In situ chemical and physical reduction of copper on bioactive glass surface

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Abstract: In this work silica-based bioactive glass powders were doped with Cu++ ions by means of ion-exchange process and subsequently they were exposed to different chemical (tannic acid, ascorbic acid and NaOH) and physical processes (UV irradiation, thermal treatment in air or argon atmosphere) to promote the in situ reduction of Cu++ ions to Cu+ or Cu0. The obtained glasses were investigated by means of structural (X-Ray diffraction - XRD), morphological and compositional analyses (scanning-transmission electron microscopy equipped with energy dispersive spectroscopy (FESEM/STEM-EDS); moreover, glasses were subjected to in vitro bioactivity test in simulated body fluid up to 14 days to investigate the influence of the performed chemical and physical treatments on glass bioactivity. At the end of incubation time glasses were analyzed by means of FESEM-EDS and Fourier transformation infrared spectroscopy (FT-IR). The obtained results evidenced that, in general, the chemical treatments are useful to induce the formation of Cu0, while thermal treatment induce the nucleation of CuO (in air) or Cu2O (in argon). All samples maintained a bioactive behavior. At least, preliminary antibacterial effect evaluation by inhibition halo test evidenced that samples containing free Cu++ ions or Cu0 nanoparticles possess the best antibacterial effect.

POLITECNICO DI TORINO
Dipartimento di Scienza Applicata e Tecnologia

Turin, March 29, 2019

Dear Editor,

I would like to submit, for a possible publication on the Journal "Applied Surface Science", the original paper:

"**In situ chemical and physical reduction of copper on bioactive glass surface**"

Submitted by:
Marta Miola[1,*] Elisa Bertone and Enrica Verné[1]

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Authors are aware of the article content and approve its submission. Authors confirm that this manuscript is original and has not been previously published in other journals, nor is it submitted to other journals at this present time and we support that do not exist conflict of interest with no academic institution or company. Moreover, authors declare that, if the article will be accepted, it will not be published elsewhere in the same form and in any language, without the written consent of the publisher.

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***Response to Reviewers**

Reviewers' comments:

Reviewer #1: the authors report on the properties of bioactive glass that was functionalized with Cu using an ion exchange procedure. They investigate for bioacticity in simulated body fluids. The methods used are logical and appear adequate for the problem. The results appear plausible, but the claims are not yet sufficiently corroborated by the data. The motivation and experimental support should be strengthened before the manuscript is suitable for APSUSC.

A: the paper has been improved according to the reviewer's comment.

The authors have remarked on the possibility of ion exchange with ambient medium. However, they have not adequately studied potential leakage of Cu ions into surrounding liquid. They should consider realistic settings in presence of high physiological protein levels (for example albumin which, after all, is a good sequester of free copper ions). Copper being highly redox active, and catalytist for denitrosylation, this should bring considerable artefacts into any biological assay.

A: the authors are aware that a more realistic settings could be of help in simulating the fate of copper eventually released in the biological fluids. However, at this step of the research, this investigation is still premature. The aim of the paper, as assessed in the manuscript, is to investigate different ways to introduce copper in a reduced form on the surface of a bioactive glass, in view of a potential application as bioactive and antibacterial bone substitute. As reported in the introduction, the bioactivity, as well as the antibacterial behavior, have been investigated preliminary, by means of commonly used standard tests and conditions, with comparative purposes among the various sets of samples.
The potential leakage of Cu ions into surrounding liquid, their redox and catalytic activities, as well as their potential impact on biological assays could be of interest to evaluate in more deep details the antibacterial effect of the materials object of the manuscript, for example to assess if a "contact killing" or a "leaching" mechanism are predominant. However, in the authors' opinion, these aspects should be explored on a selected set of samples, and are not object of investigation at this stage of the research.
The introduction and the conclusions have been improved to explain this issue.

The existing literature on Cu-glasses is not adequately reviewed, and the references should be greatly extended.
A: the paper has been improved according to the reviewer's comment.

Finally, the manuscript is difficult to read due to its defective grammar and style. The authors are emphatically advised to consult a native English speaker and revise the text in detail.
A: the paper has been improved according to the reviewer's comment.

The motivation for this particular material choice should be strengthened.
A: the paper has been improved according to the reviewer's comment.

Reviewer #2: General comments
The submitted paper reports on the synthesis and characterisation of silica-based bioactive glass powders doped with Cu++ ions by means of ion-exchange process. The influence of different chemical (tannic acid, ascorbic acid and NaOH) and physical processes (UV irradiation, thermal treatment in air or argon atmosphere) to promote the in situ reduction of Cu++ ions to Cu+ or Cu0 was investigated.
Many characterisation techniques were used, including XRD, FESEM/STEM-EDS, in vitro bioactivity test in simulated body fluid, FT-IR.
The subject of this work is appealing, of current interest and worthy of investigation and matches the aim and scope of Applied Surface Science.
The paper is well organised, but, as a general consideration, the Authors should not only describe the acquired results but also to better and more deeply discuss them and compare them with literature.

A: the paper has been improved according to the reviewer's comment, taking into account that, on the basis of authors knowledge, there are not studies regarding the reduction of copper directly on bioactive glass surface and thus, for some characterization, the comparison results difficult.

Finally, a deep and accurate revision of the English grammar and language is strongly recommended.
Detailed suggestions and remarks are reported below point by point.

A: the paper has been improved according to the reviewer's comment.

Keywords
The chosen keywords (i.e. bioactive glass; copper; in situ reduction; antibacterial) do not completely cover the manuscript content.
A: the keywords have been corrected to completely cover the manuscript content.

1. Introduction
- The Introduction section is well conceived and organized. However more recent literature references have to be added.
A: more recent references have been added

- Regarding the copper action, more recent literature references have to be added. For example, the Authors should cite some recent chapters and/or reviews where the biological role of copper, used as vicarious ions within both hydroxyapatites and glasses, is reported.
A: the paper has been improved according to the reviewer's comment.

- The following sentence "Focusing the attention on biomedical applications, copper has been included also in the composition of several bioactive glasses, a class of materials widely studied as bone substitutes for their ability to chemically bond to living bone through a peculiar surface reactivity, which implicates ion exchange between glass and biological fluids, the development of a silica-rich amorphous layer, the adsorption of calcium and phosphate ions and their crystallization into hydroxyapatite (HAp) [9,10]" has to be supported with more recent and complete suitable literature references, including "Bivalent cationic ions doped bioactive glasses: the influence of magnesium, zinc, strontium and copper on the physical and biological properties, Journal of Materials Science 52(15) (2017): 8812-8831".

A: the paper has been improved according to the reviewer's comment.

2. Materials and Methods
2.1 Synthesis and thermal characterization of Cu-containing bioactive glass
- - More details about the used reagents, such as the supplier and purity, have to be added.
- The heating rate has to be specified.
- For the milling step, the rpm and time have to be reported.

A: the paper has been improved according to the reviewer's comment.

2.2 In situ reduction processes
- Many justifications do not sound appropriate for the Experimental section.
For example, "as reported in literature [24,25] the irradiation with UV light can induce the formation of copper nanoclusters.", "since as reported by Sheng et al. [25] the thermal annealing can produce Cu nanoclusters.", and "Regarding the chemical treatments, tannic acid (SBA3-CuTA) and ascorbic acid (SBA3-CuAA) were selected for their well know ability to reduce ions, such as copper, silver or gold ions, to

nanoparticles [28-32]; moreover, they are green reducing agent that can be obtained from vegetal products" could be removed here and moved to the Introduction section or to the Discussion one.
A: the paper has been improved according to the reviewer's comment.

2.3 Glasses characterizations

-- For XRD measurements, the used <lambda>, the investigated 2Theta range, time per step and step size have to be specified.
How were the samples prepared for SEM observation? Add details.
A: the paper has been improved according to the reviewer's comment.

- Even if well know, the composition and preparation of SBF have to be briefly reported.
A: the composition of SBF and the preparation protocol are not only well known, but they are also reported in the ISO standard that has been cited in the references. To simplify the interpretation of the bioactivity test the composition of SBF has been reported qualitatively, but in the authors' opinion the description of the SBF preparation protocol, already defined in the ISO standard, is redundant and not essential for the results interpretation, so it was not reported.

- The following description "As reported in literature [35-37] the bioactivity process can be divided in five steps: the first one (Step I) consists of a rapid ions exchange between alkaline ions from the glass and hydrogen ions from the solution, in the Step II the formation of silanols occurs (Step II), then they condensate to develop a silica gel layer (Step III). Subsequently calcium, phosphate and carbonate ions are adsorbed in the silica gel (Step IV) and react, forming the hydroxyapatite (HAp) layer (Step V)" can be removed, since it is well known and does not sound proper for the Experimental section.

A: the description has been strongly reduced, according to the reviewer's comment, removing all the details that do not sound proper for the Experimental section. This description has been moved to the "Results and discussion" section, since the results of bioactivity test have been commented by referring to the four steps of bioactivity.

3. Results and Discussion
As a general comment, all the acquired data and results should be better and more deeply discussed and compared with the Literature and not only described. Thus, the Authors have to improve this section.

A: the paper has been improved according to the reviewer's comment. However, it should be noticed that, on the basis of authors knowledge, there are not papers regarding the in situ reduction of copper on bioactive glass surface; thus a deep comparison with literature results difficult.

1.1 Thermal characterization of Cu-containing bioactive glass
- In the title, please replace 1.1 with 3.1.
- The Authors should not only describe the results but also justify them, with proper literature references.
- For Figure 2a and Figure 2 b use the same scale and the same character and dimension.
A: the section has been improved according to the reviewer's comment.

1.2 Phase analysis
- In the title, please replace 1.2 with 3.2.
- The Authors have to report the used JCPDS card numbers both within the text and in the related Figures.
- Even if already reported in Ref [18], the XRD pattern of the pristine Cu-doped glass should be shown.

A: the section has been improved according to the reviewer's comment. The JCPDS card numbers have been reported in the text due to the complexity of the figure.

1.3 Morphological and compositional characterization
- In the title, please replace 1.3 with 3.3.
- The shown SEM micrographs have to be at the same magnification. Please make them comparable.
- Moreover, it is not possible to consider EDX microanalysis a quantitative characterisation. It is only possible to obtain an estimation of the amounts of the identified elements. It is necessary to use chemical analyses, such as ICP and XRF, in order to obtain quantitative data.

A: the section has been improved according to the reviewer's comment. SEM micrographs of large area have been selected at the same magnification, in the other cases (e.g:…Fig 4e,h,m,p, Fig 5b,e,h) the magnification has been selected on the basis of the details that authors wonted to highlight.

1.4 Bioactivity
- In the title, please replace 1.4 with 3.4.
- As evidence above, it is not possible to consider EDX microanalysis a quantitative characterisation. It is only possible to obtain an estimation of the amounts of the identified elements. It is necessary to use chemical analyses, such as ICP and XRF, in order to obtain quantitative data. It is strongly suggested to use ICP analyses to be applied on the SBF media at the selected times.
A: the section has been improved according to the reviewer's comment. Reviewer is right, EDS is a semi-quantitative analysis and ICP could be useful to estimate the variation of elements; however, due to the large number of samples and analyses, at this step of the research authors decided to investigated the bioactivity by means of SEM-EDS and FT-IR analysis. The bioactivity mechanism of the most promising samples will be investigated in details in future works.

In situ chemical and physical reduction of copper on bioactive glass surface

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Abstract

In this work silica-based bioactive glass powders were doped with Cu^{++} ions by means of ion-exchange process and subsequently they were exposed to different chemical (tannic acid, ascorbic acid and NaOH) and physical processes (UV irradiation, thermal treatment in air or argon atmosphere) to promote the in situ reduction of Cu^{++} ions to Cu^{+} or Cu^{0}. The obtained glasses were investigated by means of structural (X-Ray diffraction – XRD), morphological and compositional analyses (scanning-transmission electron microscopy equipped with energy dispersive spectroscopy (FESEM/STEM-EDS); moreover, glasses were subjected to *in vitro* bioactivity test in simulated body fluid up to 14 days to investigate the influence of the performed chemical and physical treatments on glass bioactivity. At the end of incubation time glasses were analyzed by means of FESEM-EDS and Fourier transformation infrared spectroscopy (FT-IR). The obtained results evidenced that, in general, the chemical treatments are useful to induce the formation of Cu^{0}, while thermal treatment induce the nucleation of CuO (in air) or Cu_2O (in argon). All samples maintained a bioactive behavior. At least, preliminary antibacterial effect evaluation by inhibition halo test evidenced that samples containing free Cu^{++} ions or Cu^{0} nanoparticles possess the best antibacterial effect.

Keywords

Bioactive glass; surface modification; copper ion exchange; in situ chemical/physical reduction; antibacterial.

1. Introduction

Copper (Cu) and its compounds have been recently reported in numerous studies as effective tools to reduce the incidence of contact-mediated infections [1-3], due to several advantages in comparison with other antimicrobial materials, such as, among others, their multitoxicity and effectiveness against multiresistant microorganisms. The mechanism of “contact killing” of bacteria recognized to metallic Cu

surfaces [4], that makes it an intrinsically antibacterial material, is gaining increasing attention in the face of growing antibiotics resistance of bacteria, even if the role of the interaction between bacteria and metal surface, media composition and metal surface chemistry on contact killing is still object of few studies. For example, the antibacterial properties of thermally generated Cu oxides (Cu_2O and CuO) in comparison to pure Cu, as well as the formation of Cu oxides on Cu during antimicrobial test, was investigated by M. Hans et al. [4] demonstrating that CuO was significantly effective against *E. hirae*, compared to pure Cu, and Cu_2O was as effective as pure Cu. The release of Cu^+ and Cu^{2+} ions from the different surfaces was highest for pure copper, followed by Cu_2O and CuO and was only approximately correlated with their antibacterial efficacy. Other researchers investigated the antibacterial activity of copper NPs related to their oxidation state and suggested that cuprous state of copper bind to the proteins and so Cu_2O NPs show high affinity to the bacterial cells, while CuO NPs effect is ascribable to the significant production of ROS (reactive oxygen species) [5] The efficacy of copper metallic nanoparticles has also been investigated by several authors [6, 7]; however, there are very few studies focused on the Cu-NPs bactericidal mechanism [7]. In general three mechanisms of action are reported in literature: i) changing of bacterial membrane permeability due to the accumulation and dissolution of NPs; ii) NPS ability to generate reactive oxygen species (ROSs) or/and their corresponding ions; iii) uptake of released metallic ions; moreover, Cu NPs seem to possess a prolonged antibacterial effect [7].

Copper at different oxidation states (Cu^{2+}, Cu^+, and Cu^0), can be added in glasses both as network modifier or nanoparticles [8-12]. Focusing the attention on biomedical applications, Cu in ionic form has been included in the composition of several bioactive glasses, a class of materials widely studied as bone substitutes for their ability to chemically bond to living bone through a peculiar surface reactivity, which implicates ion-exchange between glass and biological fluids, the development of a silica-rich amorphous layer, the adsorption of calcium and phosphate ions and their crystallization into hydroxyapatite (HAp) [13-15]. Silica-based Cu-containing bioactive glasses have been obtained by sol–gel [16-21], as well as by the traditional melt and quenching technique both for silica-based bioactive glasses [22,23] and phosphate glasses [24]. The ion-exchange technique in aqueous solution was also used to dope with Cu ions the surface of different silica-based bioactive glasses [25,26]. In all these studies the goal was to develop bioactive and antibacterial glasses without affecting the structure, morphology, composition and properties of the glasses. These studies demonstrated that the glasses doped with Cu ions significantly reduced the bacterial adhesion and proliferation, while preserving their bioactivity.

In the present paper, powders of a silica-based bioactive glass, prepared by melt and quenching route, have been doped with Cu^{++} ions by ion-exchange process in aqueous solution and, for the first time on the basis of authors knowledge, subjected to several physical and chemical treatments in order to induce the in situ reduction of Cu^{++} ions to Cu^+ or Cu^0. Thus, the first aim of this study was to investigate the best physical and/or chemical treatments able to reduce Cu^{++} ions directly on bioactive glass surface and the possible

formation of nanoparticles, in order to investigate the possibility of improving the bioactive glass surface properties with the peculiar features of copper nanoparticles, such as high surface to volume ratio, multiple mode of antibacterial action and the possibility to be further functionalized with drugs [27-30]. The second goal of this work was to verify the bioactivity and preliminary evaluate the antibacterial effect of the modified glass containing copper in different oxidation state, by means of commonly used standard tests and conditions, with comparative purposes among the various sets of samples. These preliminary characterizations are expected to be helpful in the selection of a representative set of samples, obtained by the best treatment, that could be object of future biological assays in a more complex and realistic environment.

2. Materials and Methods

2.1 Synthesis and thermal characterization of Cu-containing bioactive glass

The starting glass (named SBA3 from now on), having the molar composition 48% SiO_2, 26% Na_2O, 22% CaO, 3% P_2O_5, 0.43% B_2O_3, 0.57% Al_2O_3, was produced by melting and quenching process. All chemicals were purchased from Sigma-Aldrich (Switzerland, reagent grade > 99%). Briefly, SBA3 precursors were melted in a platinum crucible at 1450 °C for 1 h (heating rate 10 °C/min) and cooled in water to obtain a frit. Subsequently, the frit was mechanically milled into a zirconia jar (300 rpm for 1 h) and sieved to obtain glass powders with a final grain size < 20 μm. Copper was introduced in the glass powders (SBA3-Cu) by means of ion-exchange process in aqueous solution of copper acetate 0.05 M for 1 h at 37 °C and 150 rpm; at the end of the process, the solution was removed and SBA3-Cu powders were washed with bi-distilled water, filtered and dried as reported in [25]. The glasses (doped and undoped) structure, morphology, composition and antibacterial properties were evaluated in a previous work [25]. In the present work Differential Thermal Analysis (DTA, 404 PC Netzsch) was carried out in order to identify the characteristic temperatures of the glass in view of reduction processes, that involve, in some cases, a thermal treatment.

2.2 In situ reduction processes

Aiming to induce the *in situ* reduction of copper ions, SBA3-Cu powders were subjected to several physical and chemical treatments, adapted from literature [31-34], as reported in table 1.

SBA3 containing Cu^{2+} ions				
	Acronym	**UV irradiation**	**550 °C air**	**550 °C Ar**
Physical treatments	**UV**	X		
	UV+TT	X	X	
	TT		X	

	UV+TTAr	X		X
	TTAr			X
Chemical treatments	**TA**	Tannic acid		
	AA	Sodium L-ascorbate		
	NaOH	Sodium hydroxyde		

Table 1: physical and chemical treatments and samples acronym.

In particular, for what is concerning the physical treatments, SBA3-Cu glass powders were subjected to UV-light irradiation (SBA3-Cu1 sample, 20 W for 30 min) [31,32]. The glass powders were also subjected to a thermal treatment at 550 °C for 2h (with – SBA3-Cu2 and without – SBA3-Cu3 UV irradiation) [32]. The thermal treatments at 550 °C for 2 h (after UV irradiation – SBA3-Cu4 – or not – SBA3-Cu5) were also performed in argon atmosphere in order to evaluate the role of the atmosphere in the reduction process [33,34].

As far as the chemical treatments is concerned, SBA3-Cu powders were treated with a 0.01 M solution of tannic acid at 37 °C, 150 rmp for 30 minutes, while a solution 0.1 M of sodium L-ascorbate at 80 °C, 150 rmp for 30 minutes were used. The concentrations were selected on the basis of literature [35-39]. At the end of treatments glass powders were washed with bi-distilled water, filtered and dried overnight at 37 °C.

Lastly, the reducing ability of NaOH [37] was estimated by immersing SBA3-Cu in a 1 M solution of NaOH at 80 °C, 150 rpm for 1 h (SBA3-Cu8). Then, sample was washed with bi-distilled water, filtered and dried overnight at 37 °C. These treatments have been adapted from literature in order to induce the *in situ* reduction of Cu^{++} ions. The formation of Cu^{+} ions, copper oxides and Cu° nanoparticles on the glass surface has been hypothesized and verified by morphological observation, chemical and phase analysis.

Figure 1 resumes the possible reduction mechanisms involved during *in situ* chemical and physical treatments.

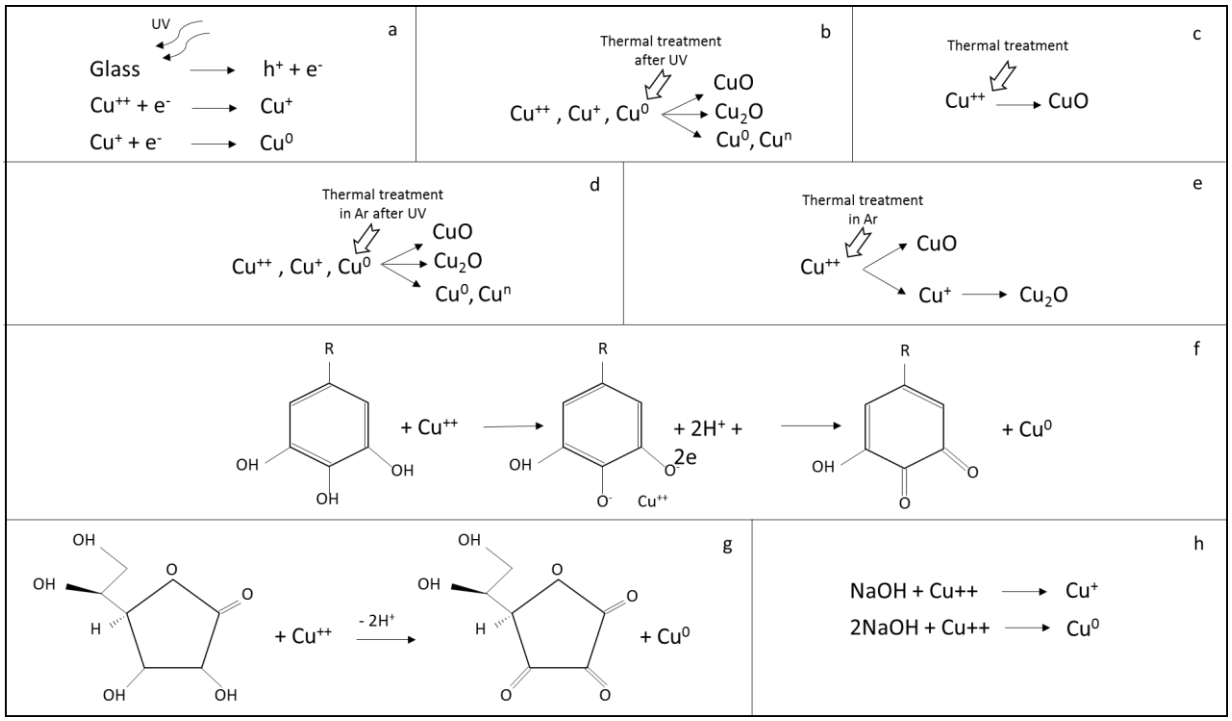

Figure 1: proposed mechanism of *in situ* reduction after a) UV irradiation (h^+ is a hole center, e^- is an electron), b) thermal treatment after UV, c) thermal treatment, d) thermal treatment in Ar after UV, e) thermal treatment in Ar, f) tannic acid treatment, g) sodium L-ascorbate treatment, h) NaOH treatment.

2.3 Glasses characterizations

All glasses exposed to the reduction process were subjected to structural analysis by means of XRD (X'Pert Philips diffractometer), using the Bragg Brentano camera geometry and the Cu-Ka incident radiation (source voltage and current set at 40 kV and 30 mA, incident wavelength λ = 1.5405 Å, step size Δ(2θ) = 0.02°, fixed counting time of 1 s per step), in order to identify the formation of new crystalline phases and in particular metallic copper. The obtained patterns were analysed with X'Pert High Score software and the PCPDF data bank.

