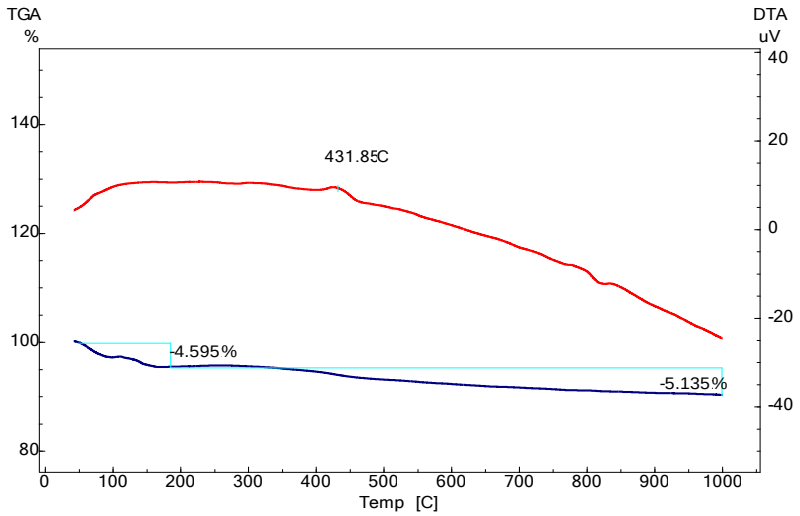


Figure 3.5 *DTA and TG runs of supercritical dried aerogel sample*.

From the TG curve an approximately 10% total weight loss can be calculated, associated with the shrinkage of the sample as revealed from diameter, height, mass and density changes during the thermal treatment. First weight loss step ~ 4.6% corresponds to desorption of water, followed by an approximately 5.2% weight loss which can be explained with the condensation of silanol groups and with the reduction of surface and collapse of the matrix.

After loading the samples with ascorbic acid, DTA/TG measurements were performed on the newly obtained samples. The results of the three AA loaded xerogel samples are presented in figure 3.6. The results show endothermic peaks in 61^{o}C – 79^{o}C temperature range corresponding to the elimination of water used in ascorbic acid loading. In TG curve this event appear with an approximately weight loss of 10%. The events noticed at 194^{o}C - X_2AA (a) and at 192^{o}C - $X_{4.5}$AA (b) are attributed to the decomposition of vitamin C, in agreement with the results obtained on pure ascorbic acid presented previously. DTA/TG results of the sample with the highest pH ($X_{7.5}$AA (c)) show 3 different events. The endothermic event at 61^{o}C associated with the elimination of water used in AA loading followed by two exothermic events at 227^{o}C and 345^{o}C also attributed to the vitamin C decomposition. In this case the decomposition of vitamin C stars later then in case of pH=2 and 4.5, and comparing the results with that obtained on pure vitamin C (described in the first part of the chapter) a possible explanation could be that the ascorbic acid decomposes with maximum decomposition rate, followed by an additional decomposition step.

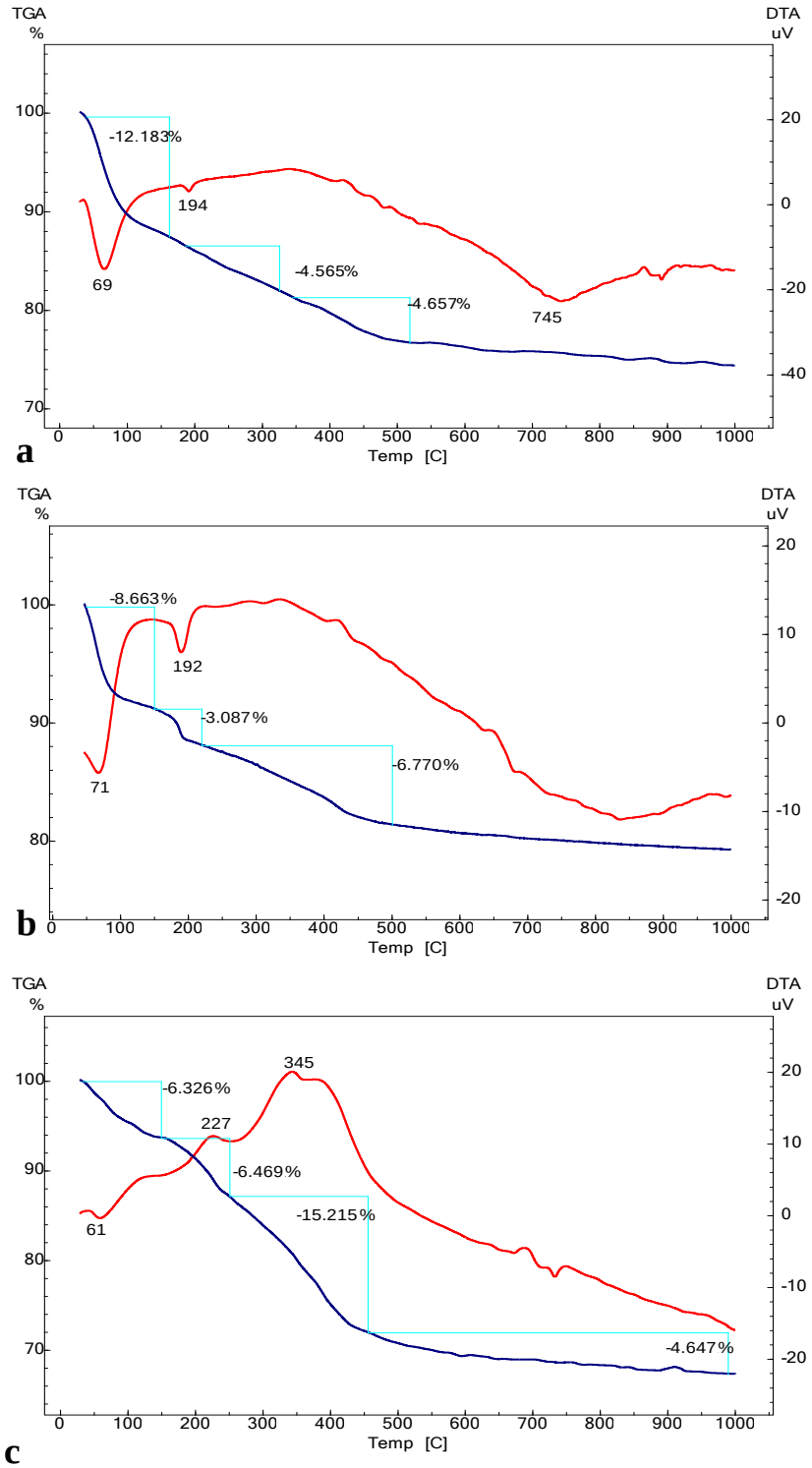


Figure 3.6. *DTA and TGA runs of sol-gel derived xerogel samples after AA loading pH=2(a), pH=4.5 (b), pH=7.5 (c)*

The Aerogel loaded sample (Figure 3.7.) present an endothermic and an exothermic event, first at 184 ^{o}C second at 346 ^{o}C attributed to the slow decomposition of vitamin C in agreement with the literature and the results obtained on pure vitamin C. In TG curve this decomposition is followed by an appropriated weight loss of 30 %.