Morphological and compositional analyses were performed by means of scanning electron microscopy equipped with energy dispersive spectroscopy (FESEM-EDS, SUPRATM 40, Zeiss) and scanning transmission electron microscopy (STEM, Merlin Gemini Zeiss) to evaluate the influence of the reduction treatments on glass powders morphology and composition and to investigate the possible formation of copper nanoparticles and their dimensional range. Samples for SEM observation were prepared by positioning glass powders on adhesive carbon tape, attached to aluminum stub. Samples were chromium-coated prior to the analysis. STEM analysis was performed by preparing a glass particles suspension and depositing a drop on a copper TEM grid with carbon film (SPI Supplies® Brand Lacey Carbon Coated 200 Mesh Copper Grids – JEOL S.p.A.).

All glasses were then subjected to *in vitro* bioactivity test in simulated body fluid (SBF), an inorganic solution developed at the Department of Material Chemistry, Graduate School of Engineering, Kyoto University, which is characterized by ion concentrations nearly equal to those of human blood plasma, buffered at pH 7.40 with 50 mM trishydroxymethylaminomethane and 45 mM hydrochloric acid at 36.5°C, as reported in literature [40]. The test was performed by dipping glass powders (100 mg) in 100 ml of SBF following the protocol of Kyoto University as suggested by the ISO standard [41] and maintaining samples at 37 °C for 3, 7 and 14 days at 150 rpm in orbital shaker (IKA® KS4000i control). As reported in literature [42-44] the bioactivity test is useful to predict the ability of bioactive glasses to bond to leaving bone by observing the growth of a hydroxyapatite (HAp) layer on their surface after soaking in SBF.

The pH of the solutions was monitored every 2-3 days. At the end of incubation time, glass powders were gently washed with bi-distilled water, filtered and dried overnight at 37 °C; subsequently, FESEM-EDS analyses were performed to verify the precipitation of hydroxyapatite or its precursor on glass powders. Moreover, Fourier transformation infrared spectroscopy (FT-IR) was used to investigate the reactivity of the glasses and accurately monitoring the formation of reaction phases during SBF immersion. FT-IR spectra were acquired in a Hyperion 2000 FT/IR (Tensor 27, Bruker Optics S.p.A, Ettlingen, Germany) from 2000 to 400 cm^{-1} and with 2 cm^{-1} resolution. OPUS software (v. 6.5, Bruker S.p.A) was used for instrumental control and spectral acquisition.

A preliminary investigation of antibacterial properties of Cu-containing glasses was carried out by means of inhibition halo test (Kirby Bauer test in accordance with National Committee for Clinical Laboratory Standards [45]). The test was performed using a standard *Staphylococcus aureus* strain (ATCC 29213), which is one of the strains most involved in infections development [46]. To realize the test, glass pellets were prepared by weight 200 mg of each glass powder and press them at 4 tons for 10 seconds in an automatic press (Graseby-Specac T-40). Then glass pellets were placed in contact with Mueller Hinton agar plates uniformly covered with bacteria, as recommended by NCCLS standard and reported in [25], and incubated overnight at 35 °C. At the end of incubation time possible formation of a halo around the sample, in which the bacteria have not proliferated was observed and measured.

3. Results and Discussion

3.1 *Thermal characterization of Cu-containing bioactive glass*

Figure 2 shows the DTA traces of SBA3 and SBA3-Cu glass powders; regarding SBA3 glass (Figure 2a), the glass transition temperature was observed at about 550 °C, while an exothermic peak was individuated at 658 °C, with an onset of crystallization at about 560 °C. The copper introduction (Figure 2b) slightly decreases both the characteristic temperatures: the glass transition temperature of SBA3-Cu is about at 510 °C, while the crystallization peak occurs at 652 °C with an onset at about 560 °C.

On the basis of the DTA analyses, the thermal treatments in air and in Ar were performed at 550 °C, the maximum temperatures that allows to avoid significant crystallization phenomena.

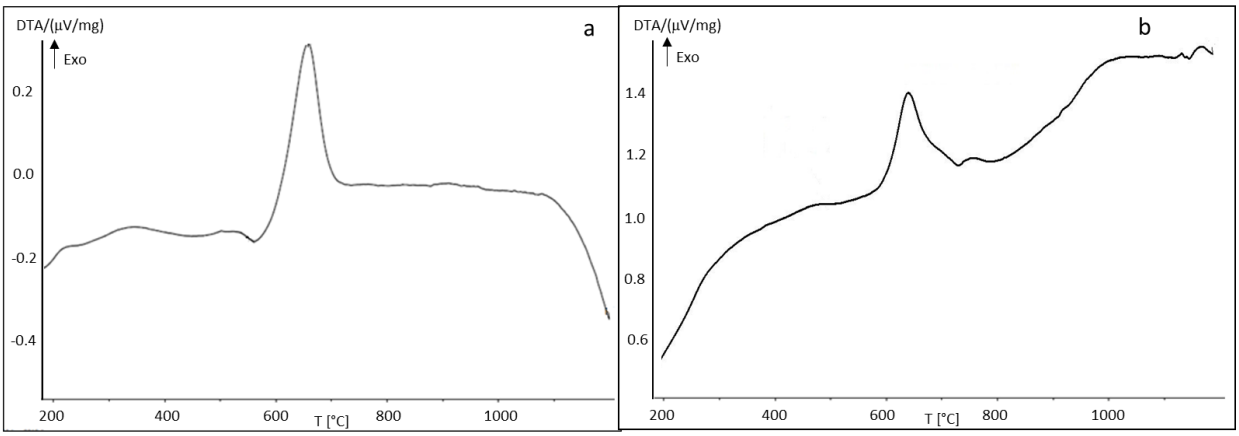

Figure 2: DTA graph of SBA3 (a) and SBA3-Cu (b) glass powders.

3.2 Phase analysis

XRD analyses, reported in figure 3, were performed to investigate the possible crystallization of new phases due to the different reducing treatments. The first reduction method investigated in this work is the irradiation with UV light, that it is reported to induce the formation of copper nanoclusters [31,32] in copper doped-silicate glass and starting from cupric oxide (CuO) and cuprous oxide (Cu_2O). However, the UV irradiation of Cu doped bioactive glass did not cause any variation in the glass structure, since the spectrum (Figure 3a) shows only a peak at about 2 theta = 29.5°, ascribable to $CaCO_3$ (JCPDS code: 01-072-1650), which was also observed on the pristine Cu-doped glass, as reported in [25] and in the inset of figure 3a. The second reduction method investigated in the present paper is the thermal annealing, that is reported by Sheng et al. [31] to be able to induce the nucleation of Cu nanoclusters in copper-doped silicate glass. In this case, the heat treatment at 550 °C in air, both with and without UV pre-treatment, (SBA3-CuUV+TT and SBA3-CuTT samples, figure 3b and 3c respectively) induced the nucleation of Na_2CaSiO_4 (JCPDS code: 01-073-1726) and the formation of CuO (JCPDS code: 01-080-1268). Realistically, since the thermal treatment has been performed at a temperature close the crystallization onset (Figure 2), the reached temperature is sufficient to allow the formation of the first crystallization nuclei of Na_2CaSiO_4, which is a biocompatible phase that can induce bone-like apatite formation in simulated body fluid [47]. Moreover, the thermal treatments in air allow the formation of CuO but not copper reduction.

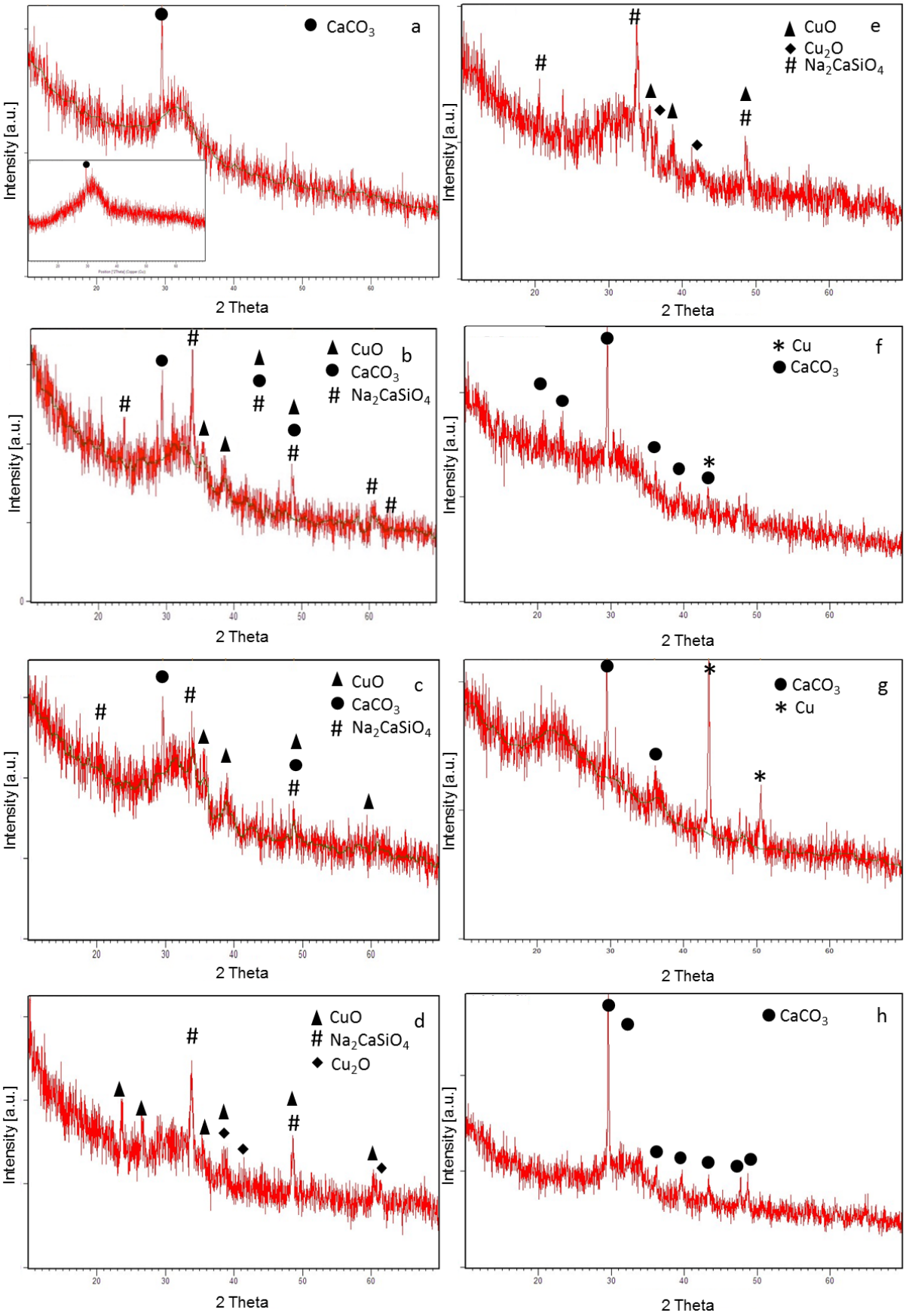

Figure 3: XRD patterns of SBA3-CuUV (a, inset: pristine Cu-doped SBA3), SBA3-CuUV+TT (b), SBA3-CuTT (c), SBA3-CuUV+TTAr (d), SBA3-CuTTAr (e), SBA3-CuTA (f), SBA3-CuAA (g), SBA3-CuNaOH (h).

Heat treatments in Ar (SBA3-CuUV+TTAr and SBA3-CuTTAr samples, figure 3d and 3e respectively) atmosphere induced the nucleation of the same crystalline phases observed after thermal treatment in air and the formation of Cu_2O (JCPDS code: 01-077-0199), supporting the hypothesis that the use of argon allows partial reduction of Cu^{2+} to Cu^{+}.

Regarding the chemical treatments, tannic acid (SBA3-CuTA) and ascorbic acid (SBA3-CuAA) were selected for their well know ability to reduce ions, such as copper, silver or gold ions, to nanoparticles [35-39], starting from metal salts (e.g. copper acetate, copper chloride, and copper sulfate); moreover, they are green reducing agent that can be obtained from vegetal products.

In the present work, the use of tannic acid allowed the copper reduction and the formation of metallic copper, as evidenced by the diffraction pattern (Figure 3f); the same result was obtained using ascorbic acid (Figure 3g): the XRD pattern of SBA3-CuAA samples shows very high peaks of metallic Cu (JCPDS code: 01-085-1326). The treatment with NaOH did not induce any particular change from structural point of view (Figure 3h).

3.3 Morphological and compositional characterization

FESEM-EDS analysis of Cu-doped glasses subjected to physical reduction processes are reported in figure 4. Figure 4a shows the of SBA3-glass for comparison. Glass powders irradiated with UV (Figures 4b and c) showed the same morphology and composition of pristine glass; this result, together with structural analysis, evidenced that the UV irradiation did not induce copper reduction nor the formation of different phases. SBA3-CuUV+TT and SBA3-CuTT samples (heat treated in air) showed a rounded shape (Figures 4d and g) and the presence of nanoparticles, which probably are the first crystallization nuclei of Na_2CaSiO_4 (Figures 4e and h), due to the thermal treatment. The same observation can be deduced for SBA3-CuUV+TTAr and SBA3-CuTTAr samples (heat treated in Ar atmosphere, figure 4l-q): also in this case the formation of the first crystallization nuclei of Na_2CaSiO_4 can be noticed. From a compositional point of view, the EDS microanalysis of area gave a semi-quantitative identification of the elements for all samples and did not show significant differences respect the pristine glass.

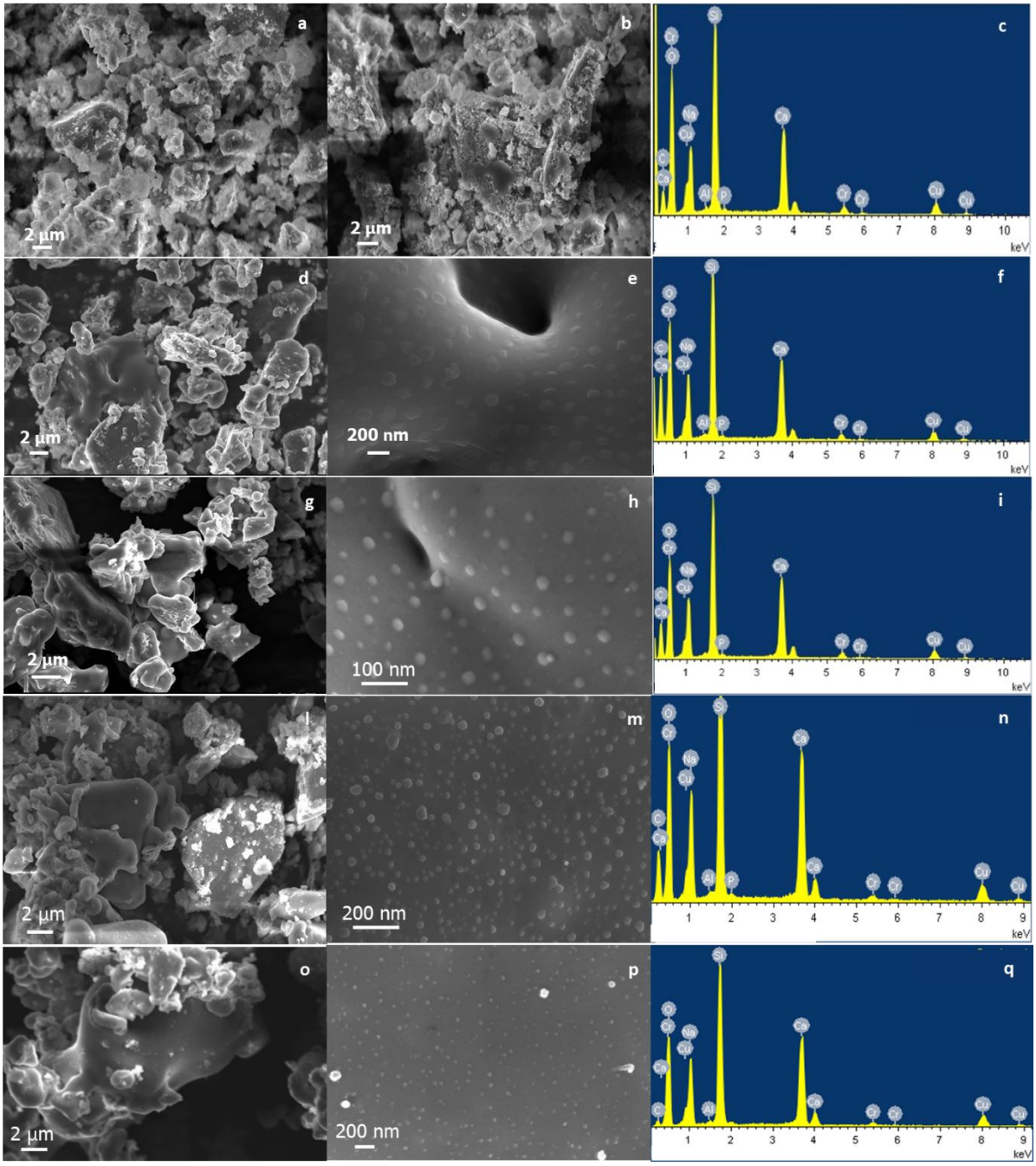

Figure 4: FESEM images and EDS of SBA3-Cu (a), SBA3-CuUV (b, c), SBA3-CuUv+TT (d-f), SBA3-CuTT (g-i), SBA3-CuUV+TTAr (l-n) and SBA3-CuTTAr (o-q).

Morphological and compositional analysis of glass powders subjected to chemical treatments are reported in figure 5. SBA3-CuTA, treated with tannic acid (Figure 5a-c), a low magnification did not show significant variations; however, at higher magnification (Figure 5b) a cracked surface was evidenced on several glass particles. Moreover, semi-quantitative area compositional analysis (Figure 5c) showed a slightly increase of Si content respect the untreated glass.

Also, the analysis at low magnification of SBA3-CuAA sample (treated with sodium L-ascorbate, figure 5d) did not evidence particular changes, but in some area of the sample some agglomerated round particles

with sub-micrometric dimension was observed (Figure 5f). As well as for SBA3-CuTA, EDS analysis showed a higher amount of Si.

FESEM-EDS analysis of SBA3-CuNaOH (Figure 5g-i) displayed the same morphology and composition of SBA3-Cu glass.

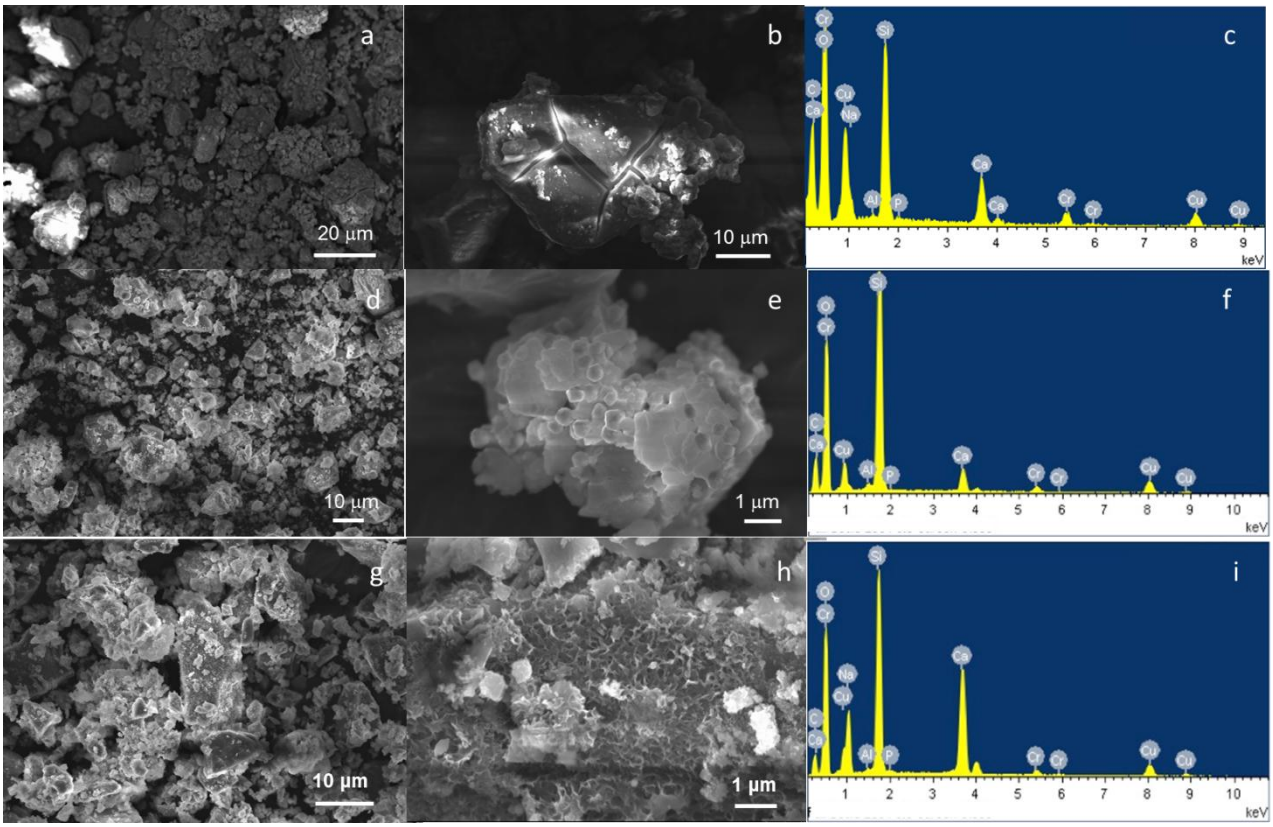

Figure 5: FESEM images and EDS of SBA3-CuTA (a-c), SBA3-CuAA (d-f) and SBA3-CuNaOH (g-i).

In order to verify the effective copper reduction, samples were also subjected to STEM-EDS analysis. Figure 6 shows the images and compositional analysis of the samples in which Cu-containing nanoparticles were evidenced. As it can be noticed, several well dispersed nanoparticles (about 100-200 nm) rich in copper were evidenced on SBA3-CuTTAr surface (Figure 6a-c), even if structural analysis (Figure 3) did not show the presence of metallic copper, but only copper oxides (as evidenced in figure 3e). STEM analysis of SBA3-CuTA evidenced the presence of nanoparticles (about 100 nm) on glass powder surface, which in some point form agglomerates (Figure 6d and e). EDS analysis performed on particles evidenced a great amount of Cu (Figure 6f). SBA3-CuAA sample show the formation of sub-micrometric particles dispersed on glass surface and rich in copper (Figure 6g-i).

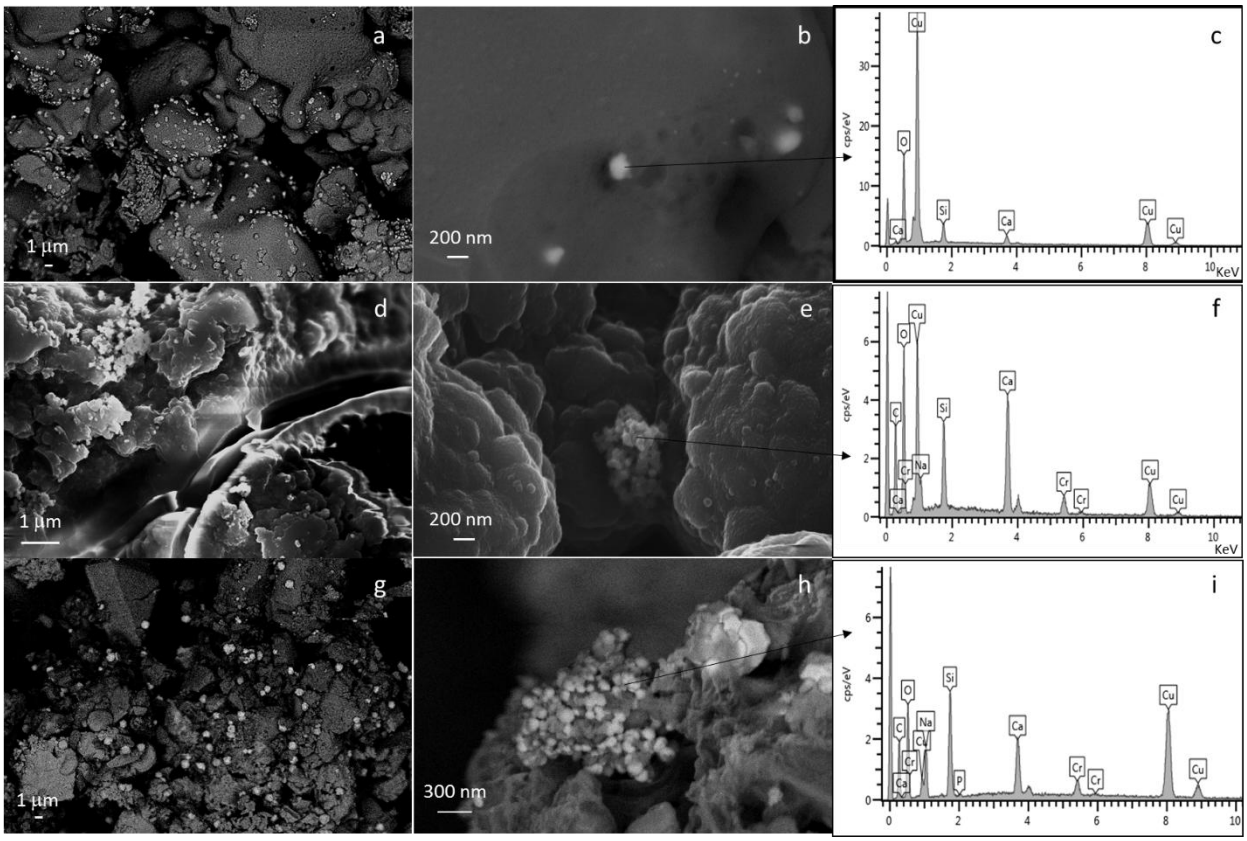

Figure 6: STEM-EDS analysis of SBA3-CuTTAr (a-c), SBA3-CuTA (d-f) and SBA3-CuAA (g-i).

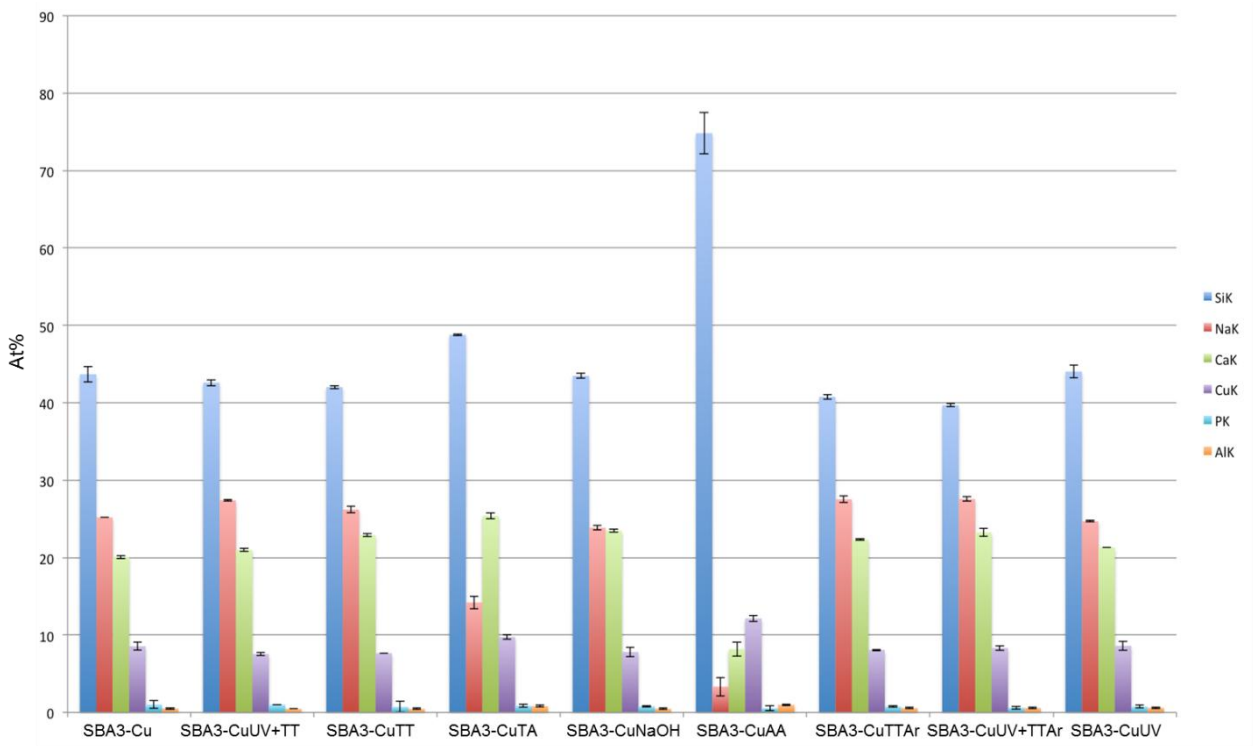

Figure 7: EDS compositional analysis of pristine SBA3-Cu and glasses subjected to physical and chemical treatments.

A resume of compositional analysis performed on the various samples is shown in Figure 7; even if EDS is a semi-quantitative analysis, significant variations in terms of at% of the elements were observed for sample treated with tannic acid and most of all for glass subjected to sodium L-ascorbate treatment. In both cases, an increase of Si content and a decrease of Na were evidenced, as regarding SBA3-Cu7 also a decrease of Ca amount was observed. These two treatments probably are a lightly aggressive and induce a release of Na and Ca.

3.4 Bioactivity

As reported in literature [42-44] the bioactivity process can be divided in five steps: the first one (Step I) consists of a rapid ions exchange between alkaline ions from the glass and hydrogen ions from the solution, in the Step II the formation of silanols occurs (Step II), then they condensate to develop a silica gel layer (Step III). Subsequently calcium, phosphate and carbonate ions are adsorbed in the silica gel (Step IV) and react, forming the hydroxyapatite (HAp) layer (Step V).

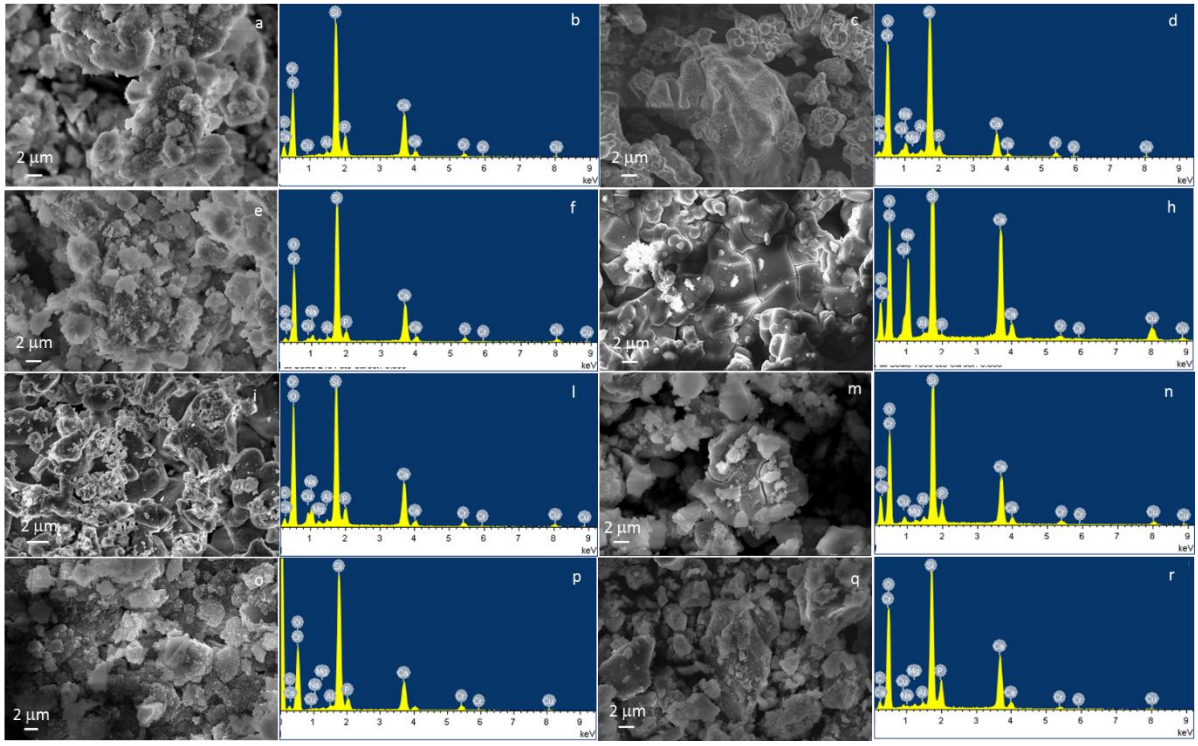

Figure 8: FESEM-EDS analysis of SBA3-CuUV (a,b), SBA3-CuUV+TT (c,d), SBA3-CuTT (e,f), SBA3-CuUV+TTAr (g,h), SBA3-CuTTAr (i,l), SBA3-CuTA (m,n), SBA3-CuAA (o,p), SBA3-CuNaOH (q,r) after 3 days of immersion in SBF

Figure 8 shows the FESEM-EDS analysis after 3 days of immersion in SBF solution of SBA3-Cu glasses subjected to physical and chemical treatments. SBA3-CuUV sample dipped in SBF for 3 days evidenced the formation of reactivity layer on its surface. EDS semi-quantitative elemental concentrations, determined over an area in different sample points, showed a decrease of Na, a high content of Si and an increase of P.

This could be ascribed to the formation of a silica gel layer (step III/IV of bioactivity mechanism) probably jet enriched by Ca and P (Figure 8a and b).

Sample subjected to thermal treatment and UV after 3 days in SBF solution showed a cracked surface due to the formation of a silica gel layer, as also confirmed by EDS analysis, which evidenced the increase of Si amount, together with a slight increase of P and a decrease of Na. Then, also in this case the material shows a surface reaction layer corresponding to the fourth step of bioactivity (Figure 8c and d). Similar results were obtained for SBA3-CuTT glass, also in this case an increase of P and most of all of Si was evidenced; moreover, in some area it was possible to notice the formation of a needle-shaped on glass surface, typical of hydroxyapatite (HAp) or its precursor (Figure 8e and f).

Sample SBA3-CuUV+TTAr (sample irradiated with UV and thermal treated in Ar), after 3 days of SBF dipping, showed a sort of film that cover glass particles, ascribable to silica gel. Moreover, in several areas of the sample, some agglomerates were noticed, some of them having the typical morphology of the HAp. EDS analysis confirmed what observed in the micrographs: compositional analysis of area evidenced a high amount of Si, but EDS analysis of the precipitates showed a very high amount of Ca and P (Figure 8g and h). The same characteristics were evidenced on sample SBA3-CuTTAr; also in this case the analysis of large area show the presence of the silica gel, but in some point of the sample a clear nucleation of HAp or its precursors was observed (Figure 8i and l).

Regarding the chemical treatments, after 3 days of SBF SBA3-CuTA glass evidenced the presence of cracks on particles, which however appeared also before the immersion in SBF. Local FESEM-EDS analysis evidenced the presence of nanometric crystals that covered glass particles. Compositional analysis showed always a high amount of Si and an increase of Ca and P (Figure 8m and n). The sample treated in sodium L-ascorbate (SBA3-CuAA) and dipped in SBF for 3 days showed a surface characterized by some cracks and in some point by the formation of needle-shaped crystals. EDS analysis evidenced a high amount of Si and a decrease of Na (whose amount however was already low for as done sample), together with an increase of Ca and P, which were decreased after the treatment in comparison with SBA3-Cu sample (Figure 8o and p). FESEM-EDS analysis of SBA3-CuNaOH showed the presence of a reaction layer and of nanometric crystals rich in Ca and P. The compositional analysis of the sample surface evidence a high content of Si and an increase of both Ca and P (Figure 8q and r).

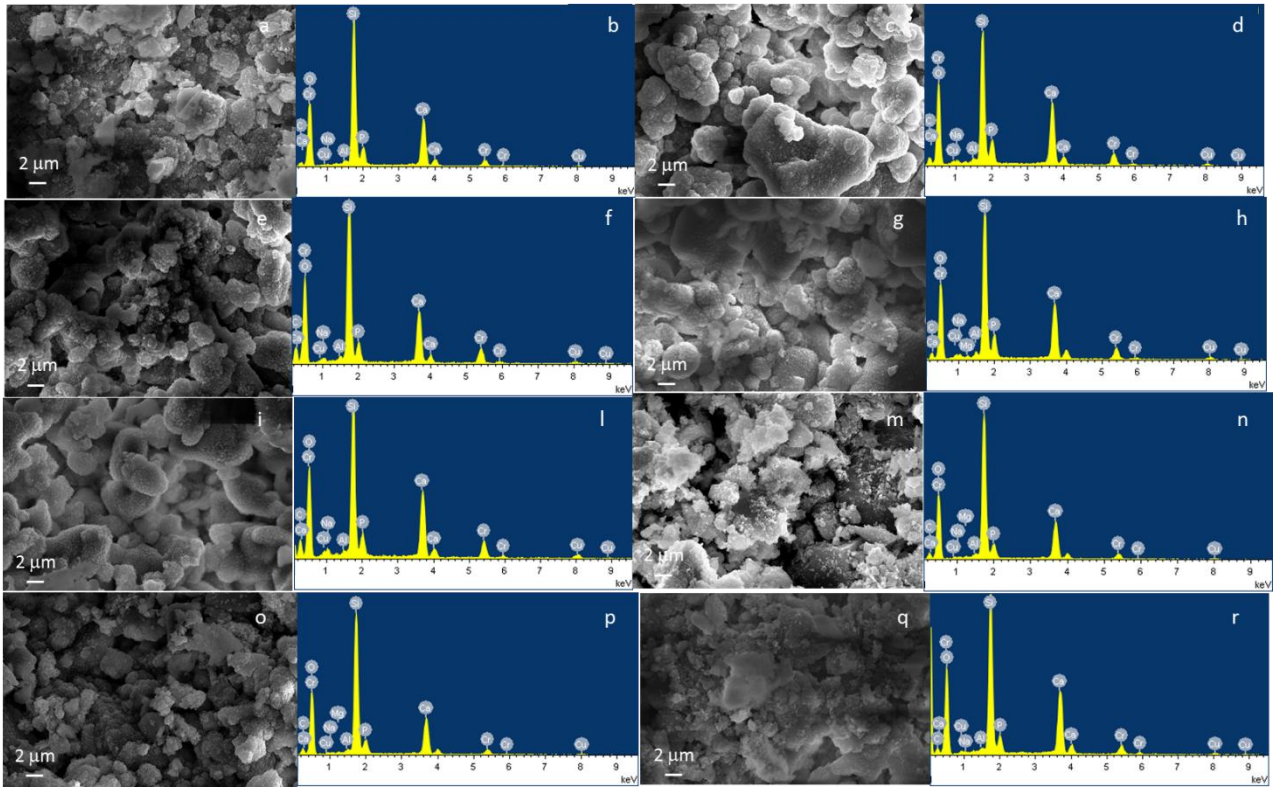

Figure 9: FESEM-EDS analysis of SBA3-CuUV (a,b), SBA3-CuUV+TT (c,d), SBA3-CuTT (e,f), SBA3-CuUV+TTAr (g,h), SBA3-CuTTAr (i,l), SBA3-CuTA (m,n), SBA3-CuAA (o,p), SBA3-CuNaOH (q,r) after 7 days of immersion in SBF solution.

Figure 9 shows the morphological and compositional analyses of samples dipped in SBF for 7 days. The formation of needle-like crystals, ascribable to HAp, is well visible on the surface of SBA3-CuUV glass (Figure 9a and b). Local EDS analysis evidenced the increase of P and Ca, confirming the precipitation of a calcium-phosphates; compositional analysis on various areas showed a slightly decrease of Si, respect the amount observed after 3 days of SBF treatment, and an increase of both Ca and P. The sample SBA3-CuUV+TT after 7 days of immersion on SBF (Figure 9 c and d) evidenced the presence of precipitates having the typical morphology of the *in vitro* grown HAp, rich in Ca and P. EDS analysis conformed the enrichment of Ca and P on glass surface. The same consideration can be done for SBA3-CuTT glass (Figure 9 e and f). Also in this case, the formation of a crystalline phase on sample surface rich in Ca and P can be noticed. Samples thermal treated in Ar atmosphere (SBA3-CuUV+TTAr and SBA3-CuTTAr) showed the formation on their surface of crystal with the typical morphology of the HAp (Figure 9g-l). The EDS analysis, performed both in large areas and locally on some glass particles, evidenced a very distinct Si peak and low amount of Ca and P. However, the quantitative analyses showed a clear increase of Ca and P together with a decrease of Na and Si, thus evidencing a progress on bioactivity process in accordance with morphological observation.

FESEM-EDS analysis of SBA3-CuTA samples dipped in SBF solution up to 7 days showed the presence of a reaction layer rich in Ca and P (Figure 9m and n), even if with the morphology is slightly different from

samples subjected to physical treatments. The semi-quantitative compositional analysis evidenced a continuous increase of Si and a stabilization of Ca and P amount. FESEM-EDS analysis of SBA3-CuAA show the presence of crystals containing Ca and P on glass powders surface, as already evidenced after 3 days. Compositional analysis evidenced a high Si peak, but reduced respect previous SBF dipping time, and an increase of Ca and P values (Figure 9o and p). Also, glass powers treated with NaOH display a morphology ascribable to the formation of HAp and, in some areas, a very intense Ca and P peaks (Figure 9q and r).

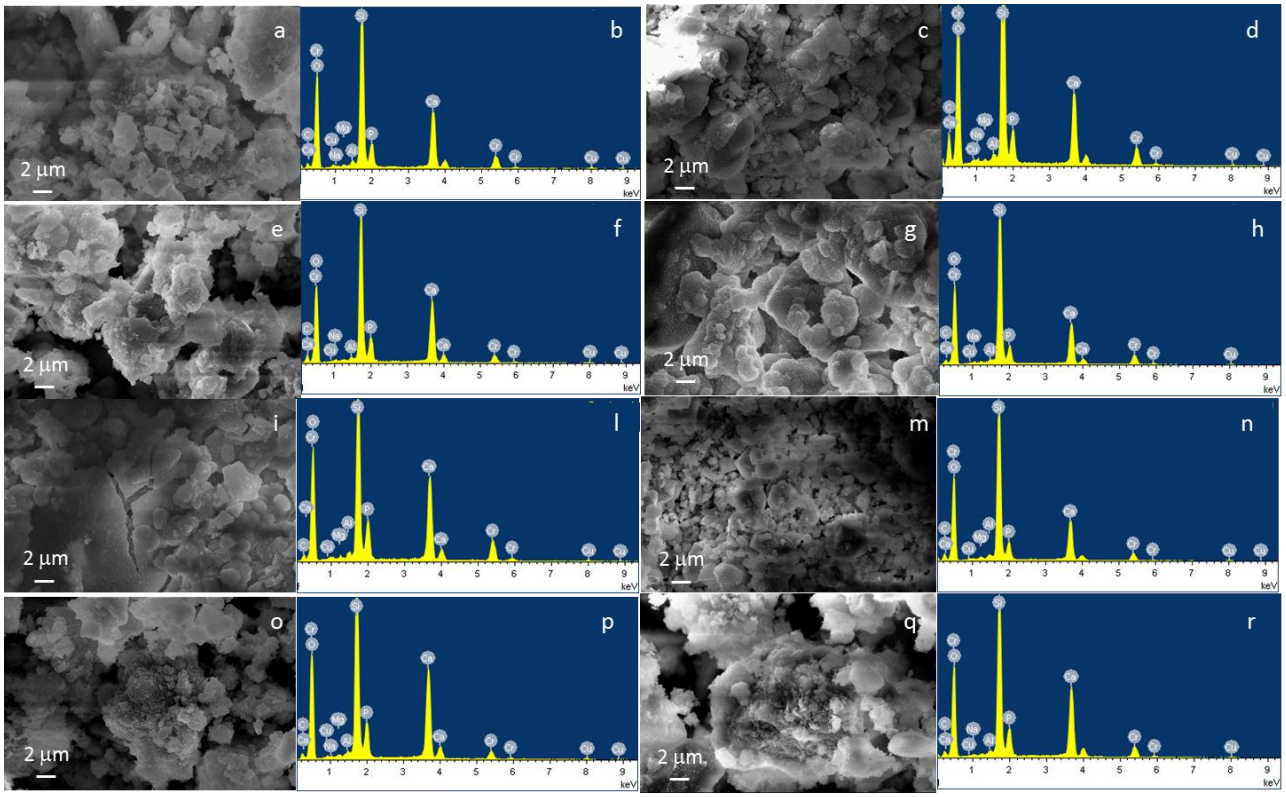

Figure 10: FESEM-EDS analysis of SBA3-CuUV (a,b), SBA3-CuUV+TT (c,d), SBA3-CuTT (e,f), SBA3-CuUV+TTAr (g,h), SBA3-CuTTAr (i,l), SBA3-CuTA (m,n), SBA3-CuAA (o,p), SBA3-CuNaOH (q,r) after 14 days of immersion in SBF solution.

The morphological-compositional characterization of samples after the immersion in SBF for 14 days is reported in figure 10. Glass UV-irradiated (Figure 10a and b) evidenced the formation of a phase rich in Ca and P with the typical morphology of HAp. Compositional analysis of area is not so different from that one after 7 days and evidenced a very low amount of Na, a high content of Si, Ca and P. Sample SBA3-CuUV+TT (Figure 10c and d) evidenced the formation of a crystalline phase rich in Ca and P, already observed after 7 days; the ration Ca/P is about 1.8, very similar to the ratio of HAp (1.67). From morphological point of view, SBA3-CuTT sample after 14 days of SBF treatment (Figure 10e and f) evidenced results very similar to what observed after 7 days and for sample SBA3-CuUV+TT: the presence of a crystalline phase rich in Ca and P, as conformed by compositional analysis. The morphological-compositional analysis of SBA3-CuUV+TTAr and SBA3-CuTTAr samples, evidenced the presence of a nanometric needle-shaped crystal phase (Figure 10g-l).

Also in this case, as it was pointed out after 7 days of SBF immersion, the EDS analysis evidenced a high amount of Si, probably due to the formation of silica-gel; however, the quantitative data and the EDS locally performed (Figure 10h and l) showed also a clear increase of Ca and P.

Samples subjected to tannic acid treatment (SBA3-CuTA) and immersed in SBF up to 14 days do not show significant differences respect the sample after 7 days (Figure 10m and n). The performed analyses displayed dissimilar results in different point: in the areas rich in crystals the amount of Ca in very high, while in the smoother areas the Si peak prevails. So, it seems that the bioactivity process did not show a uniform kinetics. SBA3-CuAA glass after 14 days of SBF treatment shows a surface covered by crystals rich in Ca and P, as already observed after 7 days. EDS analysis of area evidenced a simultaneously decrease of Si and increase of Ca and P (Figure 10o and p). NaOH-treated sample di not shows significant differences respect the 7 days-treatment: a crystalline reaction layer containing Ca and P was formed on glass powders. Moreover, quantitative EDS analysis evidenced an increase of P amount and a stabilization of Si and Ca (Figure 10q and r).