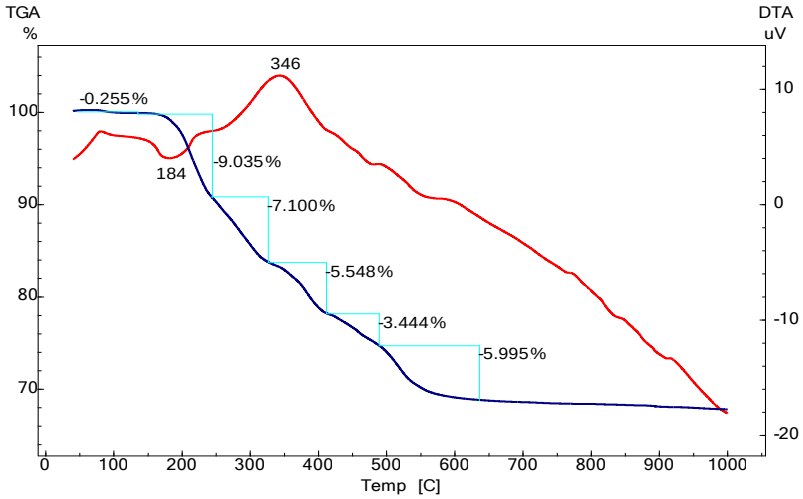


Figure 3.7 *DTA and TGA runs of sol-gel derived Aerogel sample after AA loading*

3.3.2. X-ray Diffraction results (XRD)

The X-ray diffractogram of the vitamin C (Figure 3.8(a)) presents multiple characteristic sharp peaks at 2θ varying from 5 – 80 degree which was due to the crystalline nature of ascorbic acid.

The measurements performed on the recrystallized ascorbic acid are compared with the results before dissolution and with diffraction data from the literature. Differences are observed at the characteristics diffraction peaks of ascorbic acid, at 2θ= 10.04, 24.92, 27, 28.20 and 30.10. Two peaks at 2θ = 17, 22 and 47 remains unchanged, the peaks located at 10.04, 39.46, 40 are much smaller, nearly disappeared after recrystallization. Also the small peaks located between 2θ = 50 ad 80 are less visible. These differences could be explained by the hydrogen bounding between vitamin C and solvent and the repositioning of the carbonyl (C=O) and hydroxyl (OH) groups of the vitamin C molecule [11].

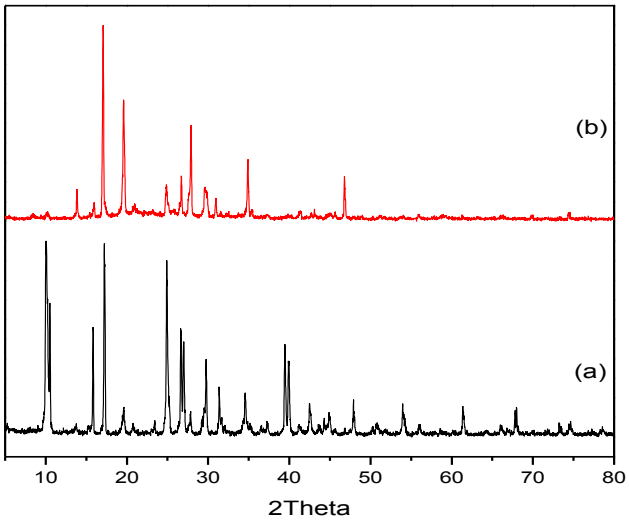


Figure 3.8 *XRD diffraction pattern of Ascorbic Acid before (a) and after recrystallization (b)*

The XRD patterns of the investigated samples displayed in figure 3.8 exhibit mainly amorphous characteristics corresponding to glass, but they show incipient crystallization of a calcium phosphate faze with the main peak centred at 2θ=32º in the form of carbonate apatite ($Ca_{10}[PO4]_6CO_3$) attributed to a carbonation process of the material, due to the high temperature treatment and atmospheric CO_2 as a consequence of the high calcium content [12, 13].

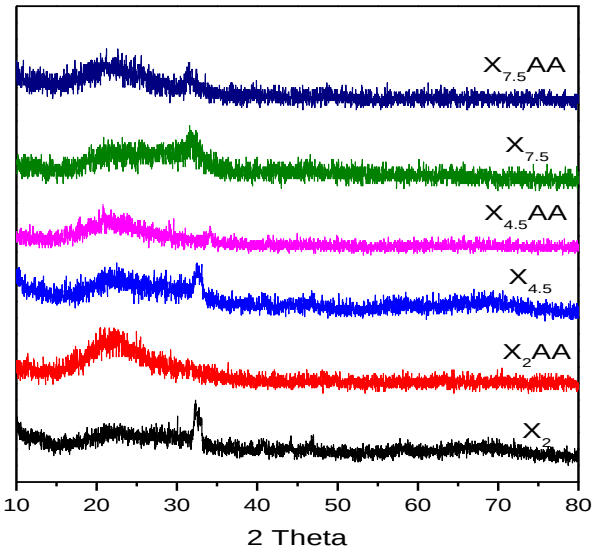


Figure 3.9 *XRD patterns for xerogel samples prepared at different pH, without and with AA*

By looking at the x-ray patterns of the loaded samples it can be observed that these do not present characteristic crystalline peaks of ascorbic acid. The sample X_2AA has no peaks from calcium phosphate, the other two samples ($X_{4.5}AA$, $X_{7.5}AA$) present small peaks of $Ca_{10}[PO_4]_6CO_3$ more visible on the sample with pH=7.5. The X-ray diffraction pattern of aerogel samples (figure 3.9) showed that both supercritical dried and 1050 ^oC thermally treated aerogels were fully amorphous, with no evidence the presence of any crystalline phase.

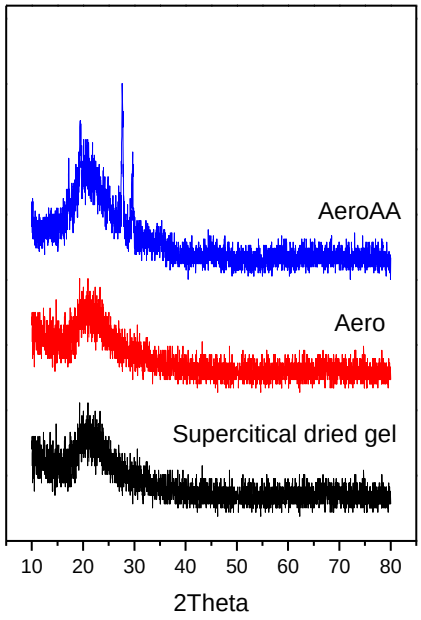


Figure 3.10 *XRD patterns of aerogel samples after supercritical drying, after thermal treatment and after AA loading*

The silica based aerogels prepared by supercritical drying are always amorphous. The sol-gel process allows an excellent control of the microstructure of silicate gels from the earliest stages of procedure. Whereas the pattern of ascorbic acid loaded aerogel indicate four peaks centred at 2θ=17.28, 19.33, 27.73, 29.78 identified as characteristic peaks for ascorbic acid described in the first part of the chapter.