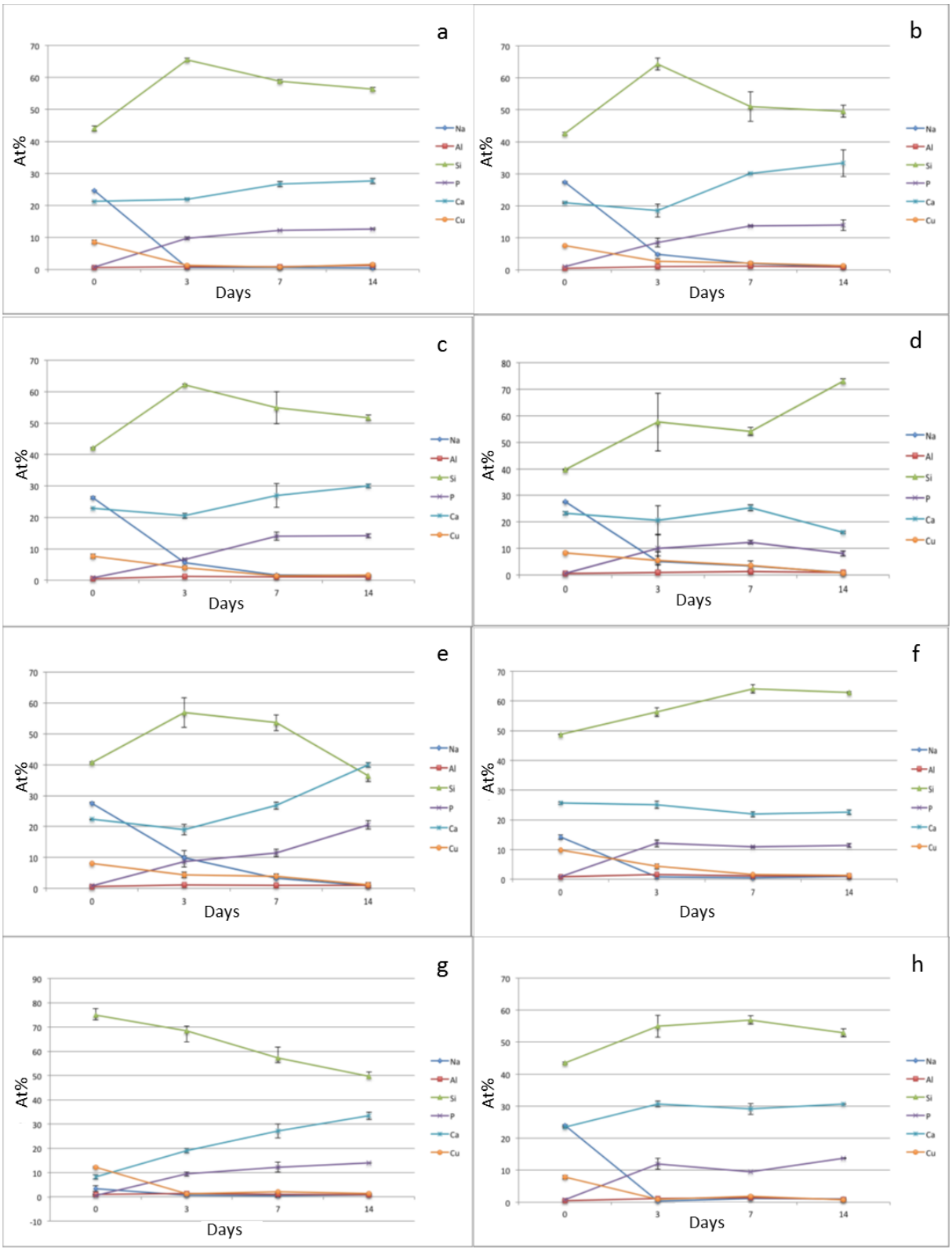

Figure 11: Atomic percentages variation (EDS, surface analysis) of SBA3-CuUV (a), SBA3-CuUV+TT (b), SBA3-CuTT (c), SBA3-CuUV+TTAr (d), SBA3-CuTTAr (e), SBA3-CuTA (f), SBA3-CuAA (g), SBA3-CuNaOH (h) during immersion in SBF up to 14 days

Figure 11 shows the atomic percentage variation of elements (EDS analysis of surface area) during the immersion in SBF solution up to 14 days. In general, an increase of Si amount can be observed during the

first days of immersion in SBF, due to the silica-gel formation, followed by a decrease due to the surface enrichment with other chemical species involved in HAp formation; only SBA3-CuAA (Figure 11g) evidenced an immediate decrease of Si content. In fact, SBA3-CuAA sample, before SBF immersion, showed higher Si content and lower Na and Ca amounts probably ascribable to the slightly aggressive treatment with ascorbic acid, as evidenced in figure 7. Regarding Ca and P trend, an increase during the SBF soaking can be noticed, confirming the results obtained with FESEM-EDS. Moreover, a fast decrease of Na content can be observed, in accordance with the bioactivity mechanism. A decrease of Cu trend can be also noticed, evidencing a release of the element during the immersion in SBF. The data of SBA3-CuUV+TTAr samples after 14 days of SBF treatment are anomalous and in contrast with what observed by FESEM-EDS analysis; this is probably ascribable to a detachment of HAp layer from powders surface during sample preparation and analysis.

The results of FTIR analysis of all glasses after SBF immersion up to 14 days are very similar. Figure 12 shows as example the FTIR spectra of SBA3-CuTA as prepared and after 3 and 14 days of SBF treatment. As it can be noticed the unsoaked glass displays two bands at around 400-500 cm^{-1} and 900-1100 cm^{-1} (centered at 1030 cm^{-1}) ascribable to ν_{sym}(Si–O–Si) and ν_{asym}(Si–O–Si) bands of SiO_4 tratrahedra [48], the shoulder at about 930 cm^{-1} is related to the SiO_{NBO} (NBO-Non-Bonding Oxygen) due to glass network modifier. After SBF immersion a new band at about 1200 cm^{-1} appeared, ascribable to the formation of new Si-O-Si bond on condensation of SiOH groups [49], while the shoulder concerning the SiO_{NBO} disappeared; the strong band centered at 1070 cm^{-1} and the sharp band at 550-600 cm^{-1}, ascribable to PO_4 symmetric stretching vibration and bending mode of P-O-P bond, confirm the HAp presence [49,50].

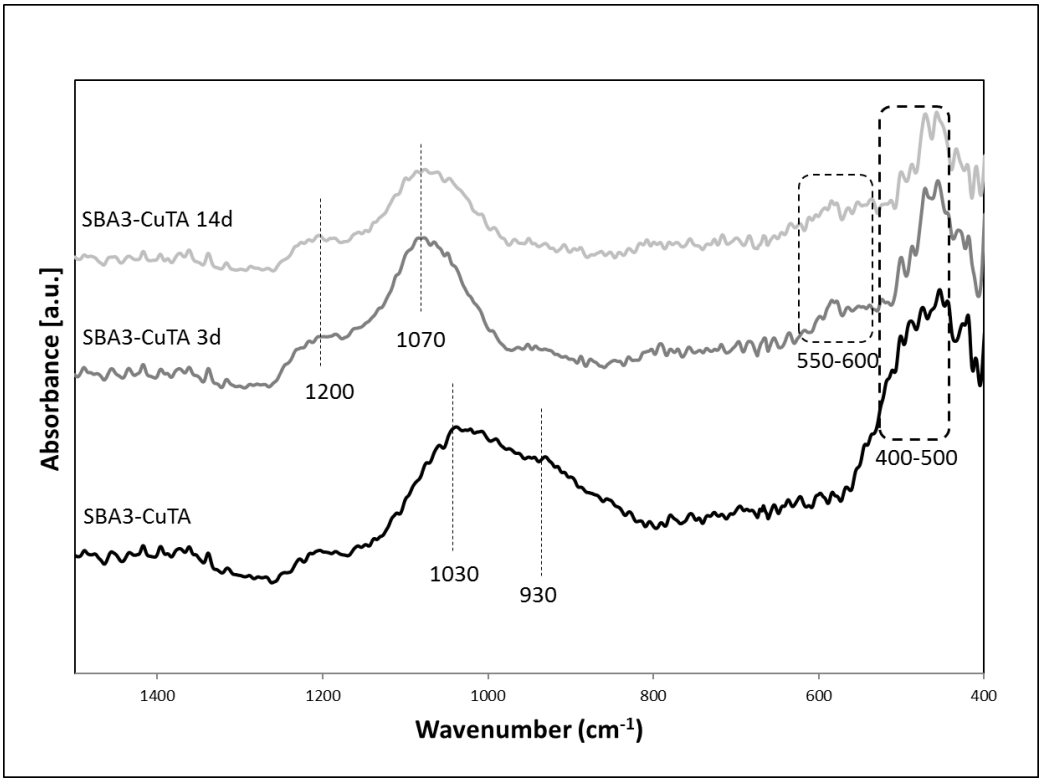

Figure 12: FTIR analysis of SBA3-CuTA

The measurement of pH evidenced a similar trend for all samples: a fast pH increase was observed during the first 3 days up to about 7.7-7.8, which is typically of bioactive glass, due to the exchange of alkaline cations with hydrogen ions from the solution [9,35]. Then, the pH values increase slowly up to 7.9-8.1 up to 14 days, remaining in the physiological tolerability range (7 ~8).

In conclusion, the bioactivity test evidenced the ability of all glasses to induce the precipitation of HAp crystals, regardless of the performed chemical or physical treatments. In literature few studies investigated the role of copper NPs in the reactivity of bioactive glass [51,52] and, on the basis of authors knowledge, there are no researches regarding the bioactive behavior of Cu-doped glasses subjected to *in situ* reducing processes. However, the similar reported studies evidenced contrasting results: Magyar et al. [51] reported that sol-gel derived bioactive glass containing Cu2ONPs up to 0.5 mol% showed an almost unchanged bioactive behavior; while Bejarano et al. [52] evidenced that the growth of apatite was inhibited on sol-gel glasses containing CuO crystals. Thus, the Cu-containing glass bioactivity can be influenced depending on glass composition and treatments.

The evaluation of the antibacterial properties was performed by means of the inhibition halo test. Figure 13 shows the images of the samples on Mueller Hinton agar plate after the incubation at 35 °C for 24 h. As it can be noticed, only SBA3-CuUV (a), SBA3-CuTA (f) and SBA3-CuNaOH (h) produced a significant and reproducible inhibition halo of about respectively 2-2.5 mm, 2 mm and 1.5 mm ± 0.5 mm. The other samples were not able to create an inhibition area; however, a no bacterial growth was observed by inspecting the back of the plate. These results let suppose that the best antibacterial effect is related to the presence of free Cu^{++} ions in the glass network, alone or in synergy with Cu^{0} nanoparticles (samples a, f and h), in good accordance with literature [4], since the presence of CuO crystals reduces the size of the inhibition halo, but a bacteriostatic effect is still visible, probably due to residual free Cu^{++} ions in the glass network or Cu_2O crystals (samples b, c, d, e, g). The treatment with ascorbic acid induced the formation of metallic copper; however, the bigger dimension of observed Cu^{0} particles (sub-micrometric) respect to SBA3-CuTA (about 100 nm) seems to reduce the antibacterial effect. Moreover all samples except SBA3-CuTA created a blue diffusion halo, due to Cu^{++} ions which diffuse in the agar, some of them more evident than the others, mainly if free Cu^{++} is still present in the glass network, as confirmed also by the blue color of the sample a and h(containing only Cu^{++}). Samples with dark color showing blue diffusion halo (b, c, d, e, g) probably still contain traces of Cu^{++} or are able to release it from CuO nanocrystals or from Cu^{0} by oxidation. On the contrary the diffusion halo of SBA3-CuTA (f) was brown, probably because this sample mainly contains copper as Cu^{+} and Cu^{0}.

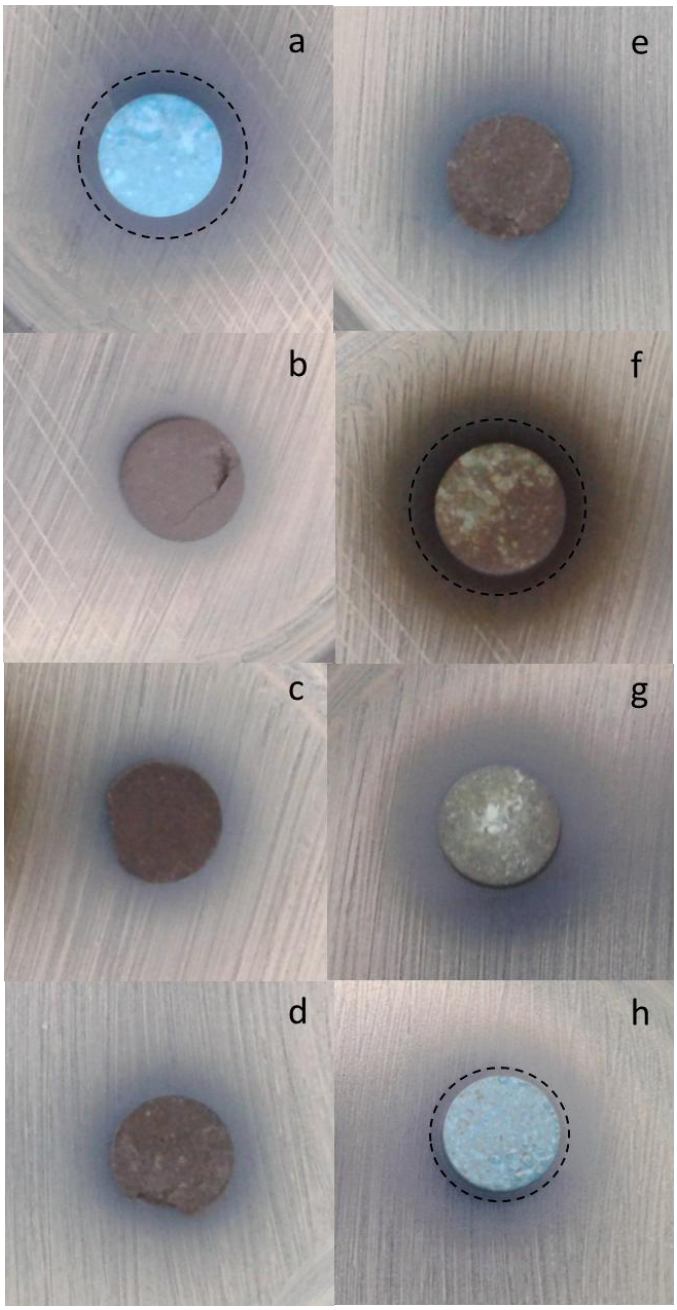

Figure 13: inhibition halo test against *S. aureus* of a) SBA3-CuUV, b) SBA3-CuUV+TT, c) SBA3-CuTT, d) SBA3-CuUV+TTAr, e) SBA3-CuTTAr, f) SBA3-CuTA, g) SBA3-CuAA and h) SBA3-CuNaOH

Conclusions

This work investigated the ability of different physical and chemical treatments to induce the *in situ* reduction of Cu^{++} ions, introduced in the outer surface layer of a bioactive glass by ion-exchange process, to Cu^{+} or Cu^{0}. The XRD, FESEM/STEM-EDS analyses demonstrated that only the chemical treatments using tannic acid and sodium L-ascorbate induce the formation of metallic copper; in particular, SBA3-CuTA sample evidenced the presence of Cu^{0} nanoparticles. The glass powders treatment with NaOH and UV irradiation did not induce structural and morphological modifications. The thermal treatment in air

promoted the nucleation of CuO, while the inert atmosphere induced the formation of Cu_2O. FESEM-EDS and FT-IR analysis after in vitro test in SBF up to 14 days demonstrated that all the investigated glasses maintained a bioactive behavior. Finally, antibacterial test towards *S. aureus* strain evidenced that the best antibacterial effect is achieved by glasses containing free Cu^{++} ions (SBA3-CuUV, SBA3-CuNaOH) and Cu^0 nanoparticles (SBA3-CuTA). An increase of Cu^0 dimension (SBA3-CuAA) or the presence of CuO and Cu_2O (formed during thermal treatments) reduced the antibacterial effect, even if a bacteriostatic effect was observed. In order to assess if the antibacterial effect is to be ascribed to a "contact killing" or a "leaching" mechanism, the potential leakage of Cu ions into biological fluid, their redox and catalytic activities, as well as their potential impact on biological assays, in presence of high physiological protein levels, could be of interest and could be object of future investigations on a selected set of samples.

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In situ chemical and physical reduction of copper on bioactive glass surface

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Abstract

In this work silica-based bioactive glass powders were doped with Cu^{++} ions by means of ion-exchange process and subsequently they were exposed to different chemical (tannic acid, ascorbic acid and NaOH) and physical processes (UV irradiation, thermal treatment in air or argon atmosphere) to promote the in situ reduction of Cu^{++} ions to Cu^{+} or Cu^{0}. The obtained glasses were investigated by means of structural (X-Ray diffraction – XRD), morphological and compositional analyses (scanning-transmission electron microscopy equipped with energy dispersive spectroscopy (FESEM/STEM-EDS); moreover, glasses were subjected to *in vitro* bioactivity test in simulated body fluid up to 14 days to investigate the influence of the performed chemical and physical treatments on glass bioactivity. At the end of incubation time glasses were analyzed by means of FESEM-EDS and Fourier transformation infrared spectroscopy (FT-IR). The obtained results evidenced that, in general, the chemical treatments are useful to induce the formation of Cu^{0}, while thermal treatment induce the nucleation of CuO (in air) or Cu_2O (in argon). All samples maintained a bioactive behavior. At least, preliminary antibacterial effect evaluation by inhibition halo test evidenced that samples containing free Cu^{++} ions or Cu^{0} nanoparticles possess the best antibacterial effect.

Keywords

Bioactive glass; surface modification; copper ion exchange; in situ chemical/physical reduction; antibacterial.

1. Introduction

Copper (Cu) and its compounds have been recently reported in numerous studies as effective tools to reduce the incidence of contact-mediated infections [1-3], due to several advantages in comparison with other antimicrobial materials, such as, among others, their multitoxicity and effectiveness against multiresistant microorganisms. The mechanism of “contact killing” of bacteria recognized to metallic Cu

surfaces [4], that makes it an intrinsically antibacterial material, is gaining increasing attention in the face of growing antibiotics resistance of bacteria, even if the role of the interaction between bacteria and metal surface, media composition and metal surface chemistry on contact killing is still object of few studies. For example, the antibacterial properties of thermally generated Cu oxides (Cu_2O and CuO) in comparison to pure Cu, as well as the formation of Cu oxides on Cu during antimicrobial test, was investigated by M. Hans et al. [4] demonstrating that CuO was significantly effective against *E. hirae*, compared to pure Cu, and Cu_2O was as effective as pure Cu. The release of Cu^+ and Cu^{2+} ions from the different surfaces was highest for pure copper, followed by Cu_2O and CuO and was only approximately correlated with their antibacterial efficacy. Other researchers investigated the antibacterial activity of copper NPs related to their oxidation state and suggested that cuprous state of copper bind to the proteins and so Cu_2O NPs show high affinity to the bacterial cells, while CuO NPs effect is ascribable to the significant production of ROS (reactive oxygen species) [5] The efficacy of copper metallic nanoparticles has also been investigated by several authors [6, 7]; however, there are very few studies focused on the Cu-NPs bactericidal mechanism [7]. In general three mechanisms of action are reported in literature: i) changing of bacterial membrane permeability due to the accumulation and dissolution of NPs; ii) NPS ability to generate reactive oxygen species (ROSs) or/and their corresponding ions; iii) uptake of released metallic ions; moreover, Cu NPs seem to possess a prolonged antibacterial effect [7].

Copper at different oxidation states (Cu^{2+}, Cu^+, and Cu^0), can be added in glasses both as network modifier or nanoparticles [8-12]. Focusing the attention on biomedical applications, Cu in ionic form has been included in the composition of several bioactive glasses, a class of materials widely studied as bone substitutes for their ability to chemically bond to living bone through a peculiar surface reactivity, which implicates ion-exchange between glass and biological fluids, the development of a silica-rich amorphous layer, the adsorption of calcium and phosphate ions and their crystallization into hydroxyapatite (HAp) [13-15]. Silica-based Cu-containing bioactive glasses have been obtained by sol–gel [16-21], as well as by the traditional melt and quenching technique both for silica-based bioactive glasses [22,23] and phosphate glasses [24]. The ion-exchange technique in aqueous solution was also used to dope with Cu ions the surface of different silica-based bioactive glasses [25,26]. In all these studies the goal was to develop bioactive and antibacterial glasses without affecting the structure, morphology, composition and properties of the glasses. These studies demonstrated that the glasses doped with Cu ions significantly reduced the bacterial adhesion and proliferation, while preserving their bioactivity.

In the present paper, powders of a silica-based bioactive glass, prepared by melt and quenching route, have been doped with Cu^{++} ions by ion-exchange process in aqueous solution and, for the first time on the basis of authors knowledge, subjected to several physical and chemical treatments in order to induce the in situ reduction of Cu^{++} ions to Cu^+ or Cu^0. Thus, the first aim of this study was to investigate the best physical and/or chemical treatments able to reduce Cu^{++} ions directly on bioactive glass surface and the possible

formation of nanoparticles, in order to investigate the possibility of improving the bioactive glass surface properties with the peculiar features of copper nanoparticles, such as high surface to volume ratio, multiple mode of antibacterial action and the possibility to be further functionalized with drugs [27-30]. The second goal of this work was to verify the bioactivity and preliminary evaluate the antibacterial effect of the modified glass containing copper in different oxidation state, by means of commonly used standard tests and conditions, with comparative purposes among the various sets of samples. These preliminary characterizations are expected to be helpful in the selection of a representative set of samples, obtained by the best treatment, that could be object of future biological assays in a more complex and realistic environment.

2. Materials and Methods

2.1 Synthesis and thermal characterization of Cu-containing bioactive glass

The starting glass (named SBA3 from now on), having the molar composition 48% SiO_2, 26% Na_2O, 22% CaO, 3% P_2O_5, 0.43% B_2O_3, 0.57% Al_2O_3, was produced by melting and quenching process. All chemicals were purchased from Sigma-Aldrich (Switzerland, reagent grade > 99%). Briefly, SBA3 precursors were melted in a platinum crucible at 1450 °C for 1 h (heating rate 10 °C/min) and cooled in water to obtain a frit. Subsequently, the frit was mechanically milled into a zirconia jar (300 rpm for 1 h) and sieved to obtain glass powders with a final grain size < 20 μm. Copper was introduced in the glass powders (SBA3-Cu) by means of ion-exchange process in aqueous solution of copper acetate 0.05 M for 1 h at 37 °C and 150 rpm; at the end of the process, the solution was removed and SBA3-Cu powders were washed with bi-distilled water, filtered and dried as reported in [25]. The glasses (doped and undoped) structure, morphology, composition and antibacterial properties were evaluated in a previous work [25]. In the present work Differential Thermal Analysis (DTA, 404 PC Netzsch) was carried out in order to identify the characteristic temperatures of the glass in view of reduction processes, that involve, in some cases, a thermal treatment.

2.2 In situ reduction processes

Aiming to induce the *in situ* reduction of copper ions, SBA3-Cu powders were subjected to several physical and chemical treatments, adapted from literature [31-34], as reported in table 1.

SBA3 containing Cu^{2+} ions				
	Acronym	**UV irradiation**	**550 °C air**	**550 °C Ar**
Physical treatments	**UV**	X		
	UV+TT	X	X	
	TT		X	

	UV+TTAr	X		X
	TTAr			X
Chemical treatments	**TA**	Tannic acid		
	AA	Sodium L-ascorbate		
	NaOH	Sodium hydroxyde		

Table 1: physical and chemical treatments and samples acronym.

In particular, for what is concerning the physical treatments, SBA3-Cu glass powders were subjected to UV-light irradiation (SBA3-Cu1 sample, 20 W for 30 min) [31,32]. The glass powders were also subjected to a thermal treatment at 550 °C for 2h (with – SBA3-Cu2 and without – SBA3-Cu3 UV irradiation) [32]. The thermal treatments at 550 °C for 2 h (after UV irradiation – SBA3-Cu4 – or not – SBA3-Cu5) were also performed in argon atmosphere in order to evaluate the role of the atmosphere in the reduction process [33,34].

As far as the chemical treatments is concerned, SBA3-Cu powders were treated with a 0.01 M solution of tannic acid at 37 °C, 150 rmp for 30 minutes, while a solution 0.1 M of sodium L-ascorbate at 80 °C, 150 rmp for 30 minutes were used. The concentrations were selected on the basis of literature [35-39]. At the end of treatments glass powders were washed with bi-distilled water, filtered and dried overnight at 37 °C.

Lastly, the reducing ability of NaOH [37] was estimated by immersing SBA3-Cu in a 1 M solution of NaOH at 80 °C, 150 rpm for 1 h (SBA3-Cu8). Then, sample was washed with bi-distilled water, filtered and dried overnight at 37 °C. These treatments have been adapted from literature in order to induce the *in situ* reduction of Cu^{++} ions. The formation of Cu^{+} ions, copper oxides and Cu° nanoparticles on the glass surface has been hypothesized and verified by morphological observation, chemical and phase analysis.

Figure 1 resumes the possible reduction mechanisms involved during *in situ* chemical and physical treatments.

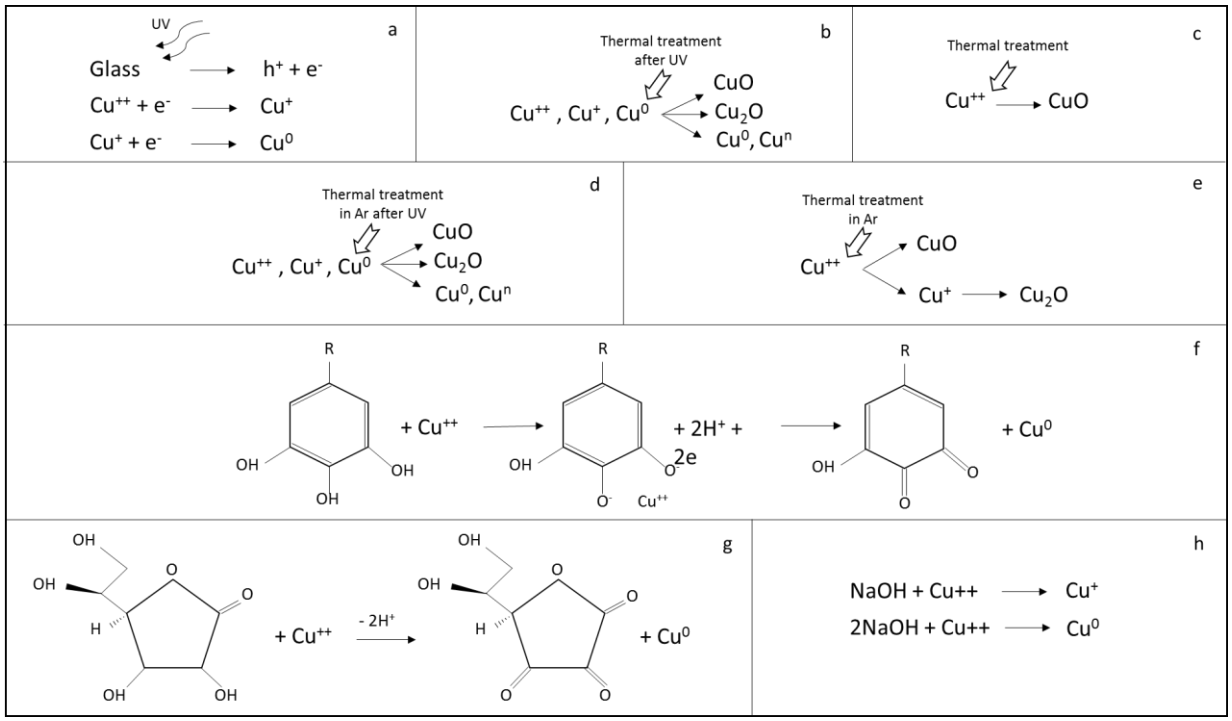

Figure 1: proposed mechanism of *in situ* reduction after a) UV irradiation (h^+ is a hole center, e^- is an electron), b) thermal treatment after UV, c) thermal treatment, d) thermal treatment in Ar after UV, e) thermal treatment in Ar, f) tannic acid treatment, g) sodium L-ascorbate treatment, h) NaOH treatment.

2.3 Glasses characterizations

All glasses exposed to the reduction process were subjected to structural analysis by means of XRD (X'Pert Philips diffractometer), using the Bragg Brentano camera geometry and the Cu-Ka incident radiation (source voltage and current set at 40 kV and 30 mA, incident wavelength λ = 1.5405 Å, step size Δ(2θ) = 0.02°, fixed counting time of 1 s per step), in order to identify the formation of new crystalline phases and in particular metallic copper. The obtained patterns were analysed with X'Pert High Score software and the PCPDF data bank.

Morphological and compositional analyses were performed by means of scanning electron microscopy equipped with energy dispersive spectroscopy (FESEM-EDS, SUPRATM 40, Zeiss) and scanning transmission electron microscopy (STEM, Merlin Gemini Zeiss) to evaluate the influence of the reduction treatments on glass powders morphology and composition and to investigate the possible formation of copper nanoparticles and their dimensional range. Samples for SEM observation were prepared by positioning glass powders on adhesive carbon tape, attached to aluminum stub. Samples were chromium-coated prior to the analysis. STEM analysis was performed by preparing a glass particles suspension and depositing a drop on a copper TEM grid with carbon film (SPI Supplies® Brand Lacey Carbon Coated 200 Mesh Copper Grids – JEOL S.p.A.).

All glasses were then subjected to *in vitro* bioactivity test in simulated body fluid (SBF), an inorganic solution developed at the Department of Material Chemistry, Graduate School of Engineering, Kyoto University, which is characterized by ion concentrations nearly equal to those of human blood plasma, buffered at pH 7.40 with 50 mM trishydroxymethylaminomethane and 45 mM hydrochloric acid at 36.5°C, as reported in literature [40]. The test was performed by dipping glass powders (100 mg) in 100 ml of SBF following the protocol of Kyoto University as suggested by the ISO standard [41] and maintaining samples at 37 °C for 3, 7 and 14 days at 150 rpm in orbital shaker (IKA® KS4000i control). As reported in literature [42-44] the bioactivity test is useful to predict the ability of bioactive glasses to bond to leaving bone by observing the growth of a hydroxyapatite (HAp) layer on their surface after soaking in SBF.

The pH of the solutions was monitored every 2-3 days. At the end of incubation time, glass powders were gently washed with bi-distilled water, filtered and dried overnight at 37 °C; subsequently, FESEM-EDS analyses were performed to verify the precipitation of hydroxyapatite or its precursor on glass powders. Moreover, Fourier transformation infrared spectroscopy (FT-IR) was used to investigate the reactivity of the glasses and accurately monitoring the formation of reaction phases during SBF immersion. FT-IR spectra were acquired in a Hyperion 2000 FT/IR (Tensor 27, Bruker Optics S.p.A, Ettlingen, Germany) from 2000 to 400 cm^{-1} and with 2 cm^{-1} resolution. OPUS software (v. 6.5, Bruker S.p.A) was used for instrumental control and spectral acquisition.

A preliminary investigation of antibacterial properties of Cu-containing glasses was carried out by means of inhibition halo test (Kirby Bauer test in accordance with National Committee for Clinical Laboratory Standards [45]). The test was performed using a standard *Staphylococcus aureus* strain (ATCC 29213), which is one of the strains most involved in infections development [46]. To realize the test, glass pellets were prepared by weight 200 mg of each glass powder and press them at 4 tons for 10 seconds in an automatic press (Graseby-Specac T-40). Then glass pellets were placed in contact with Mueller Hinton agar plates uniformly covered with bacteria, as recommended by NCCLS standard and reported in [25], and incubated overnight at 35 °C. At the end of incubation time possible formation of a halo around the sample, in which the bacteria have not proliferated was observed and measured.

3. Results and Discussion

3.1 *Thermal characterization of Cu-containing bioactive glass*

Figure 2 shows the DTA traces of SBA3 and SBA3-Cu glass powders; regarding SBA3 glass (Figure 2a), the glass transition temperature was observed at about 550 °C, while an exothermic peak was individuated at 658 °C, with an onset of crystallization at about 560 °C. The copper introduction (Figure 2b) slightly decreases both the characteristic temperatures: the glass transition temperature of SBA3-Cu is about at 510 °C, while the crystallization peak occurs at 652 °C with an onset at about 560 °C.

On the basis of the DTA analyses, the thermal treatments in air and in Ar were performed at 550 °C, the maximum temperatures that allows to avoid significant crystallization phenomena.

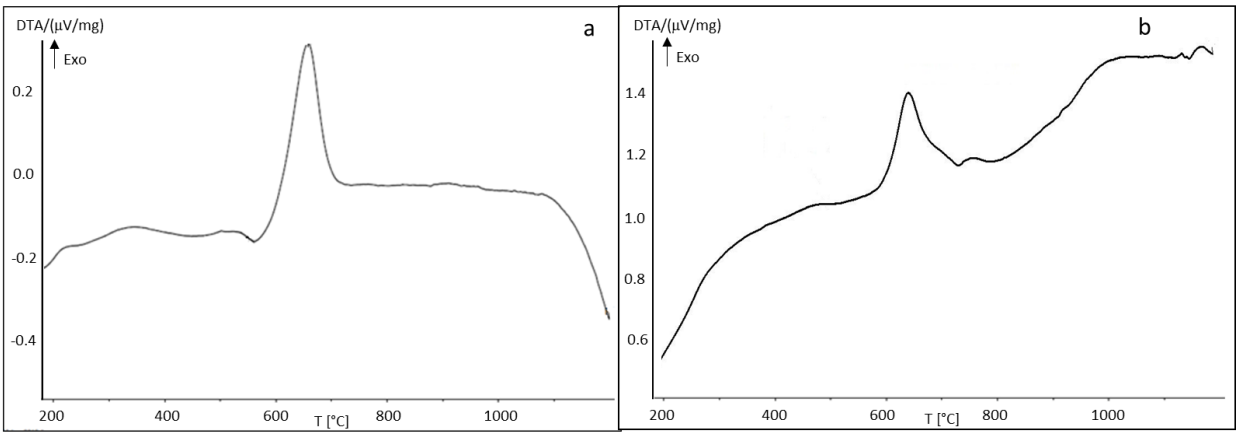

Figure 2: DTA graph of SBA3 (a) and SBA3-Cu (b) glass powders.

3.2 Phase analysis

XRD analyses, reported in figure 3, were performed to investigate the possible crystallization of new phases due to the different reducing treatments. The first reduction method investigated in this work is the irradiation with UV light, that it is reported to induce the formation of copper nanoclusters [31,32] in copper doped-silicate glass and starting from cupric oxide (CuO) and cuprous oxide (Cu_2O). However, the UV irradiation of Cu doped bioactive glass did not cause any variation in the glass structure, since the spectrum (Figure 3a) shows only a peak at about 2 theta = 29.5°, ascribable to $CaCO_3$ (JCPDS code: 01-072-1650), which was also observed on the pristine Cu-doped glass, as reported in [25] and in the inset of figure 3a. The second reduction method investigated in the present paper is the thermal annealing, that is reported by Sheng et al. [31] to be able to induce the nucleation of Cu nanoclusters in copper-doped silicate glass. In this case, the heat treatment at 550 °C in air, both with and without UV pre-treatment, (SBA3-CuUV+TT and SBA3-CuTT samples, figure 3b and 3c respectively) induced the nucleation of Na_2CaSiO_4 (JCPDS code: 01-073-1726) and the formation of CuO (JCPDS code: 01-080-1268). Realistically, since the thermal treatment has been performed at a temperature close the crystallization onset (Figure 2), the reached temperature is sufficient to allow the formation of the first crystallization nuclei of Na_2CaSiO_4, which is a biocompatible phase that can induce bone-like apatite formation in simulated body fluid [47]. Moreover, the thermal treatments in air allow the formation of CuO but not copper reduction.

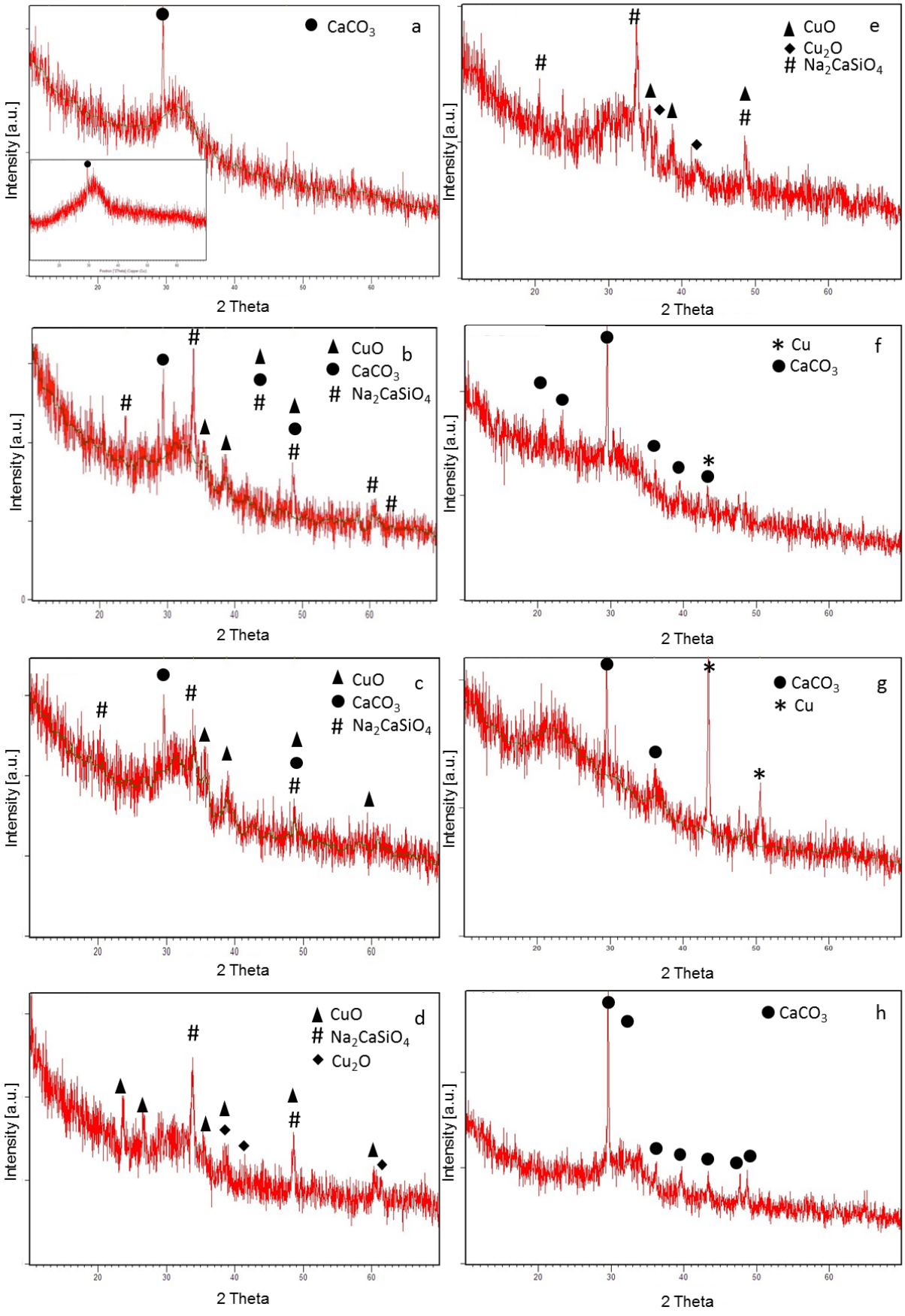

Figure 3: XRD patterns of SBA3-CuUV (a, inset: pristine Cu-doped SBA3), SBA3-CuUV+TT (b), SBA3-CuTT (c), SBA3-CuUV+TTAr (d), SBA3-CuTTAr (e), SBA3-CuTA (f), SBA3-CuAA (g), SBA3-CuNaOH (h).

Heat treatments in Ar (SBA3-CuUV+TTAr and SBA3-CuTTAr samples, figure 3d and 3e respectively) atmosphere induced the nucleation of the same crystalline phases observed after thermal treatment in air and the formation of Cu_2O (JCPDS code: 01-077-0199), supporting the hypothesis that the use of argon allows partial reduction of Cu^{2+} to Cu^{+}.

Regarding the chemical treatments, tannic acid (SBA3-CuTA) and ascorbic acid (SBA3-CuAA) were selected for their well know ability to reduce ions, such as copper, silver or gold ions, to nanoparticles [35-39], starting from metal salts (e.g. copper acetate, copper chloride, and copper sulfate); moreover, they are green reducing agent that can be obtained from vegetal products.

In the present work, the use of tannic acid allowed the copper reduction and the formation of metallic copper, as evidenced by the diffraction pattern (Figure 3f); the same result was obtained using ascorbic acid (Figure 3g): the XRD pattern of SBA3-CuAA samples shows very high peaks of metallic Cu (JCPDS code: 01-085-1326). The treatment with NaOH did not induce any particular change from structural point of view (Figure 3h).

3.3 Morphological and compositional characterization

FESEM-EDS analysis of Cu-doped glasses subjected to physical reduction processes are reported in figure 4. Figure 4a shows the of SBA3-glass for comparison. Glass powders irradiated with UV (Figures 4b and c) showed the same morphology and composition of pristine glass; this result, together with structural analysis, evidenced that the UV irradiation did not induce copper reduction nor the formation of different phases. SBA3-CuUV+TT and SBA3-CuTT samples (heat treated in air) showed a rounded shape (Figures 4d and g) and the presence of nanoparticles, which probably are the first crystallization nuclei of Na_2CaSiO_4 (Figures 4e and h), due to the thermal treatment. The same observation can be deduced for SBA3-CuUV+TTAr and SBA3-CuTTAr samples (heat treated in Ar atmosphere, figure 4l-q): also in this case the formation of the first crystallization nuclei of Na_2CaSiO_4 can be noticed. From a compositional point of view, the EDS microanalysis of area gave a semi-quantitative identification of the elements for all samples and did not show significant differences respect the pristine glass.

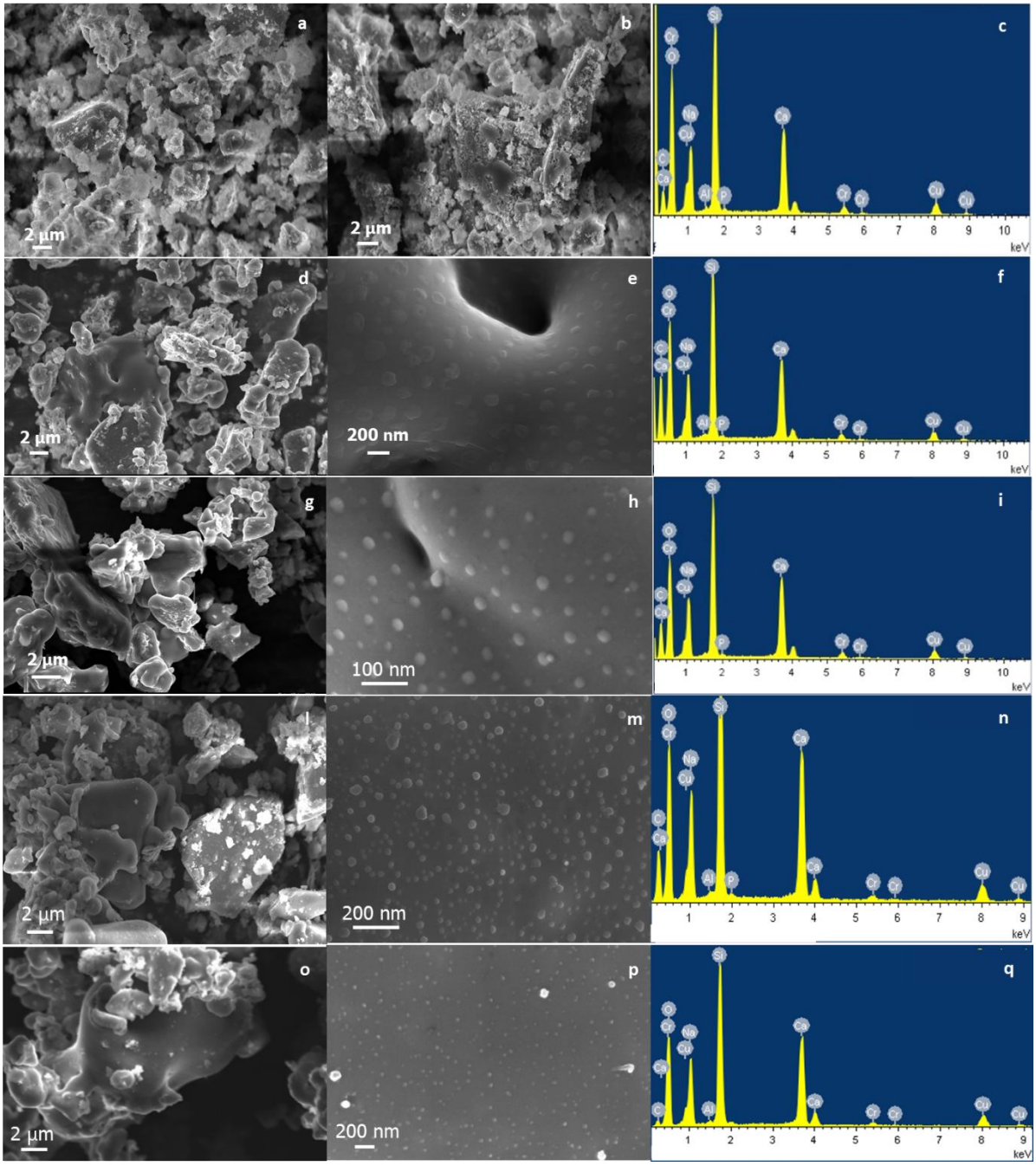

Figure 4: FESEM images and EDS of SBA3-Cu (a), SBA3-CuUV (b, c), SBA3-CuUv+TT (d-f), SBA3-CuTT (g-i), SBA3-CuUV+TTAr (l-n) and SBA3-CuTTAr (o-q).

Morphological and compositional analysis of glass powders subjected to chemical treatments are reported in figure 5. SBA3-CuTA, treated with tannic acid (Figure 5a-c), a low magnification did not show significant variations; however, at higher magnification (Figure 5b) a cracked surface was evidenced on several glass particles. Moreover, semi-quantitative area compositional analysis (Figure 5c) showed a slightly increase of Si content respect the untreated glass.

Also, the analysis at low magnification of SBA3-CuAA sample (treated with sodium L-ascorbate, figure 5d) did not evidence particular changes, but in some area of the sample some agglomerated round particles

with sub-micrometric dimension was observed (Figure 5f). As well as for SBA3-CuTA, EDS analysis showed a higher amount of Si.

FESEM-EDS analysis of SBA3-CuNaOH (Figure 5g-i) displayed the same morphology and composition of SBA3-Cu glass.