3.3.3. FT-IR spectroscopy results

The FTIR spectrum of ascorbic acid (Figure 3.11) shows the following characteristic peaks 3527 cm^{-1}, 3412 cm^{-1}, 1755 cm^{-1}, 1669 cm^{-1}, 1320 cm^{-1}, 1195 cm^{-1}, 1121 $cm^{-1}$and 1024 $cm^{-}1$. The peaks at 1195 cm^{-1}, 1320 cm^{-1} and 1755 cm^{-1} were assigned to stretching and bending vibration of C=O and CH groups present on L-Ascorbic acid [14]. The band observed at 984 cm^{-1} is related to C-H bending vibrations, and that at 1669 cm^{-1} is associated to C=C ring stretching vibration of Ascorbic Acid [13,15]

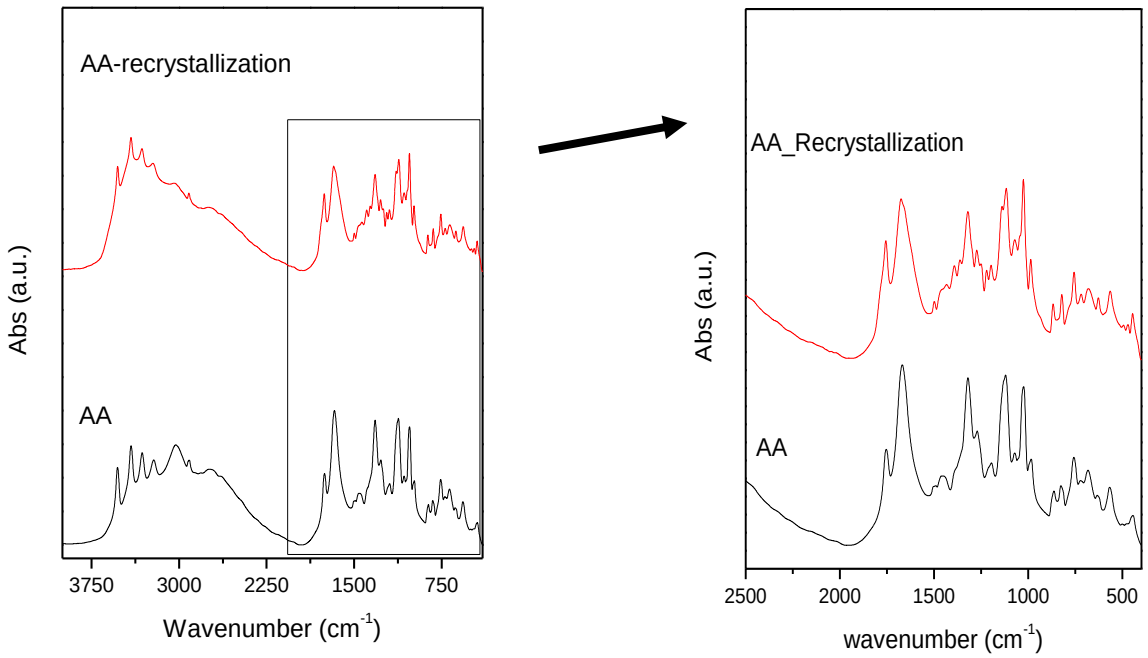


Figure 3.11 *The FT-IR spectra of pharmaceutical Ascorbic Acid before and after recrystallization*

Comparing the ascorbic acid IR spectra with the recrystallized one, from 400 cm-^{1} to 2500 cm^{-1} (Figure 3.11), small shift can be observed toward higher value at 1755 cm-1 associated with C=O stretching mode, this shift is also visible between 2900 cm^{-1} – 4000 cm^{-1} related to OH and CH groups.

The FT-IR spectra of the thermally treated xerogels before and after loading with Ascorbic Acid are presented in Figures 3.12

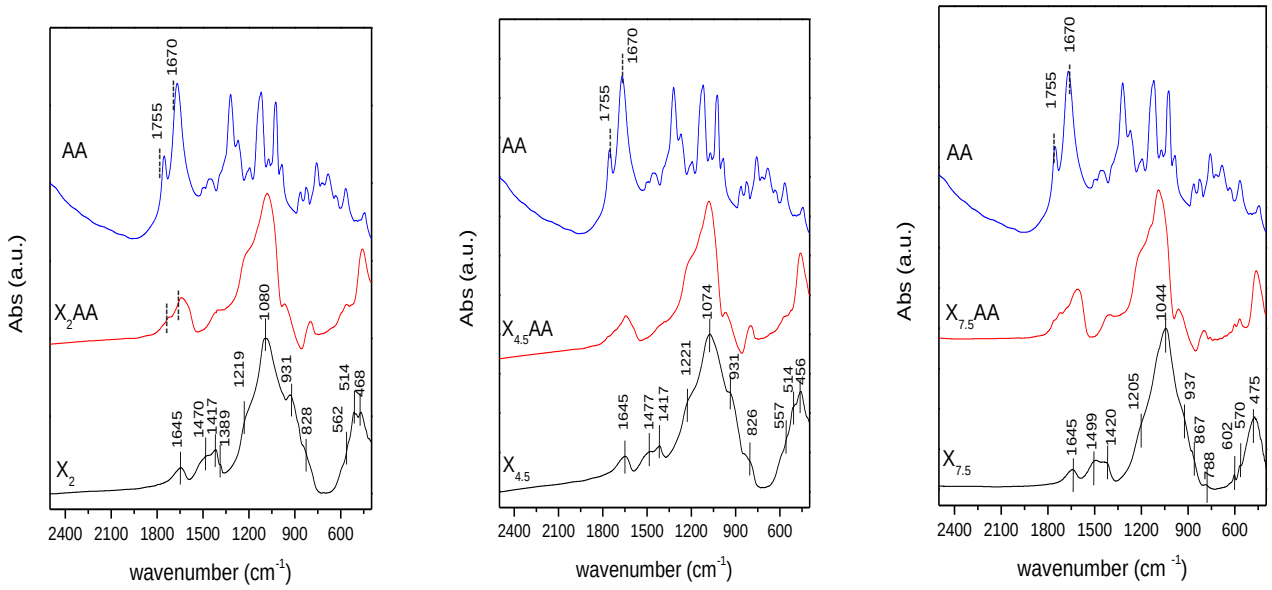


Figure 3.12 *FT-IR spectra of xerogel samples prepared at different pH without and with Ascorbic Acid*

After stabilization at 700 ^{o}C, the presence of most important characteristic peaks corresponding to Si-O-Si and O-Si-O asymmetric vibration modes in the 1000 – 1200 cm^{-1} spectral range can observed [16]. A small band appears in the 780 – 830 cm^{-1} spectral range corresponds to the C-O [17] stretching vibration while the absorption band among 860 – 930 cm^{-1} is attributed to the Si-O-Si vibration modes. The vibration at 468 cm^{-1} (X_2 (a)) 458 cm^{-1} ($X_{4.5}$(b)) and 475 cm^{-1} ($X_{7.5}$(c)) is attributed to bending vibration of Si-O. Additionally, peaks in the 500 – 602 cm^{-1}spectral range are attributed to the bending of P-O vibrational mode, resulted from a pre-existent Ca-P phase, visible also in XRD [18]. In agreement with the literature [11, 19] the peaks in the range of 1400 – 1630 cm^{-1} can be attributed to symmetrical and asymmetrical stretching modes of the $(CO_3)^{-2}$ groups. The presence of this group is due to a carboxylation process of the samples induced by thermal treatment and the atmospheric CO_2 as a consequence of high calcium content. [19,20]. After loading the samples with ascorbic acid, beside the characteristic Si-O-Si and O-Si-O bending, stretching symmetric and asymmetric vibration, the band located around 1670 cm^{-1} and 1755 cm^{-1} associated with C=C and C=O stretching vibration form the vitamin C. A small shift in 1000 – 1200 cm^{-1} spectral range is also visible and modification between 500 and 1000 cm^{-1} spectral area.

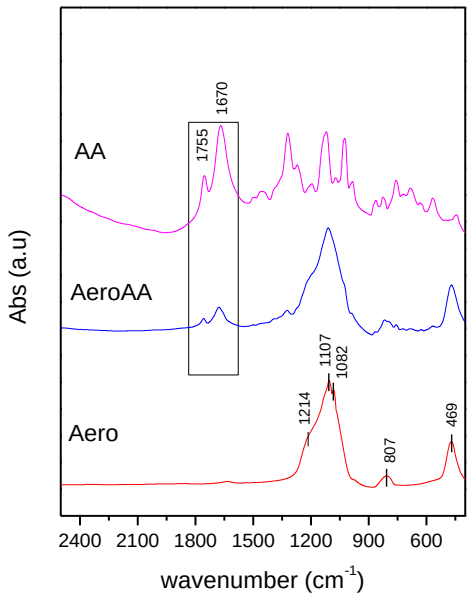


Figure 3.13*FT-IR spectra of Aerogel samples without and with Ascorbic Acid*

The FT-IR result obtained on the aerogel loaded and unloaded sample (Figure 3.13) shows absorption bands characteristic to Si-O-Si and O-Si-O stretching vibration modes in the 1100 – 1200 cm^{-1} spectral range. The bands located at 807 cm^{-1} and 467 cm^{-1}, respectively are identified as symmetric stretching vibration and bending vibration modes of Si-O units [21]. After ascorbic acid loading the characteristic peaks associated with C=C and C=O vibration can be clearly identified at 1650 and 1760 cm^{-1}, also weak peaks from ascorbic acid are observed in 550 – 750 cm^{-1} spectral range (these are completely missing before loading).

3.3.4. BET surface area and pore volume

The textural properties of the xerogel and aerogel samples were determined with a Qsurf Surface analyser based on Brunauer–Emmett–Teller (BET) method using N_2-adsorption–desorption. The obtained results (pore volume and surface area) are presented in Table 3.1.

Table 3.1 *BET surface area and pore volume of the samples with and without Ascorbic Acid*

Sample code	Specific surface area (m^2/g)	Pore volume (ml/g)
X2	153.82	0.33
X2AA	253.28	0.43
X4.5	164.30	0.32
X4.5AA	271.54	0.43
X7.5	89.09	0.4
X7.5AA	113.00	0.48
Aero	121.56	0.34
AeroAA	70.40	0.29

The specific surface area of pH=2 sample (~154 m^2/g) is similar with the value obtained for the sample with pH=4.5 (~164 m^2/g), but after increasing the pH to 7.5 the area decreases to 89 m^2/g. The pore volume for pH=2 and 4.5 is around 0.32 ml/g, after increasing the pH to 7.5 the pore volume shows an increase of 0.07ml/g. After immersion in ascorbic acid the surface areas and pore volumes of the three xerogel sample show a significant increase. The explanation for these modifications may related to the formation of Ascorbate complexes form the reaction of ascorbic acid and Calcium ions found on the sample surface in form of $Ca_{10}[PO_4]_6CO_3$ [22].

What regards the aerogel sample the BET results show a specific surface area of 121.56 m^2/g with a pore volume of 0.34 ml/g and after loading with ascorbic acid the surface area and pore volume decreases. The explanation for this phenomenon could be that important "open" nature and interconnectedness aspect of the aerogel. In a closed-pore material, gases or liquids cannot enter the pore without breaking the pore walls. This is not the case with an open-pore structure, when the sample is immersed in vitamin C containing solution, the liquid can flow from pore to pore [23] filling the pores and during drying the vitamin C recrystallize inside the pores.

4. In-vitro studies Of Xerogel and Aerogel Ascorbic acid loaded Samples

The bioactive properties of the xerogel and aerogel SBF incubated samples were investigated by XRD, SEM, and to identify the presence of specific functional groups FT-IR were used. Protein attachment was studied using FTIR and SEM.

4.1. Bioactivity study

The bioactivity study of the aerogel and xerogel samples were made in *in vitro* condition in simulated body fluid for different periods of time (3, 7 and 14 day) under renewal conditions (48 hours) at 36.5ºC. After immersion the samples were rinsed with distilled water and dried at room temperature. The X-ray patterns of the ascorbic acid loaded and unloaded samples after 14 days of immersion are presented in figure 4.1. After immersion in SBF for 3 and 7 days, the surface of aerogel sample shows no peaks that evidence the presence of apatite like phase. As it was shown in the first part of this chapter carbonated hydroxyapatite phase appears after thermal treatment at 700^{o}C. In case of xerogel samples without AA, weak peaks appear after 14 days of immersion at 2θ = 32. Comparable hydroxyapatite crystallinity was observed on irregularly shaped xerogel samples with pH=7.5 loaded with ascorbic acid after about one 14 days of immersion.