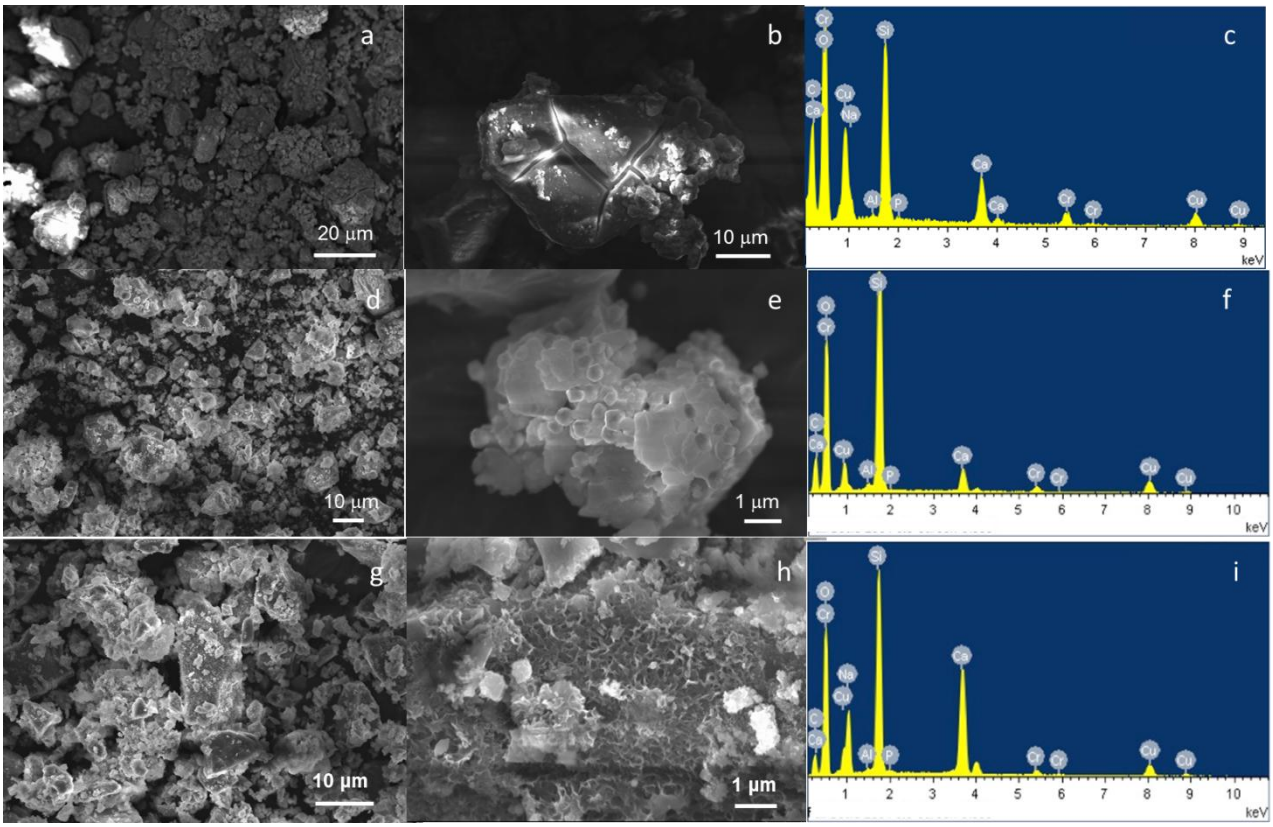

Figure 5: FESEM images and EDS of SBA3-CuTA (a-c), SBA3-CuAA (d-f) and SBA3-CuNaOH (g-i).

In order to verify the effective copper reduction, samples were also subjected to STEM-EDS analysis. Figure 6 shows the images and compositional analysis of the samples in which Cu-containing nanoparticles were evidenced. As it can be noticed, several well dispersed nanoparticles (about 100-200 nm) rich in copper were evidenced on SBA3-CuTTAr surface (Figure 6a-c), even if structural analysis (Figure 3) did not show the presence of metallic copper, but only copper oxides (as evidenced in figure 3e). STEM analysis of SBA3-CuTA evidenced the presence of nanoparticles (about 100 nm) on glass powder surface, which in some point form agglomerates (Figure 6d and e). EDS analysis performed on particles evidenced a great amount of Cu (Figure 6f). SBA3-CuAA sample show the formation of sub-micrometric particles dispersed on glass surface and rich in copper (Figure 6g-i).

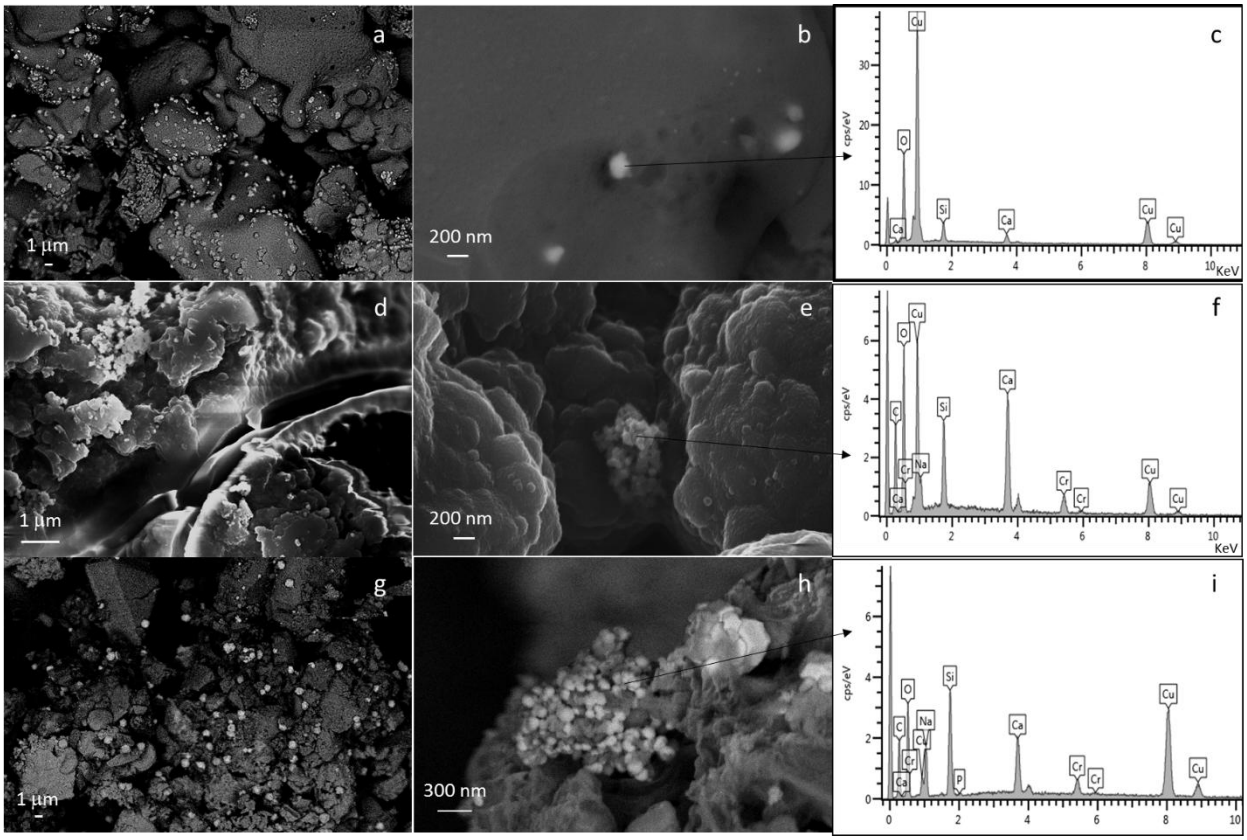

Figure 6: STEM-EDS analysis of SBA3-CuTTAr (a-c), SBA3-CuTA (d-f) and SBA3-CuAA (g-i).

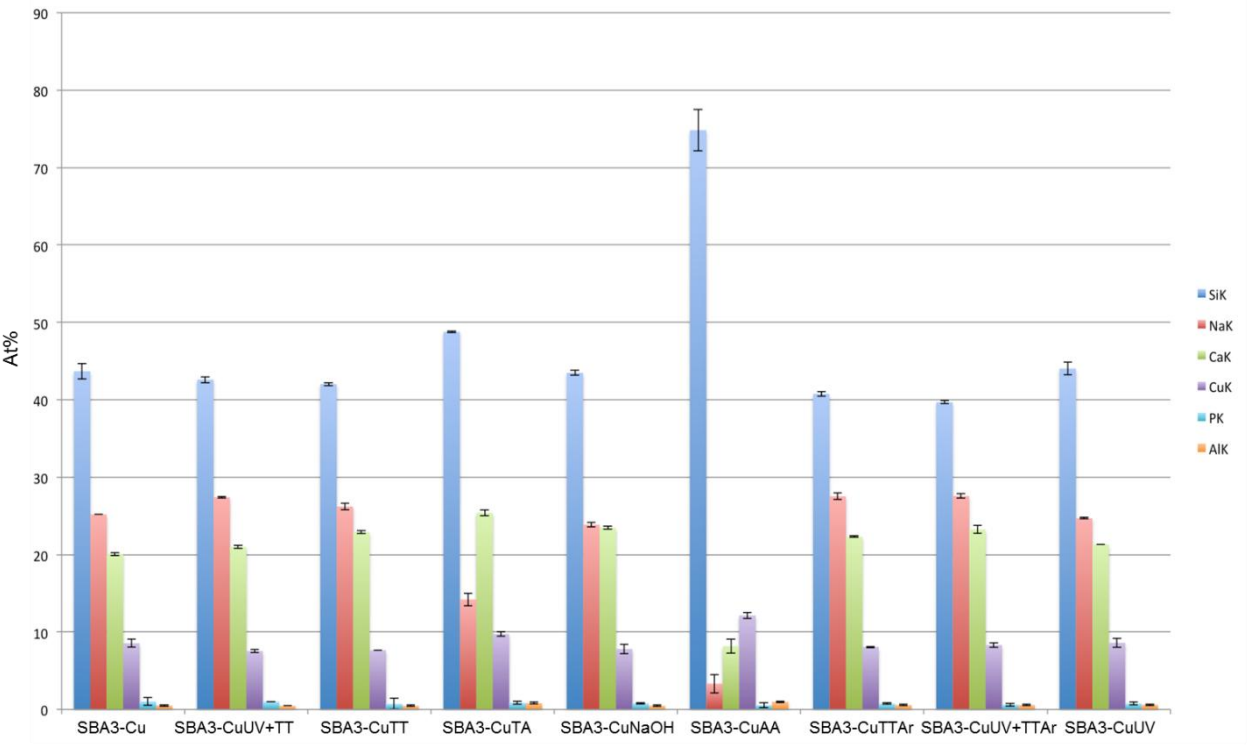

Figure 7: EDS compositional analysis of pristine SBA3-Cu and glasses subjected to physical and chemical treatments.

A resume of compositional analysis performed on the various samples is shown in Figure 7; even if EDS is a semi-quantitative analysis, significant variations in terms of at% of the elements were observed for sample treated with tannic acid and most of all for glass subjected to sodium L-ascorbate treatment. In both cases, an increase of Si content and a decrease of Na were evidenced, as regarding SBA3-Cu7 also a decrease of Ca amount was observed. These two treatments probably are a lightly aggressive and induce a release of Na and Ca.

3.4 Bioactivity

As reported in literature [42-44] the bioactivity process can be divided in five steps: the first one (Step I) consists of a rapid ions exchange between alkaline ions from the glass and hydrogen ions from the solution, in the Step II the formation of silanols occurs (Step II), then they condensate to develop a silica gel layer (Step III). Subsequently calcium, phosphate and carbonate ions are adsorbed in the silica gel (Step IV) and react, forming the hydroxyapatite (HAp) layer (Step V).

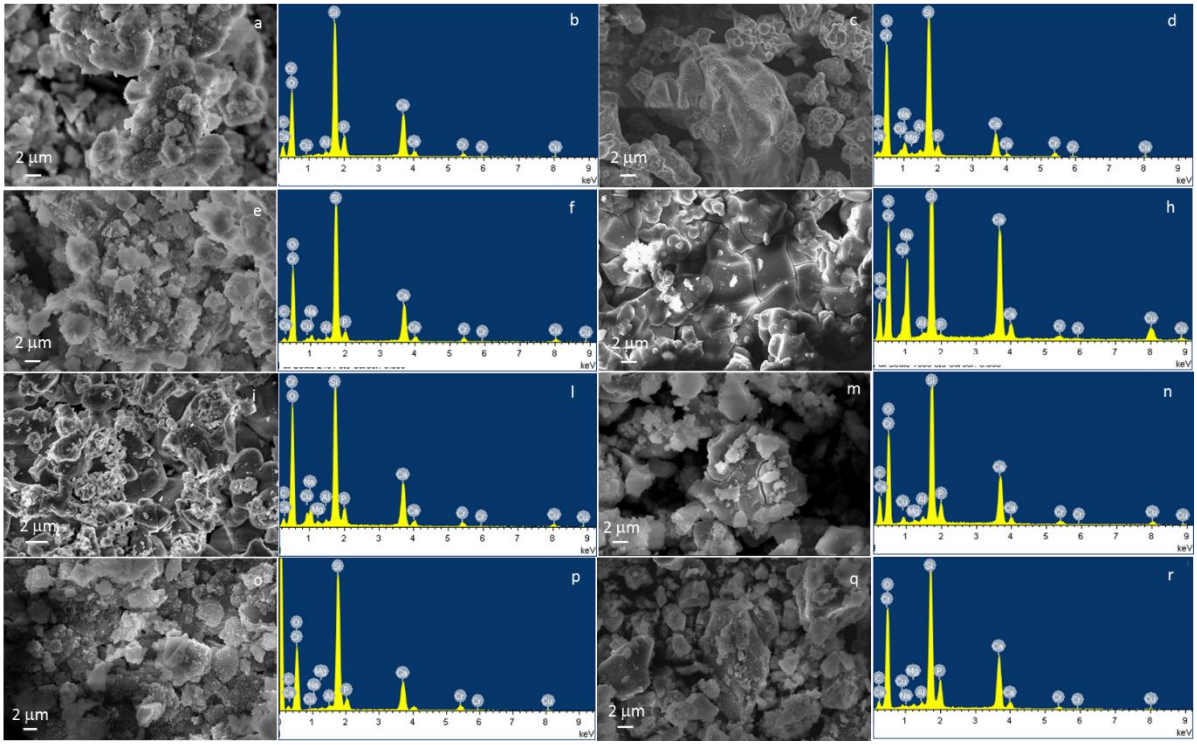

Figure 8: FESEM-EDS analysis of SBA3-CuUV (a,b), SBA3-CuUV+TT (c,d), SBA3-CuTT (e,f), SBA3-CuUV+TTAr (g,h), SBA3-CuTTAr (i,l), SBA3-CuTA (m,n), SBA3-CuAA (o,p), SBA3-CuNaOH (q,r) after 3 days of immersion in SBF

Figure 8 shows the FESEM-EDS analysis after 3 days of immersion in SBF solution of SBA3-Cu glasses subjected to physical and chemical treatments. SBA3-CuUV sample dipped in SBF for 3 days evidenced the formation of reactivity layer on its surface. EDS semi-quantitative elemental concentrations, determined over an area in different sample points, showed a decrease of Na, a high content of Si and an increase of P.

This could be ascribed to the formation of a silica gel layer (step III/IV of bioactivity mechanism) probably jet enriched by Ca and P (Figure 8a and b).

Sample subjected to thermal treatment and UV after 3 days in SBF solution showed a cracked surface due to the formation of a silica gel layer, as also confirmed by EDS analysis, which evidenced the increase of Si amount, together with a slight increase of P and a decrease of Na. Then, also in this case the material shows a surface reaction layer corresponding to the fourth step of bioactivity (Figure 8c and d). Similar results were obtained for SBA3-CuTT glass, also in this case an increase of P and most of all of Si was evidenced; moreover, in some area it was possible to notice the formation of a needle-shaped on glass surface, typical of hydroxyapatite (HAp) or its precursor (Figure 8e and f).

Sample SBA3-CuUV+TTAr (sample irradiated with UV and thermal treated in Ar), after 3 days of SBF dipping, showed a sort of film that cover glass particles, ascribable to silica gel. Moreover, in several areas of the sample, some agglomerates were noticed, some of them having the typical morphology of the HAp. EDS analysis confirmed what observed in the micrographs: compositional analysis of area evidenced a high amount of Si, but EDS analysis of the precipitates showed a very high amount of Ca and P (Figure 8g and h). The same characteristics were evidenced on sample SBA3-CuTTAr; also in this case the analysis of large area show the presence of the silica gel, but in some point of the sample a clear nucleation of HAp or its precursors was observed (Figure 8i and l).

Regarding the chemical treatments, after 3 days of SBF SBA3-CuTA glass evidenced the presence of cracks on particles, which however appeared also before the immersion in SBF. Local FESEM-EDS analysis evidenced the presence of nanometric crystals that covered glass particles. Compositional analysis showed always a high amount of Si and an increase of Ca and P (Figure 8m and n). The sample treated in sodium L-ascorbate (SBA3-CuAA) and dipped in SBF for 3 days showed a surface characterized by some cracks and in some point by the formation of needle-shaped crystals. EDS analysis evidenced a high amount of Si and a decrease of Na (whose amount however was already low for as done sample), together with an increase of Ca and P, which were decreased after the treatment in comparison with SBA3-Cu sample (Figure 8o and p). FESEM-EDS analysis of SBA3-CuNaOH showed the presence of a reaction layer and of nanometric crystals rich in Ca and P. The compositional analysis of the sample surface evidence a high content of Si and an increase of both Ca and P (Figure 8q and r).

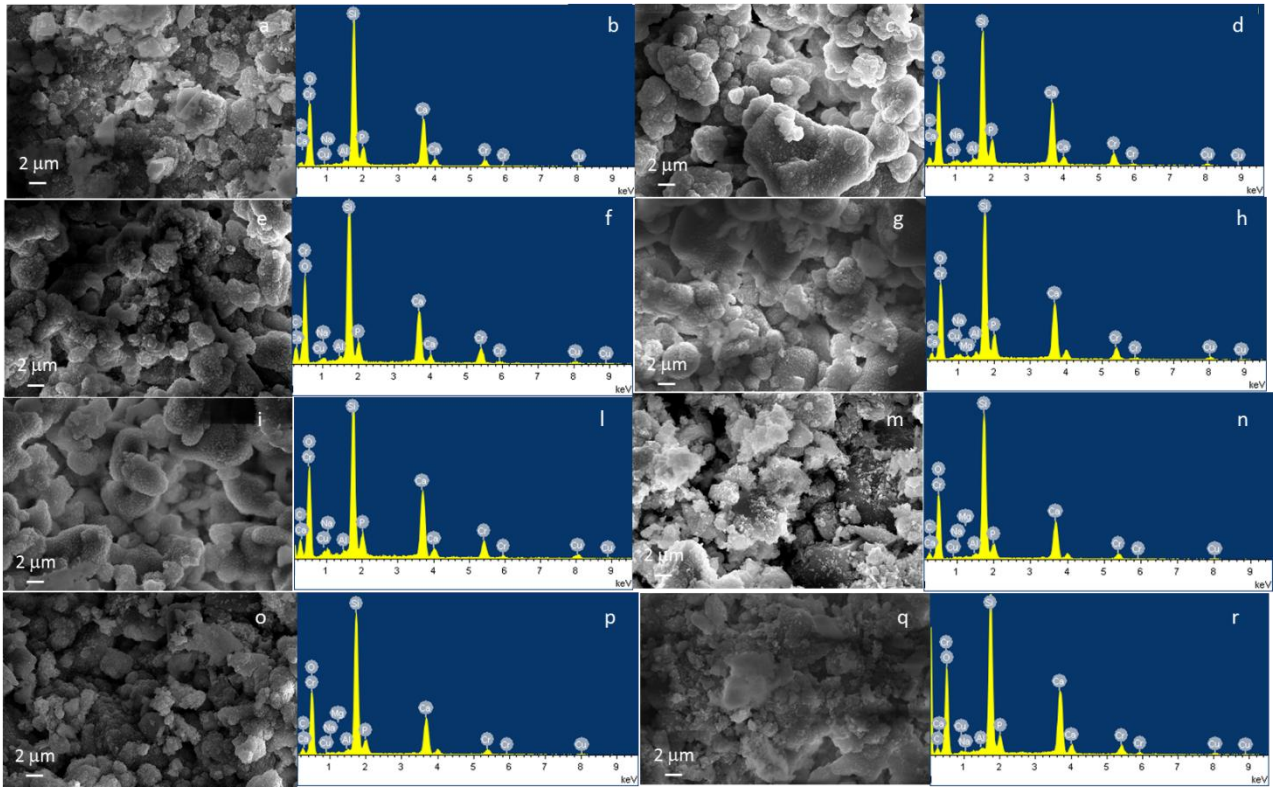

Figure 9: FESEM-EDS analysis of SBA3-CuUV (a,b), SBA3-CuUV+TT (c,d), SBA3-CuTT (e,f), SBA3-CuUV+TTAr (g,h), SBA3-CuTTAr (i,l), SBA3-CuTA (m,n), SBA3-CuAA (o,p), SBA3-CuNaOH (q,r) after 7 days of immersion in SBF solution.

Figure 9 shows the morphological and compositional analyses of samples dipped in SBF for 7 days. The formation of needle-like crystals, ascribable to HAp, is well visible on the surface of SBA3-CuUV glass (Figure 9a and b). Local EDS analysis evidenced the increase of P and Ca, confirming the precipitation of a calcium-phosphates; compositional analysis on various areas showed a slightly decrease of Si, respect the amount observed after 3 days of SBF treatment, and an increase of both Ca and P. The sample SBA3-CuUV+TT after 7 days of immersion on SBF (Figure 9 c and d) evidenced the presence of precipitates having the typical morphology of the *in vitro* grown HAp, rich in Ca and P. EDS analysis conformed the enrichment of Ca and P on glass surface. The same consideration can be done for SBA3-CuTT glass (Figure 9 e and f). Also in this case, the formation of a crystalline phase on sample surface rich in Ca and P can be noticed. Samples thermal treated in Ar atmosphere (SBA3-CuUV+TTAr and SBA3-CuTTAr) showed the formation on their surface of crystal with the typical morphology of the HAp (Figure 9g-l). The EDS analysis, performed both in large areas and locally on some glass particles, evidenced a very distinct Si peak and low amount of Ca and P. However, the quantitative analyses showed a clear increase of Ca and P together with a decrease of Na and Si, thus evidencing a progress on bioactivity process in accordance with morphological observation.

FESEM-EDS analysis of SBA3-CuTA samples dipped in SBF solution up to 7 days showed the presence of a reaction layer rich in Ca and P (Figure 9m and n), even if with the morphology is slightly different from

samples subjected to physical treatments. The semi-quantitative compositional analysis evidenced a continuous increase of Si and a stabilization of Ca and P amount. FESEM-EDS analysis of SBA3-CuAA show the presence of crystals containing Ca and P on glass powders surface, as already evidenced after 3 days. Compositional analysis evidenced a high Si peak, but reduced respect previous SBF dipping time, and an increase of Ca and P values (Figure 9o and p). Also, glass powers treated with NaOH display a morphology ascribable to the formation of HAp and, in some areas, a very intense Ca and P peaks (Figure 9q and r).