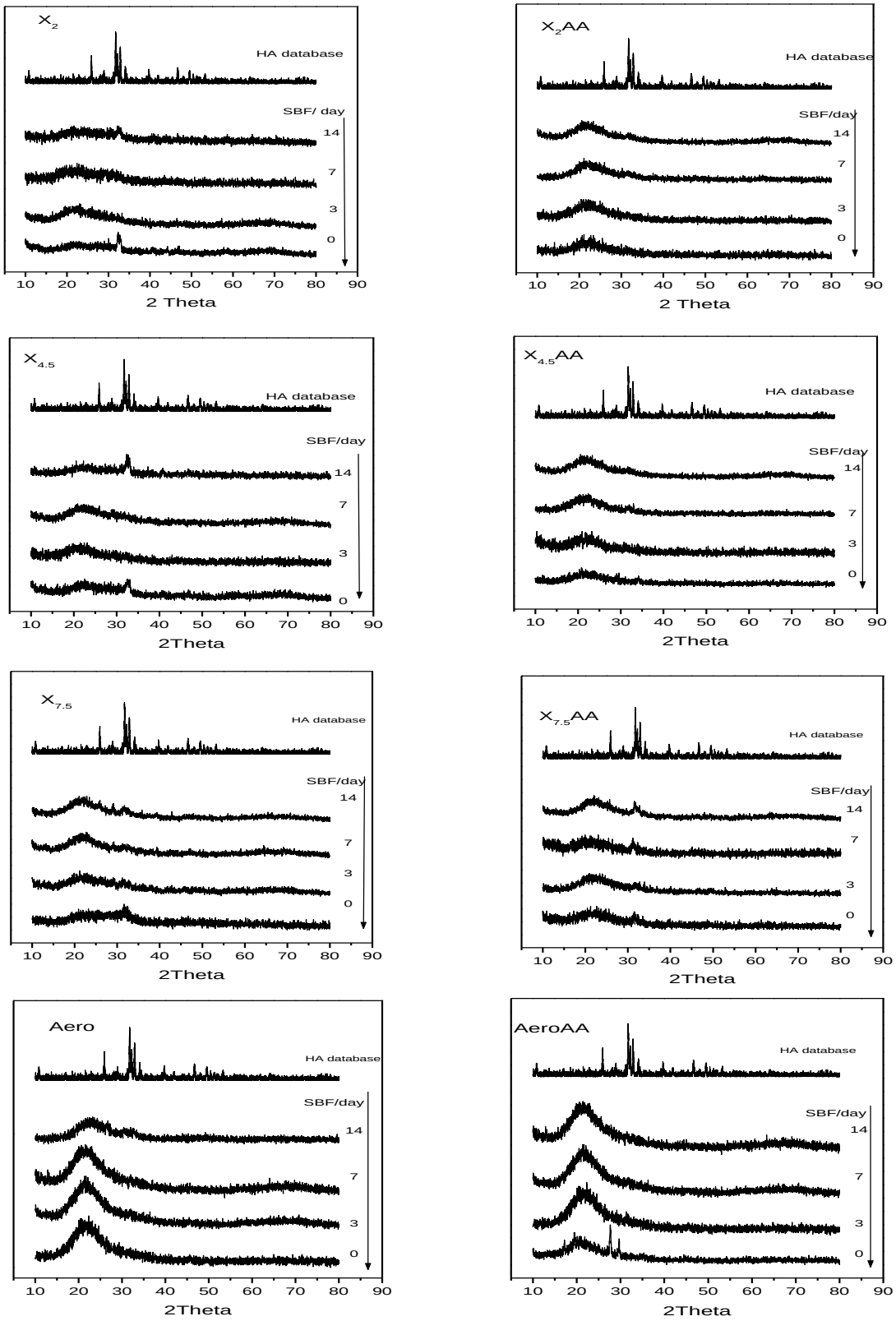


Figure 4.1 *XRD patterns of ascorbic acid loaded and unloaded samples after 14 days of soaking in SBF*

The XRD method is widely used for apatite characterization, for it provide data about the crystal structure of materials and its phase composition, however it's not conventional to determine [OH] and [CO_3] groups in apatite, using FT-IR method samples can be characterized considering the location of characteristic chemical groups for HAp [11]. The FT- IR results obtained on ascorbic acid contains sample and those of free ones are presented in Figure 4.2. After 14 days of soaking in SBF, the appeared absorption bands confirm the presence of the apatite layer on the surface which could not been identified with XRD. Looking at the results it is visible that the formation of apatite like phase is pH dependent.

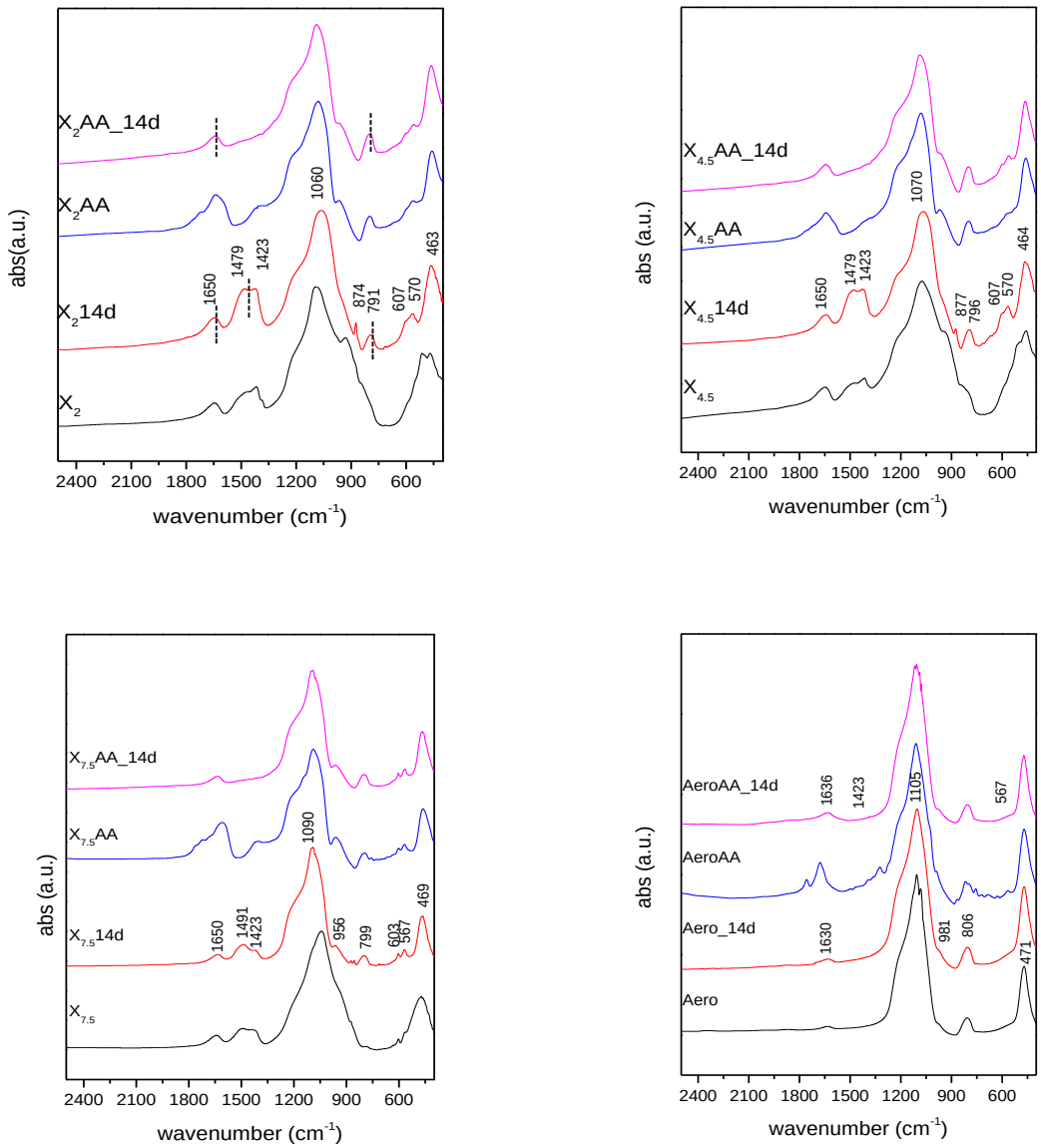


Figure 4.2. *FT-IR results of ascorbic acid loaded and unloaded samples after 14 days of soaking in SBF*

Comparing the results with those from the typical data for calcium phosphate from the literature the doublet absorption band at 570cm^{-1} and 607cm^{-1} corresponds to a bending vibrational mode of PO_4^{3-} units. The bands located at 791 cm^{-1} and the more intensive peaks between 1420 and 1650 cm^{-1}composed : the double peak at 1420 and 1479 cm^{-1} assigned to the symmetric vibrational mode of CO_3^{2-} group indicating the formation of an amorphous Ca-P phase, and a single peak at 1650 cm^{-1} allocated also to the CO_3^{2-} group characteristic for apatite [18].The intensive absorption band located between 1060- 1090 cm^{-1} (X_2, $X_{4.5}$, $X_{7.5}$) and between 1080 – 1100 cm^{-1} (X_2AA, $X_{4.5}$AA, $X_{7.}$5AA) correspond to the vibration mode of Si-O-Si are shifted to higher values. The absorption bands of the AA loaded and unloaded aerogel sample show the same characteristic vibration. A weak band 566 cm cm^{-1} identified as PO_4^{3-} vibration, and the band at 1632 cm^{-1} allocated to the CO_3^{2-} vibrational mode [24].

SEM images taken after 14 days immersion in SBF from xerogel samples prepared with and without ascorbic acid (Figure 4.3.) reveal the presence on their surface of shaped agglomerates that grow perpendicular to the substrate. This is a good proof for apatite like phases self-assembling in SBF [25]. It can be concluded from the SEM images that ascorbic acid containing samples has a positive effect for apatite formation. On the other hand, the pH increasing during samples synthesis has favoured the formation of apatite layer on their surface, which is also visible from the XRD results. After 14 day of immersion in SBF, the cauliflower-like apatite cluster formed on the surface of X7.5 AA sample.

SEM images of aerogel surface after incubation in SBF for 14 days with and without ascorbic acid are also presented in figure 2.19. Apatite layer formation on the surface of aerogel specimens are in correlation with IR results. After two weeks the deposition of sponge-wig shaped hydroxyapatite (HAp) layer on the surface are clearly visible and the entire surface was almost covered in 14 days [24].

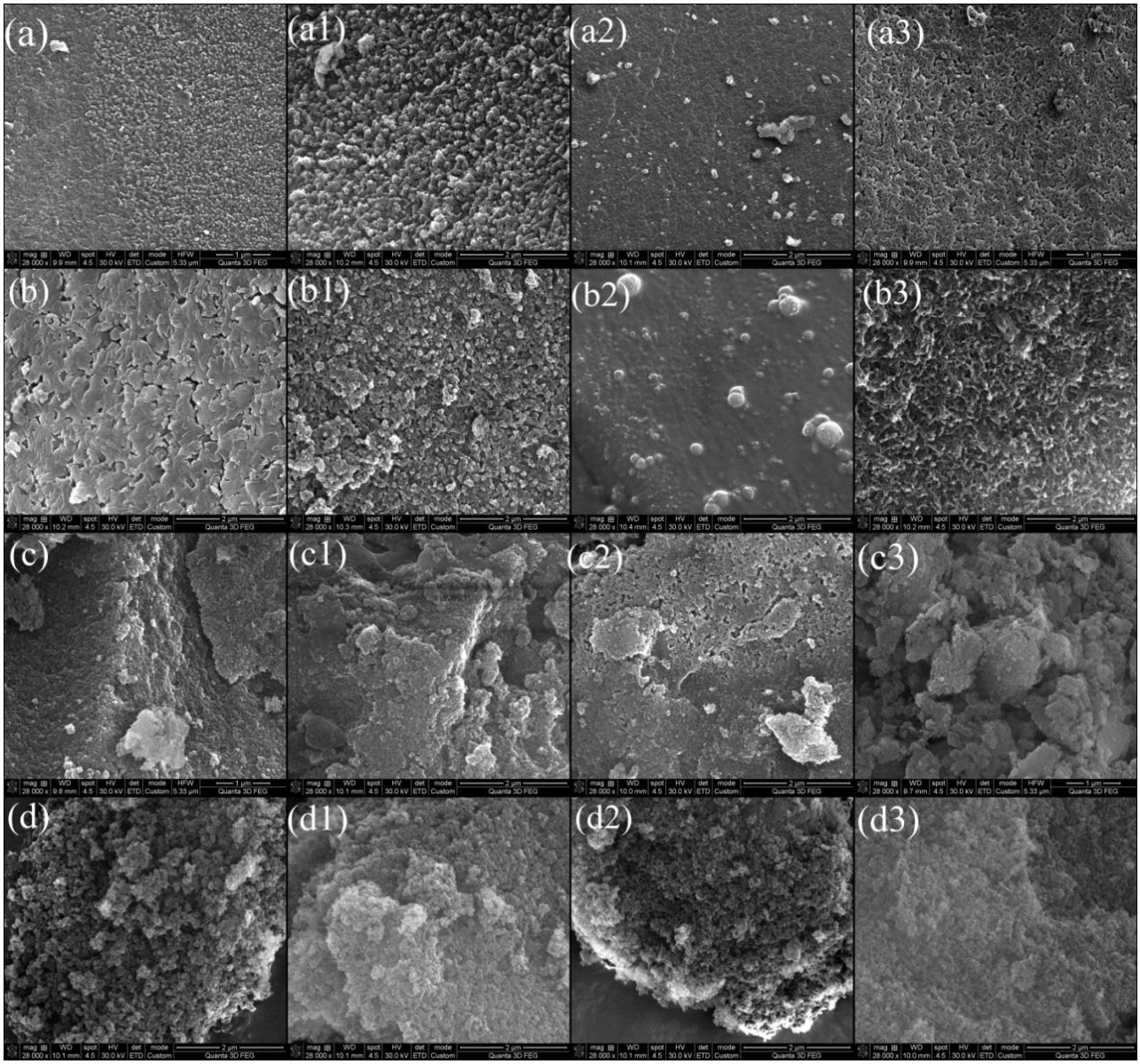


Figure 4.3 *SEM image of samples with and without AA after 14 day of SBF (mag: 28000) Where: (a) sample with pH=2; (a_1) X_2 after 14 day of SBF; (a_2) X_2 loaded with AA and (a_3) X_2AA after 14 day of SBF; (b) sample with pH=4.5 (b_1) $X_{4.5}$ after 14 day of SBF;(b_2) $X_{4.5}$ AA; (b_3) $X_{4.5}$AA after 14 day of SBF; (c) $X_{7.5}$; (c_1) $X_{7.5}$ SBF; (c_2) $X_{7.5}$AA; (c_3) $X_{7.5}$AA SBF (d) Aero; (d_1) Aero SBF; (d_2) Aero AA; (d_3) Aero AA SBF*

4.2. Protein Adsorption Study

The protein binding capacity of xerogel and aerogel ascorbic acid loaded and unloaded sample was investigated using Bovine Serum Albumin protein, an amount of 0.04g from each AA unloaded and loaded sample was immersed in 2 ml of phosphate puffer solution (PBS) with pH = 7.4 in which previously 0.1 g of BSA was dissolved, and incubated at 36.5^{o}C temperature of human body.

Before this investigation, knowing that ascorbate has a very high affinity to BSA [26], the interaction between Ascorbic Acid and Bovine Serum Albumin (BSA) was also studied. For this study three different amounts (1, 0.5, 0.25 grams) of Vitamin C were dissolved in distillate water (5ml), after that BSA was added to the solutions in an appropriated concentration (0.0033mg/dl) to those of albumin in human blood (3.5 - 5.0 g/dl) [27]. Fluorescence spectra of BSA and BSA with Ascorbic Acid were recorded in the range of 275- 500 nm and the results are shown in Figure 4.4. The excitation wavelength was at 290 nm, knowing that human serum albumin and bovine serum albumin excited at 290 emit fluorescence attributable mainly to tryptophan residue