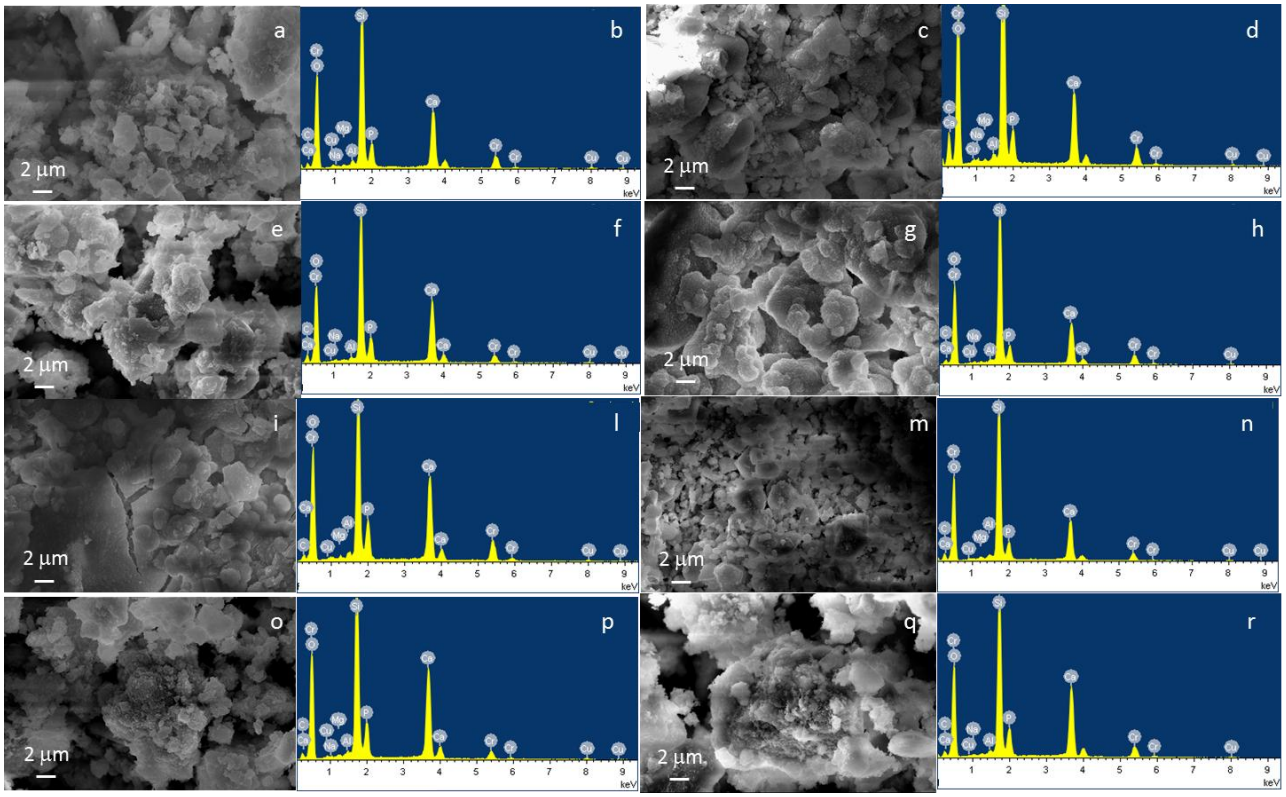

Figure 10: FESEM-EDS analysis of SBA3-CuUV (a,b), SBA3-CuUV+TT (c,d), SBA3-CuTT (e,f), SBA3-CuUV+TTAr (g,h), SBA3-CuTTAr (i,l), SBA3-CuTA (m,n), SBA3-CuAA (o,p), SBA3-CuNaOH (q,r) after 14 days of immersion in SBF solution.

The morphological-compositional characterization of samples after the immersion in SBF for 14 days is reported in figure 10. Glass UV-irradiated (Figure 10a and b) evidenced the formation of a phase rich in Ca and P with the typical morphology of HAp. Compositional analysis of area is not so different from that one after 7 days and evidenced a very low amount of Na, a high content of Si, Ca and P. Sample SBA3-CuUV+TT (Figure 10c and d) evidenced the formation of a crystalline phase rich in Ca and P, already observed after 7 days; the ration Ca/P is about 1.8, very similar to the ratio of HAp (1.67). From morphological point of view, SBA3-CuTT sample after 14 days of SBF treatment (Figure 10e and f) evidenced results very similar to what observed after 7 days and for sample SBA3-CuUV+TT: the presence of a crystalline phase rich in Ca and P, as conformed by compositional analysis. The morphological-compositional analysis of SBA3-CuUV+TTAr and SBA3-CuTTAr samples, evidenced the presence of a nanometric needle-shaped crystal phase (Figure 10g-l).

Also in this case, as it was pointed out after 7 days of SBF immersion, the EDS analysis evidenced a high amount of Si, probably due to the formation of silica-gel; however, the quantitative data and the EDS locally performed (Figure 10h and l) showed also a clear increase of Ca and P.

Samples subjected to tannic acid treatment (SBA3-CuTA) and immersed in SBF up to 14 days do not show significant differences respect the sample after 7 days (Figure 10m and n). The performed analyses displayed dissimilar results in different point: in the areas rich in crystals the amount of Ca in very high, while in the smoother areas the Si peak prevails. So, it seems that the bioactivity process did not show a uniform kinetics. SBA3-CuAA glass after 14 days of SBF treatment shows a surface covered by crystals rich in Ca and P, as already observed after 7 days. EDS analysis of area evidenced a simultaneously decrease of Si and increase of Ca and P (Figure 10o and p). NaOH-treated sample di not shows significant differences respect the 7 days-treatment: a crystalline reaction layer containing Ca and P was formed on glass powders. Moreover, quantitative EDS analysis evidenced an increase of P amount and a stabilization of Si and Ca (Figure 10q and r).

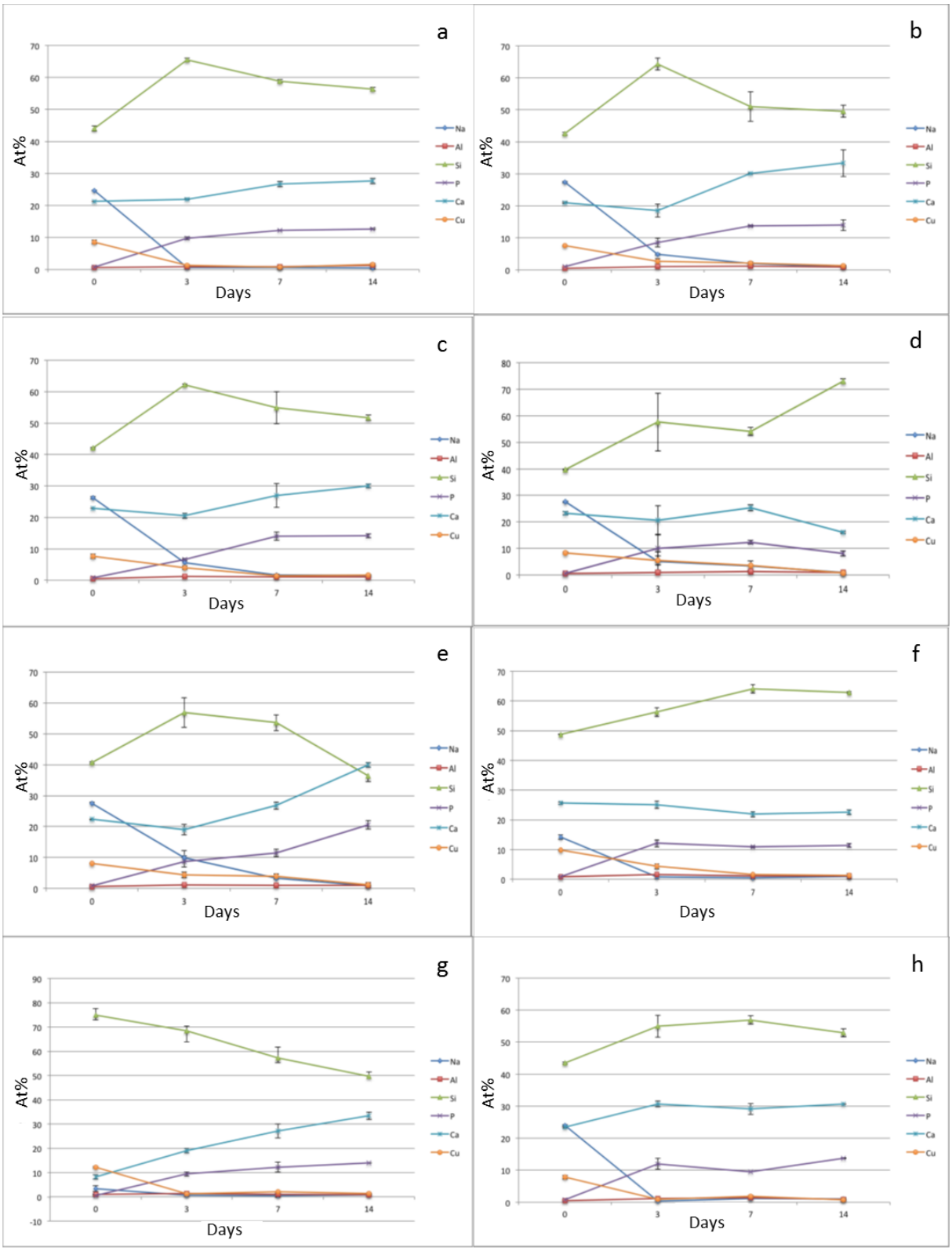

Figure 11: Atomic percentages variation (EDS, surface analysis) of SBA3-CuUV (a), SBA3-CuUV+TT (b), SBA3-CuTT (c), SBA3-CuUV+TTAr (d), SBA3-CuTTAr (e), SBA3-CuTA (f), SBA3-CuAA (g), SBA3-CuNaOH (h) during immersion in SBF up to 14 days

Figure 11 shows the atomic percentage variation of elements (EDS analysis of surface area) during the immersion in SBF solution up to 14 days. In general, an increase of Si amount can be observed during the

first days of immersion in SBF, due to the silica-gel formation, followed by a decrease due to the surface enrichment with other chemical species involved in HAp formation; only SBA3-CuAA (Figure 11g) evidenced an immediate decrease of Si content. In fact, SBA3-CuAA sample, before SBF immersion, showed higher Si content and lower Na and Ca amounts probably ascribable to the slightly aggressive treatment with ascorbic acid, as evidenced in figure 7. Regarding Ca and P trend, an increase during the SBF soaking can be noticed, confirming the results obtained with FESEM-EDS. Moreover, a fast decrease of Na content can be observed, in accordance with the bioactivity mechanism. A decrease of Cu trend can be also noticed, evidencing a release of the element during the immersion in SBF. The data of SBA3-CuUV+TTAr samples after 14 days of SBF treatment are anomalous and in contrast with what observed by FESEM-EDS analysis; this is probably ascribable to a detachment of HAp layer from powders surface during sample preparation and analysis.

The results of FTIR analysis of all glasses after SBF immersion up to 14 days are very similar. Figure 12 shows as example the FTIR spectra of SBA3-CuTA as prepared and after 3 and 14 days of SBF treatment. As it can be noticed the unsoaked glass displays two bands at around 400-500 cm^{-1} and 900-1100 cm^{-1} (centered at 1030 cm^{-1}) ascribable to ν_{sym}(Si–O–Si) and ν_{asym}(Si–O–Si) bands of SiO_4 tratrahedra [48], the shoulder at about 930 cm^{-1} is related to the SiO_{NBO} (NBO-Non-Bonding Oxygen) due to glass network modifier. After SBF immersion a new band at about 1200 cm^{-1} appeared, ascribable to the formation of new Si-O-Si bond on condensation of SiOH groups [49], while the shoulder concerning the SiO_{NBO} disappeared; the strong band centered at 1070 cm^{-1} and the sharp band at 550-600 cm^{-1}, ascribable to PO_4 symmetric stretching vibration and bending mode of P-O-P bond, confirm the HAp presence [49,50].

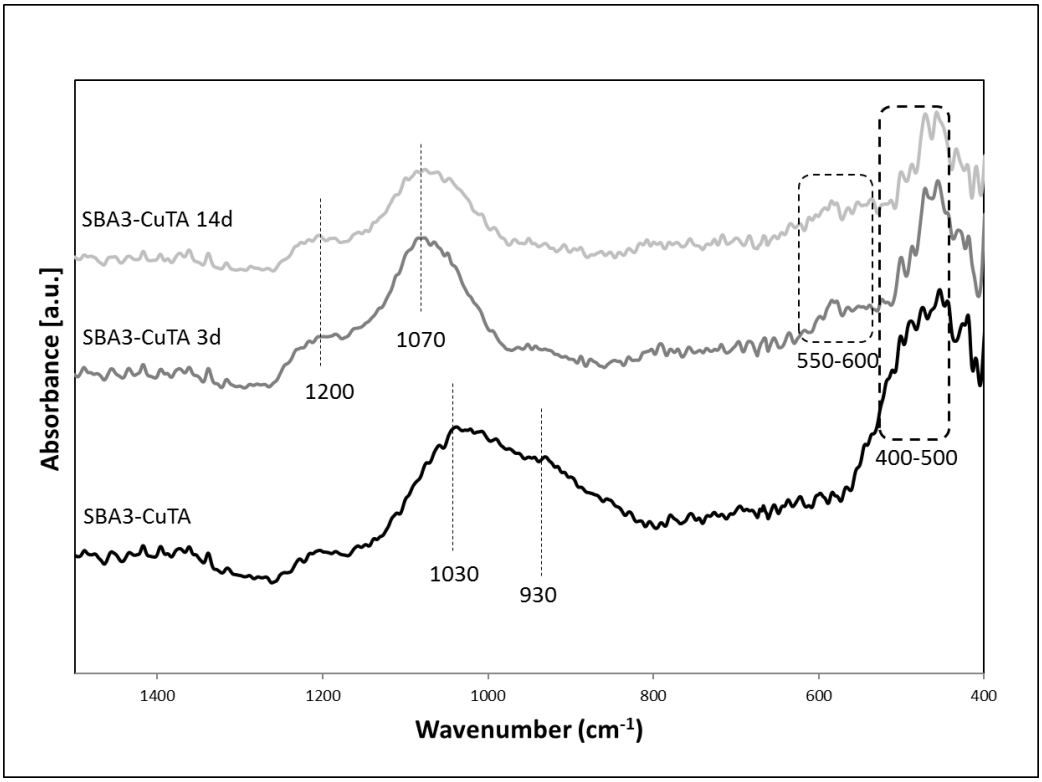

Figure 12: FTIR analysis of SBA3-CuTA

The measurement of pH evidenced a similar trend for all samples: a fast pH increase was observed during the first 3 days up to about 7.7-7.8, which is typically of bioactive glass, due to the exchange of alkaline cations with hydrogen ions from the solution [9,35]. Then, the pH values increase slowly up to 7.9-8.1 up to 14 days, remaining in the physiological tolerability range (7 ~8).

In conclusion, the bioactivity test evidenced the ability of all glasses to induce the precipitation of HAp crystals, regardless of the performed chemical or physical treatments. In literature few studies investigated the role of copper NPs in the reactivity of bioactive glass [51,52] and, on the basis of authors knowledge, there are no researches regarding the bioactive behavior of Cu-doped glasses subjected to *in situ* reducing processes. However, the similar reported studies evidenced contrasting results: Magyar et al. [51] reported that sol-gel derived bioactive glass containing Cu2ONPs up to 0.5 mol% showed an almost unchanged bioactive behavior; while Bejarano et al. [52] evidenced that the growth of apatite was inhibited on sol-gel glasses containing CuO crystals. Thus, the Cu-containing glass bioactivity can be influenced depending on glass composition and treatments.

The evaluation of the antibacterial properties was performed by means of the inhibition halo test. Figure 13 shows the images of the samples on Mueller Hinton agar plate after the incubation at 35 °C for 24 h. As it can be noticed, only SBA3-CuUV (a), SBA3-CuTA (f) and SBA3-CuNaOH (h) produced a significant and reproducible inhibition halo of about respectively 2-2.5 mm, 2 mm and 1.5 mm ± 0.5 mm. The other samples were not able to create an inhibition area; however, a no bacterial growth was observed by inspecting the back of the plate. These results let suppose that the best antibacterial effect is related to the presence of free Cu^{++} ions in the glass network, alone or in synergy with Cu^0 nanoparticles (samples a, f and h), in good accordance with literature [4], since the presence of CuO crystals reduces the size of the inhibition halo, but a bacteriostatic effect is still visible, probably due to residual free Cu^{++} ions in the glass network or Cu_2O crystals (samples b, c, d, e, g). The treatment with ascorbic acid induced the formation of metallic copper; however, the bigger dimension of observed Cu^0 particles (sub-micrometric) respect to SBA3-CuTA (about 100 nm) seems to reduce the antibacterial effect. Moreover all samples except SBA3-CuTA created a blue diffusion halo, due to Cu^{++} ions which diffuse in the agar, some of them more evident than the others, mainly if free Cu^{++} is still present in the glass network, as confirmed also by the blue color of the sample a and h(containing only Cu^{++}). Samples with dark color showing blue diffusion halo (b, c, d, e, g) probably still contain traces of Cu^{++} or are able to release it from CuO nanocrystals or from Cu^0 by oxidation. On the contrary the diffusion halo of SBA3-CuTA (f) was brown, probably because this sample mainly contains copper as Cu^+ and Cu^0.

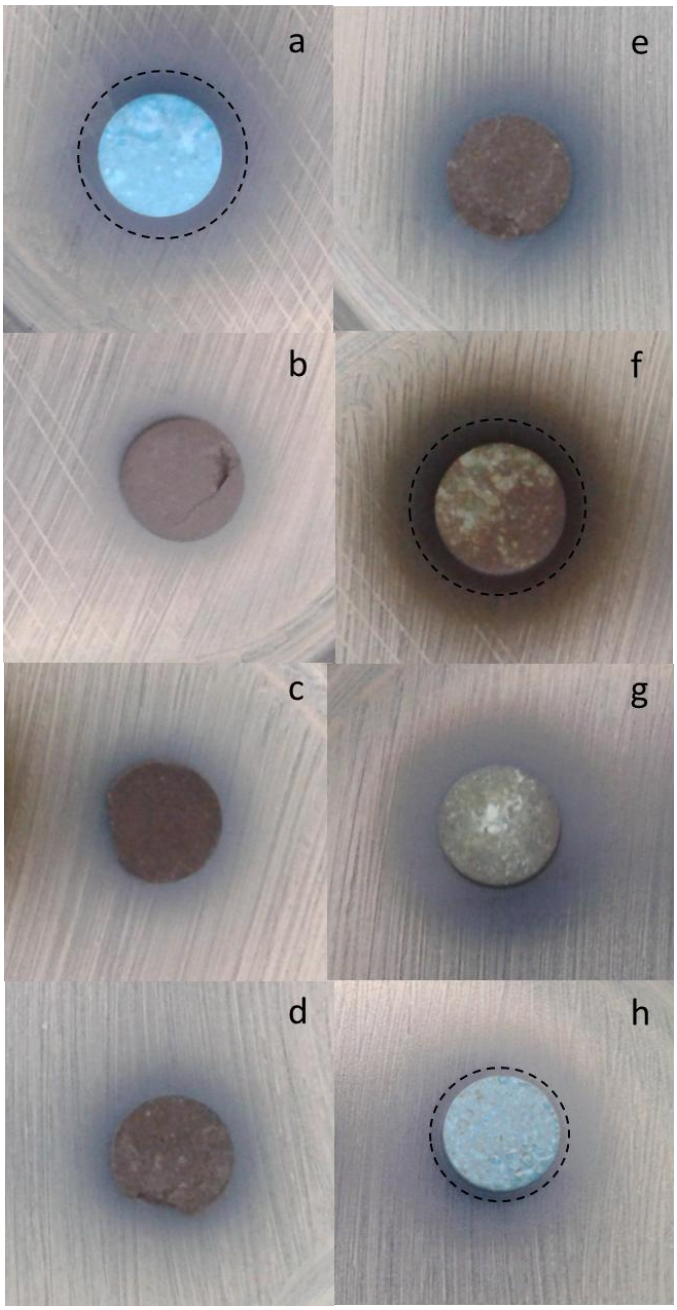

Figure 13: inhibition halo test against *S. aureus* of a) SBA3-CuUV, b) SBA3-CuUV+TT, c) SBA3-CuTT, d) SBA3-CuUV+TTAr, e) SBA3-CuTTAr, f) SBA3-CuTA, g) SBA3-CuAA and h) SBA3-CuNaOH

Conclusions

This work investigated the ability of different physical and chemical treatments to induce the *in situ* reduction of Cu^{++} ions, introduced in the outer surface layer of a bioactive glass by ion-exchange process, to Cu^{+} or Cu^{0}. The XRD, FESEM/STEM-EDS analyses demonstrated that only the chemical treatments using tannic acid and sodium L-ascorbate induce the formation of metallic copper; in particular, SBA3-CuTA sample evidenced the presence of Cu^{0} nanoparticles. The glass powders treatment with NaOH and UV irradiation did not induce structural and morphological modifications. The thermal treatment in air

promoted the nucleation of CuO, while the inert atmosphere induced the formation of Cu_2O. FESEM-EDS and FT-IR analysis after in vitro test in SBF up to 14 days demonstrated that all the investigated glasses maintained a bioactive behavior. Finally, antibacterial test towards *S. aureus* strain evidenced that the best antibacterial effect is achieved by glasses containing free Cu^{++} ions (SBA3-CuUV, SBA3-CuNaOH) and Cu^0 nanoparticles (SBA3-CuTA). An increase of Cu^0 dimension (SBA3-CuAA) or the presence of CuO and Cu_2O (formed during thermal treatments) reduced the antibacterial effect, even if a bacteriostatic effect was observed. In order to assess if the antibacterial effect is to be ascribed to a "contact killing" or a "leaching" mechanism, the potential leakage of Cu ions into biological fluid, their redox and catalytic activities, as well as their potential impact on biological assays, in presence of high physiological protein levels, could be of interest and could be object of future investigations on a selected set of samples.

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Table

Tables

SBA3 containing Cu^{2+} ions				
	Acronym	**UV irradiation**	**550 °C air**	**550 °C Ar**
Physical treatments	**UV**	X		
	UV+TT	X	X	
	TT		X	
	UV+TTAr	X		X
	TTAr			X
Chemical treatments	**TA**	Tannic acid		
	AA	Sodium L-ascorbate		
	NaOH	Sodium hydroxyde		

Table 1: physical and chemical treatments and samples acronym.