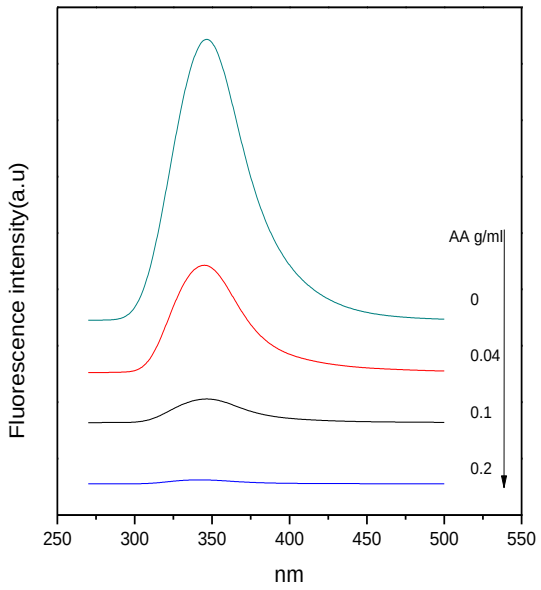
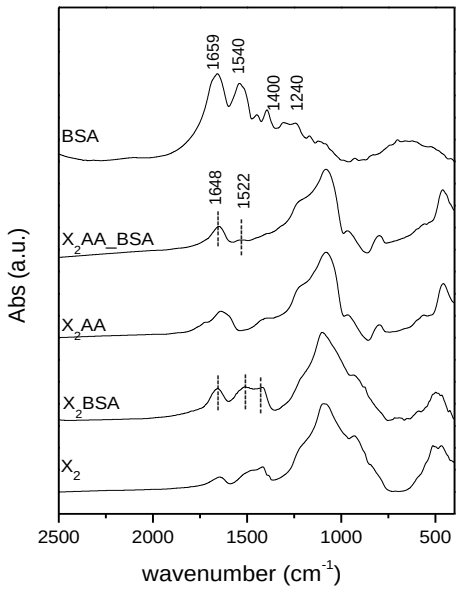
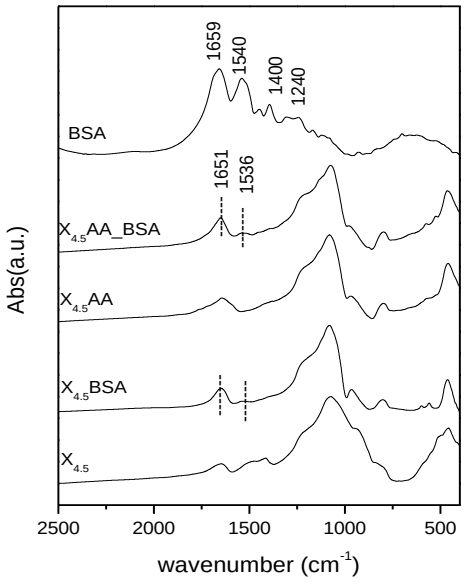


Figure 4.4 *Fluorescence spectra of BSA in presence of different concentration of vitamin C.*

The results indicate that the fluorescence intensity of BSA decrease with the increasing concentration of Ascorbic Acid, but no significant shifts can be observed that indicate that ascorbic acid interacts with BSA and quenches its intrinsic fluorescence [27]. Ascorbic Acid is a compound having two ionisable –OH groups that usually appear in the form of ascorbate anion, and because of having carbonyl groups shows a strong reaction with amino groups and therefore strongly react with amino groups of protein [28].

The FT-IR absorption spectrum of bovine serum albumin (BSA) protein is in agreement with the literature and shows the characteristic vibrational modes. The peak at 1659 cm^{-1} (figure 4.5) is the amide I band the most dominant band component, usually attributed to α-helices, and results from the C=O stretching vibrations of the peptide bond. Similarly, the peak at 1540 cm^{-1} attributed to the N-H plane bending vibration and C-N stretching vibration and a small contribution from C=O in-plane bending and NC stretching, is identified as Amide II [29,30]. The peak located at 1400 cm^{-1} to result from protein side-chain COO-, and 1240 cm^{-1} (C-N stretching vibration/N-H bending vibration) are called the amide III bands, respectively as it was reported before in this work.





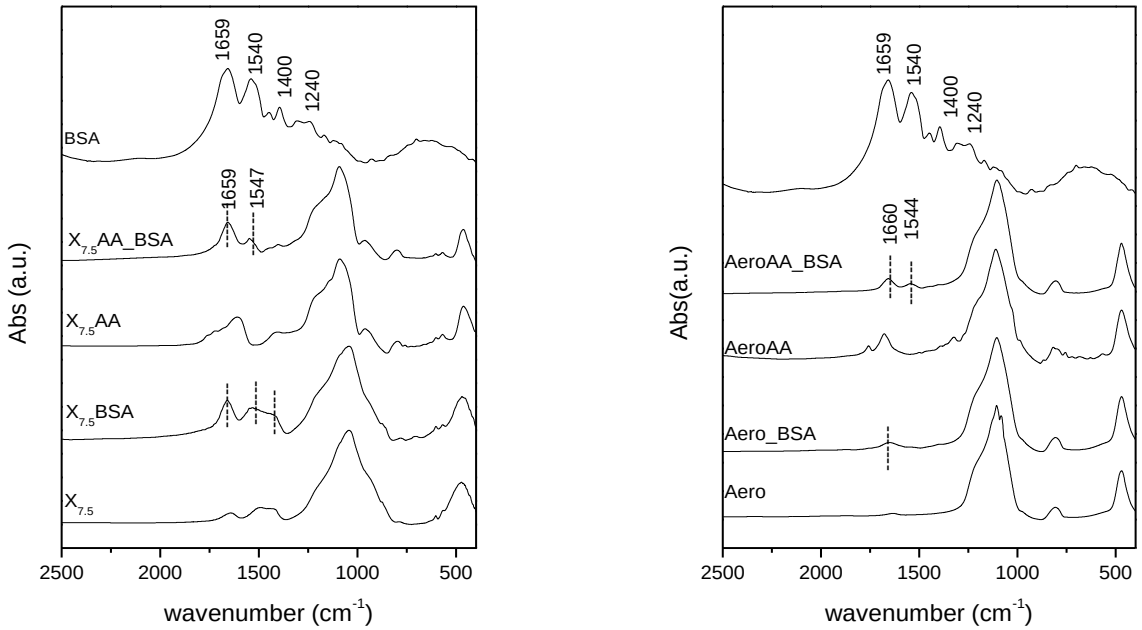


Figure 4.5 *FT-IR results of pure BSA and samples without and with AA after protein attachment*

Based on previous studies [31] protein adsorption/desorption onto bioactive glass surface depends on protein size and shape, concentration, substrate microstructure, hydrophobic behaviour, pH, ionic strength, temperature, competitive adsorption with other proteins and ions, as well as the solvent motion relative to the substrate.

The FT-IR spectra of xerogel samples (different pH) and aerogel samples before and after BSA adsorption are shown in Figure 4.5. Looking at the obtained results it could be noticed that the CO_3^{2-} band in 1400 – 1630 cm^{-1} spectral region disappear after protein adsorption. Difference can be observed between the samples X_2BSA and $X_{4.5}$BSA , the vibrational bands located at 1540 cm^{-1} and at 1400 cm^{-1}, are not so visible at sample with pH=4.5. According to literature [32, 33] this could be possible because BSA has a pH-dependent isomeric conformation and the changes observed reflect its multidomain structure.

The results obtained on sample containing ascorbic acid show the clear presence of amide I vibrational band at 1648 cm^{-1}(X_2), 1651 cm^{-1} ($X_{4.5}$) and at 1659 cm^{-1} ($X_{7.5}$) respectively. The amide II vibrational band can be identified also at 1522 cm^{-1} (X_2), 1536 cm^{-1} ($X_{4.5}$), and at 1547 cm^{-1} ($X_{7.5}$). In case of aerogel sample without AA after BSA adsorption only the amide I band at 1660 cm^{-1} could be identified. The FT-IR results of aerogel with AA reveal the presence of the second amide band at 1544 cm^{-1} spectral region, confirming that the presence of ascorbic acid does not have negative influence on protein attachment.

The adsorption of BSA on to xerogel and aerogel sample surface is confirmed also by SEM images. The obtained pictures are presented in figure 4.6. The results of protein attachment on to the sample surface reveal two distinct structures, on xerogel samples without AA globular structure can be identified, instead on those with AA polymerized structure can be found. This structure is also present in case of aerogel samples.

An explanation could come form that fact that the conformations of BSA are pH dependent. Between pH 4.5-7, BSA is in its normal (N) conformation, the most compact structure. However, between pH 4.5 and 3.5, BSA changes to the F (Fast) conformation by unfolding of domain III. Foster (1960) found that when BSA is in the F form, its viscosity increases whereas its solubility and helical content decreases. At pH lower than 3.5, BSA changes to the expansion (E) conformation characterized by a high intrinsic viscosity (least ordered structure) and an increase in its hydrodynamic axial ratio to more than twice the value of the N conformation. In this case it is important to notice that the isoelectric point of albumin is at pH=5.2 [34].

The second explanation, for the difference between the samples with and without AA, come from the important antioxidant characteristics of Ascorbic acid which keeps most of metal cofactors in reduction status, and in physiological conditions it appears in the form of dehydroascorbic acid and can react with amino groups and form Schiff bases, as it was mentioned before in this work.

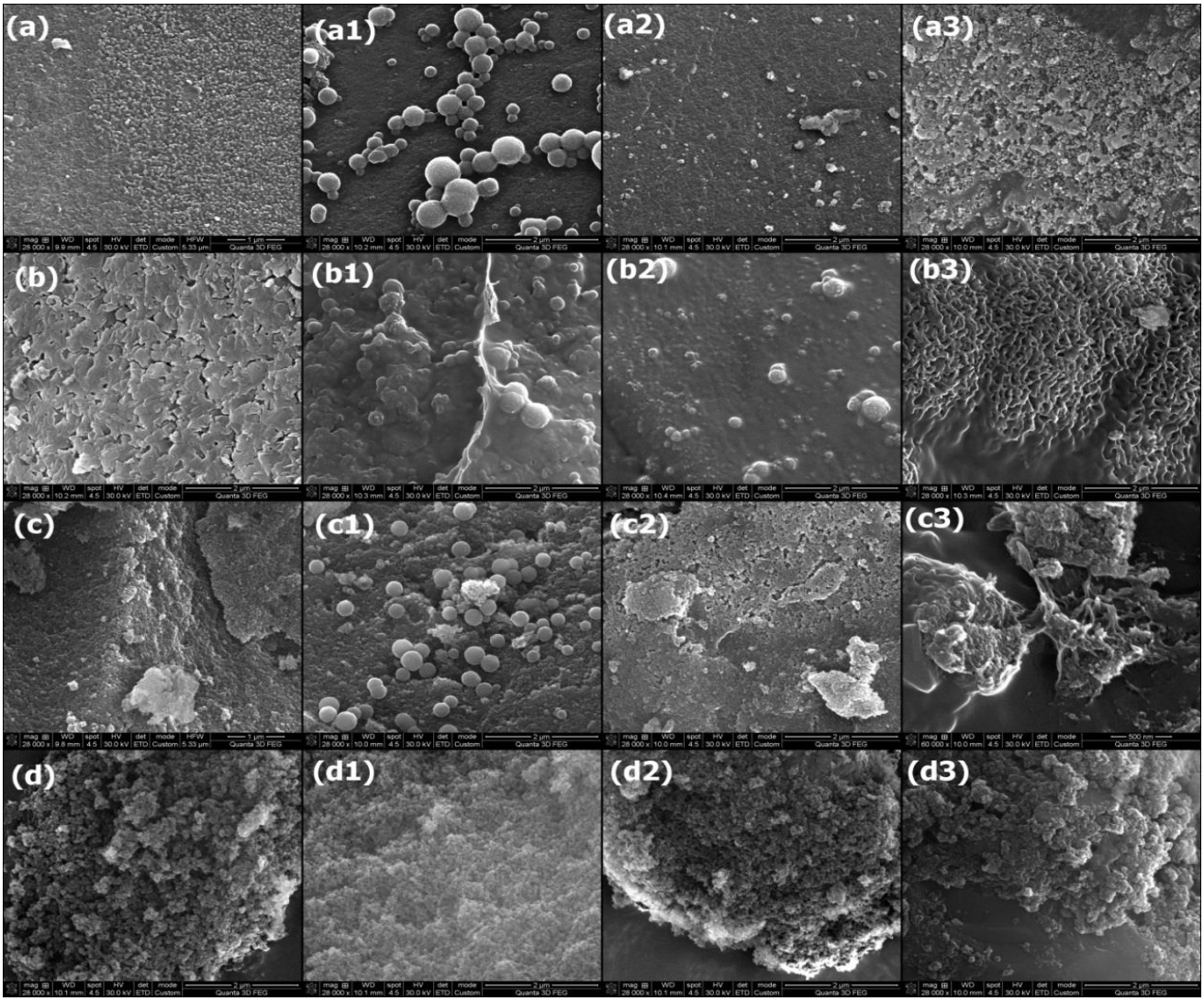


Figure 4.6 *SEM image of protein loaded sample without and with AA (mag: 28000) (Where: (a) sample with pH=2; (a_1) X_2 with BSA; (a_2) X_2 loaded with AA and (a_3) X_2AA with BSA (b) sample with pH=4.5 (b_1) $X_{4.5}$ with BSA;(b_2) $X_{4.5}$AA; (b_3) $X_{4.5}$AA with BSA; (c) $X_{7.5}$; (c_1) $X_{7.5}$ BSA; (c_2) $X_{7.5}$AA; (c_3) $X_{7.5}$AA BSA; (d) Aero; (d_1) Aero BSA; (d_2) Aero BSA; (d_3) Aero AA BSA)*

Conclusion

- $SiO_2 – CaO – P_2O_5$ based glass ceramics were successfully synthesized via sol-gel route. The Xerogel sample were prepared at different pH (2, 4.5, and 7.5) during the sol synthesis and the Aerogel samples were dried super-critically using CO_2 and loaded with Ascorbic Acid.
- The incorporation of Ascorbic Acid was realized:
  - immersing the xerogel (with different pH) and aerogel samples under argon atmosphere in ascorbic acid solution.
  - The results show that argon atmosphere is necessary to prevent ascorbic acid decomposition, and samples oxidation.
- The large mass loss recorded in 50-250 ºC temperature range for the dried xerogel sample is related to removal of residues from the preparation, and retained water in cavities that suggest porosity, also expected for these materials, confirmed with BET method to. Aerogel samples present no significant event on the DTA curve after supercritical drying; the mass loss registered on the TG shows the surface reduction at high temperature. DTA results also confirm the presence of ascorbic acid in samples
- The XRD results:
  - of the Xerogel samples after thermal treatment at 700^oC shows the presence of $Ca_{10}[PO_4]_6CO_3$ crystalline phase, while the aerogel sample shows amorphous structure even after 1050^oC.
- BET results:
  - show an increase in surface area and pore volume of xerogel samples after ascorbic acid incorporation under argon atmosphere, and a decrease in case of aerogel samples.
- FTIR analyses show the characteristic vibrational modes for AA presence after loading.
- SEM analysis reveals that the vitamin C dose not influence negative the porous structure of the obtained samples
- The bioactivity and protein attachment:
  - XRD, FTIR, and SEM results reveal that the addition of Ascorbic Acid to $SiO_2 – CaO – P_2O_5$ samples favours the formation of amorphous HA and HCA.
  - FTIR and SEM results show that AA incorporation in Xerogel and Aerogel samples under argon atmosphere have an important effect in improvement of protein affinity toward.
- Fluorescence measurement shows that AA has a high affinity to BSA.

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"Life is a wondrous phenomenon. I can only hope that someday man will achieve a deeper insight into its nature and its guiding principles and will be able to express them in more exact terms…To express the marvels of nature in the language of science is one of man's noblest endeavours."
– Albert Szent-Györgyi.

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