Figure1

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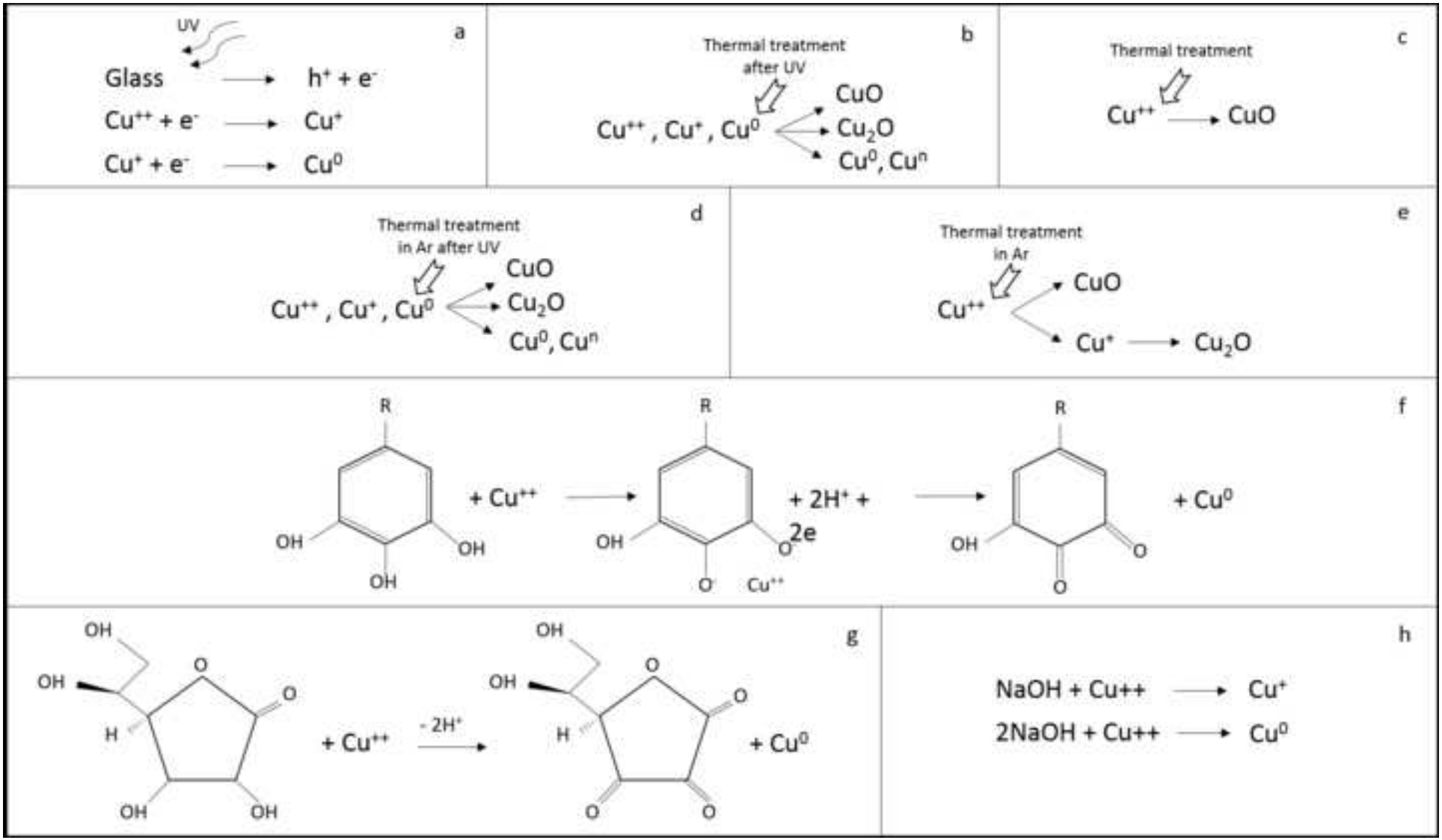

Figure2 R1

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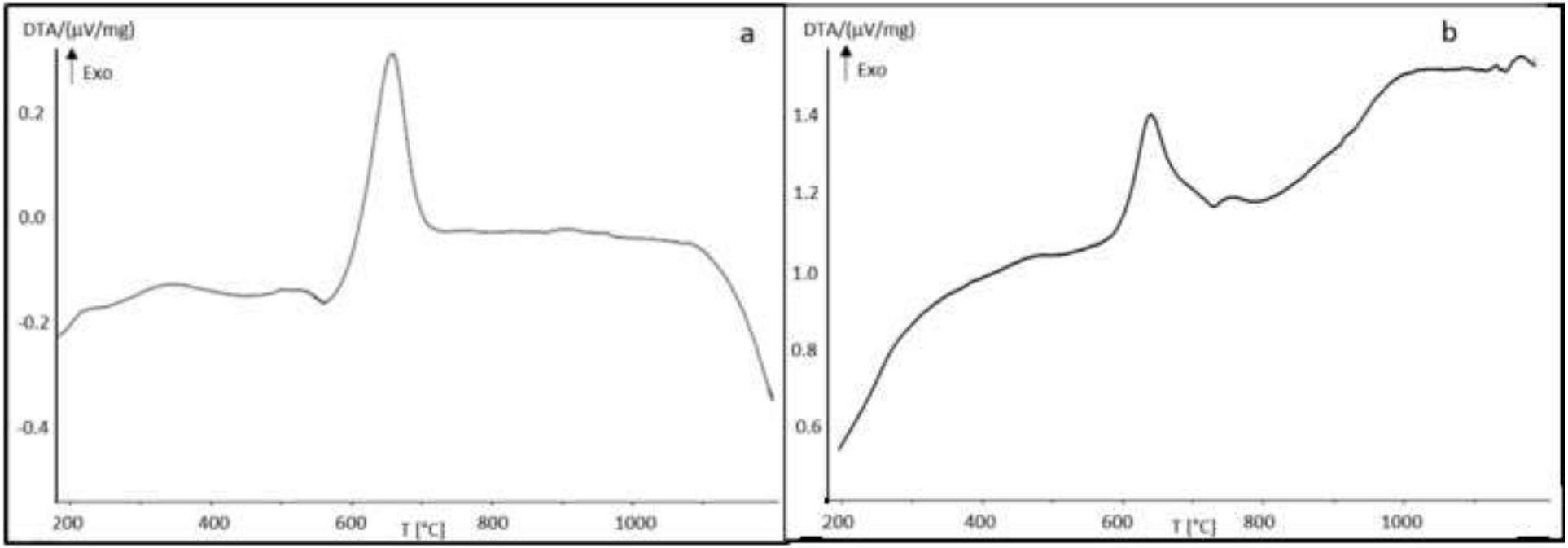

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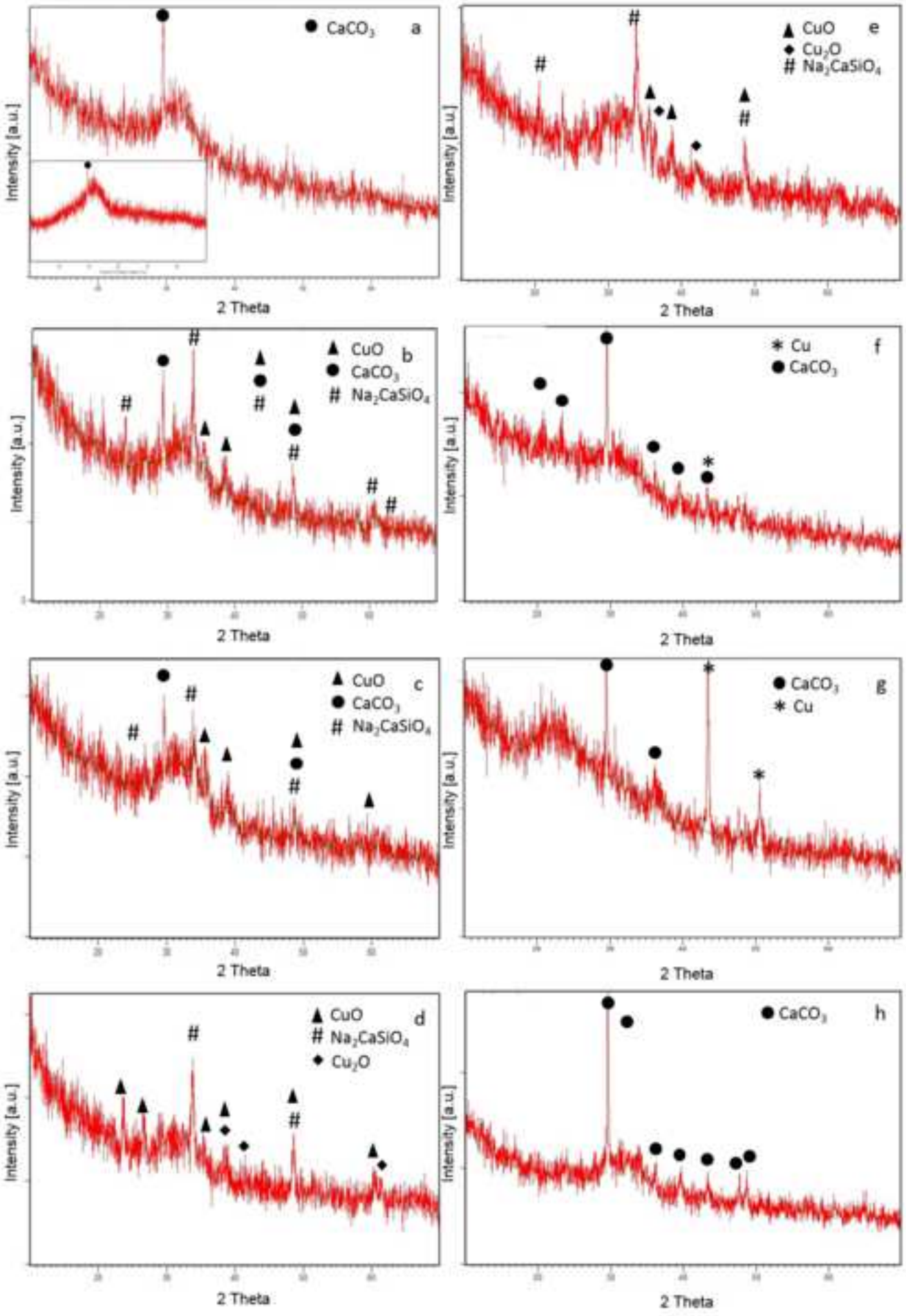

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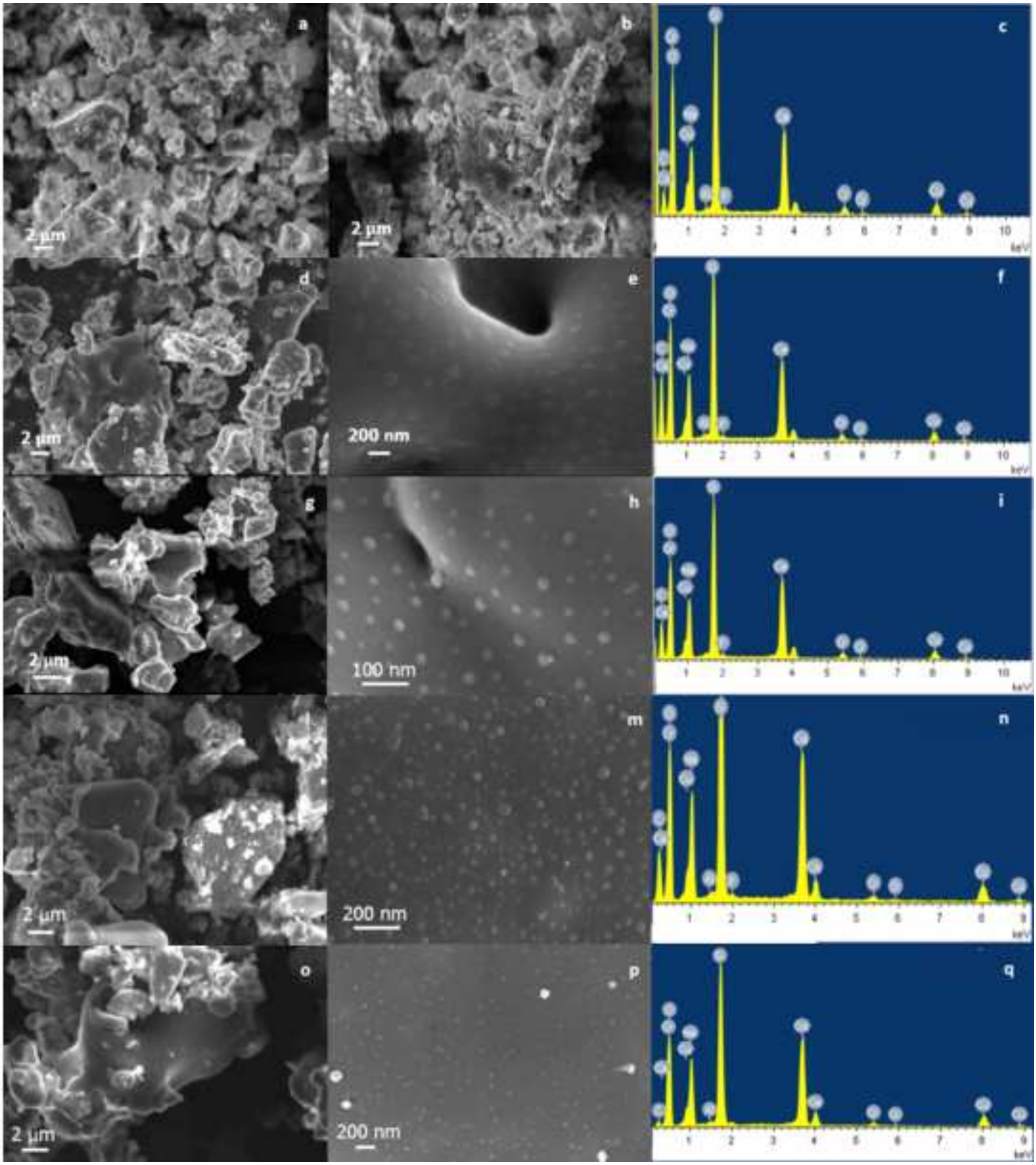

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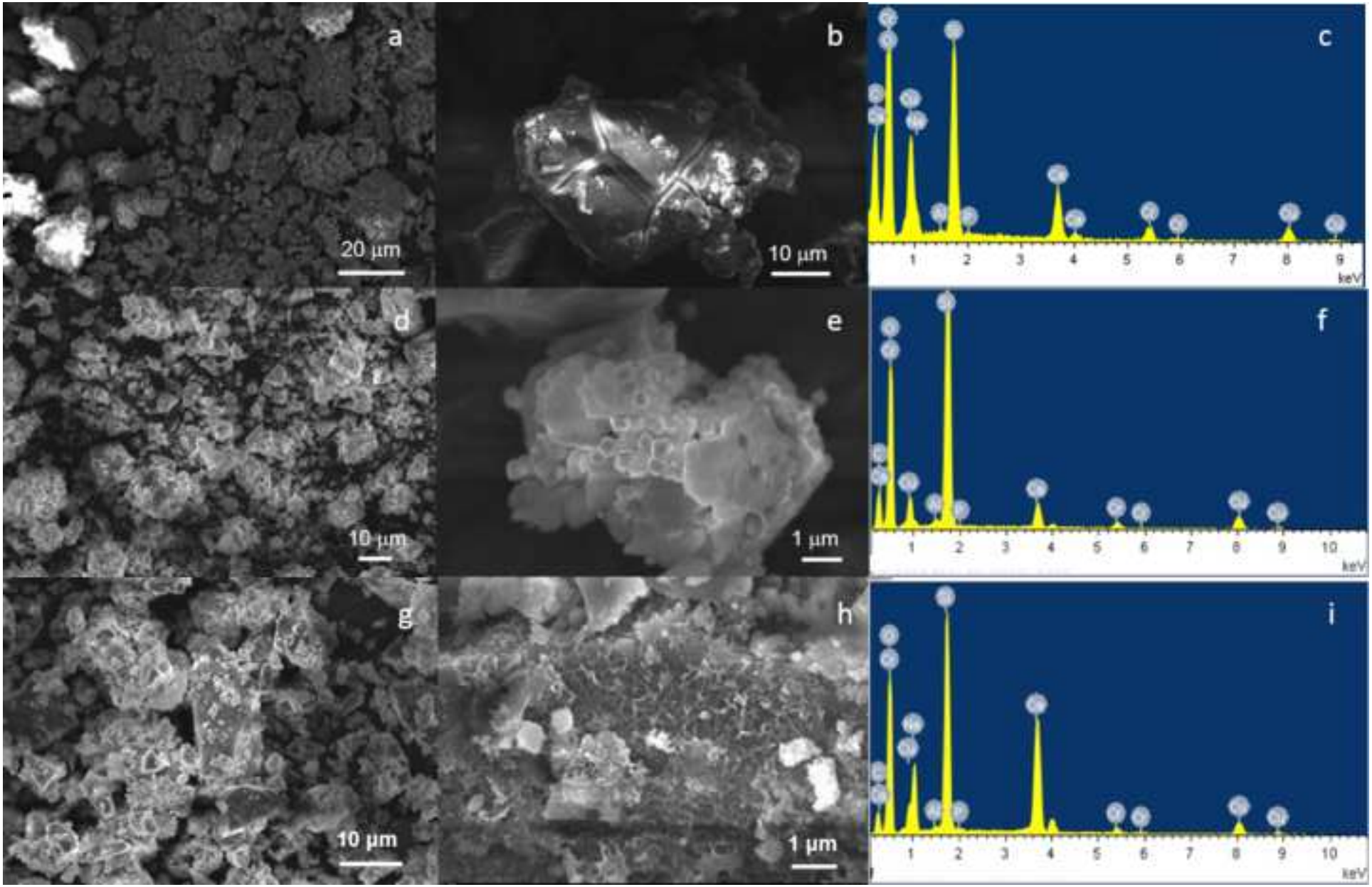

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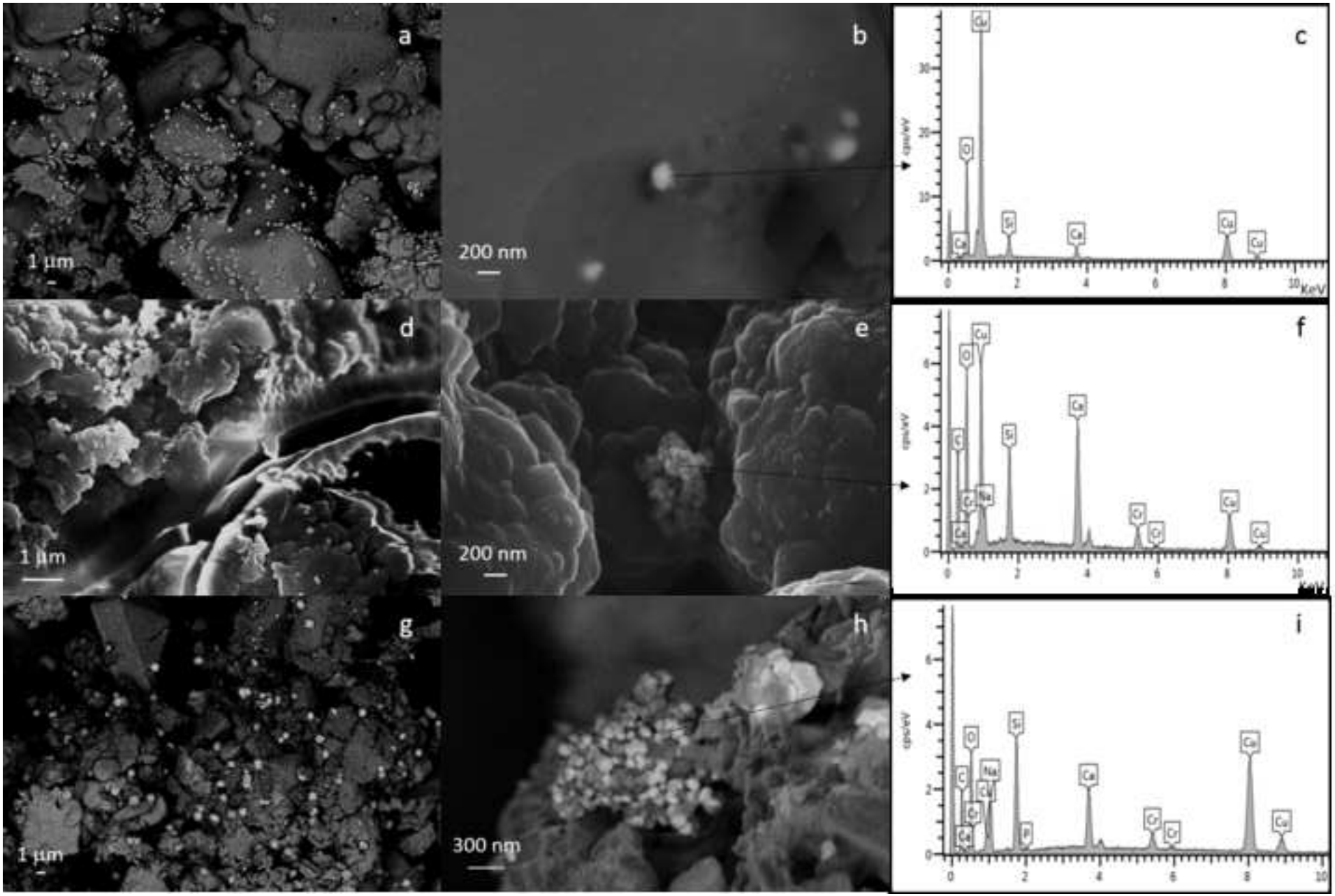

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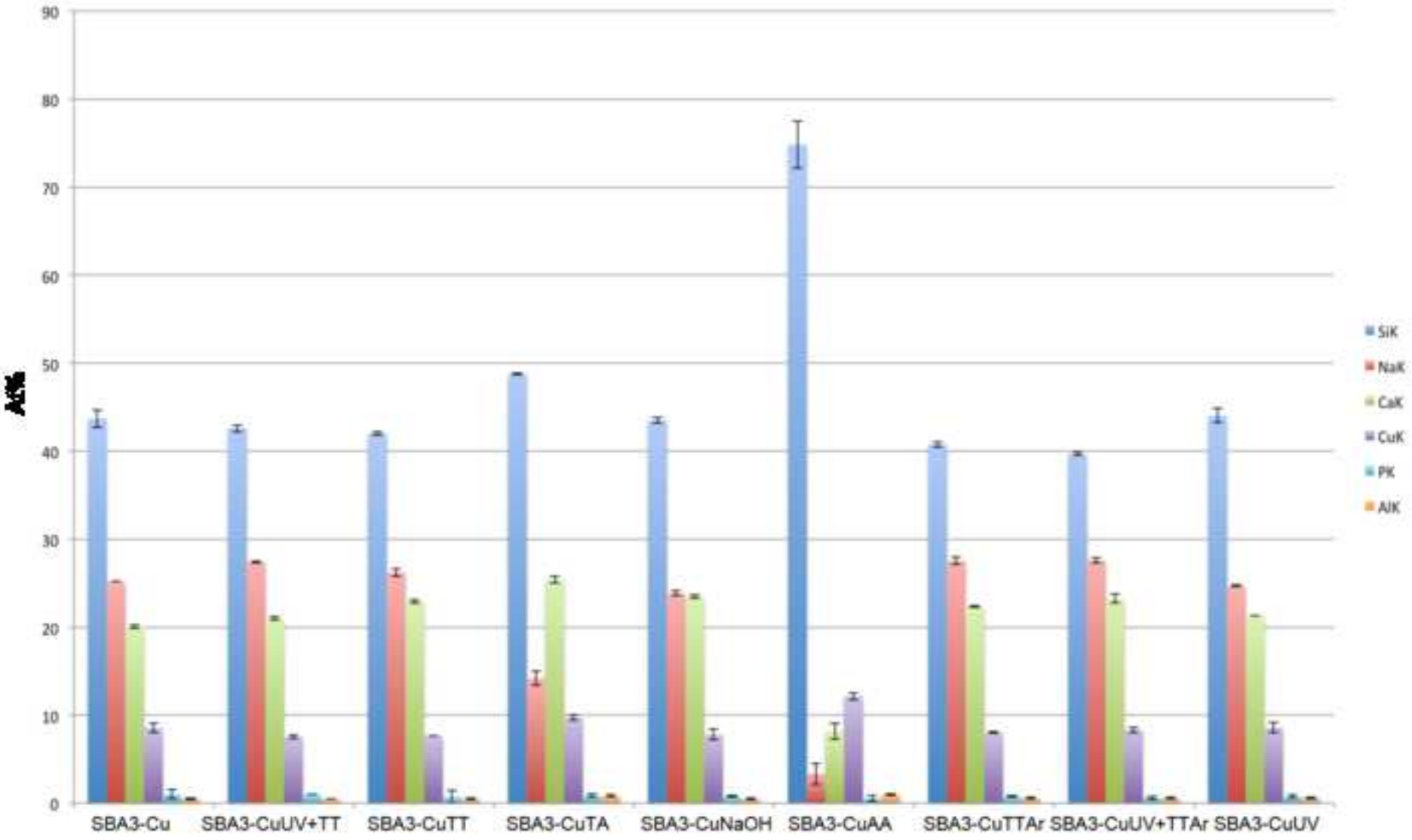

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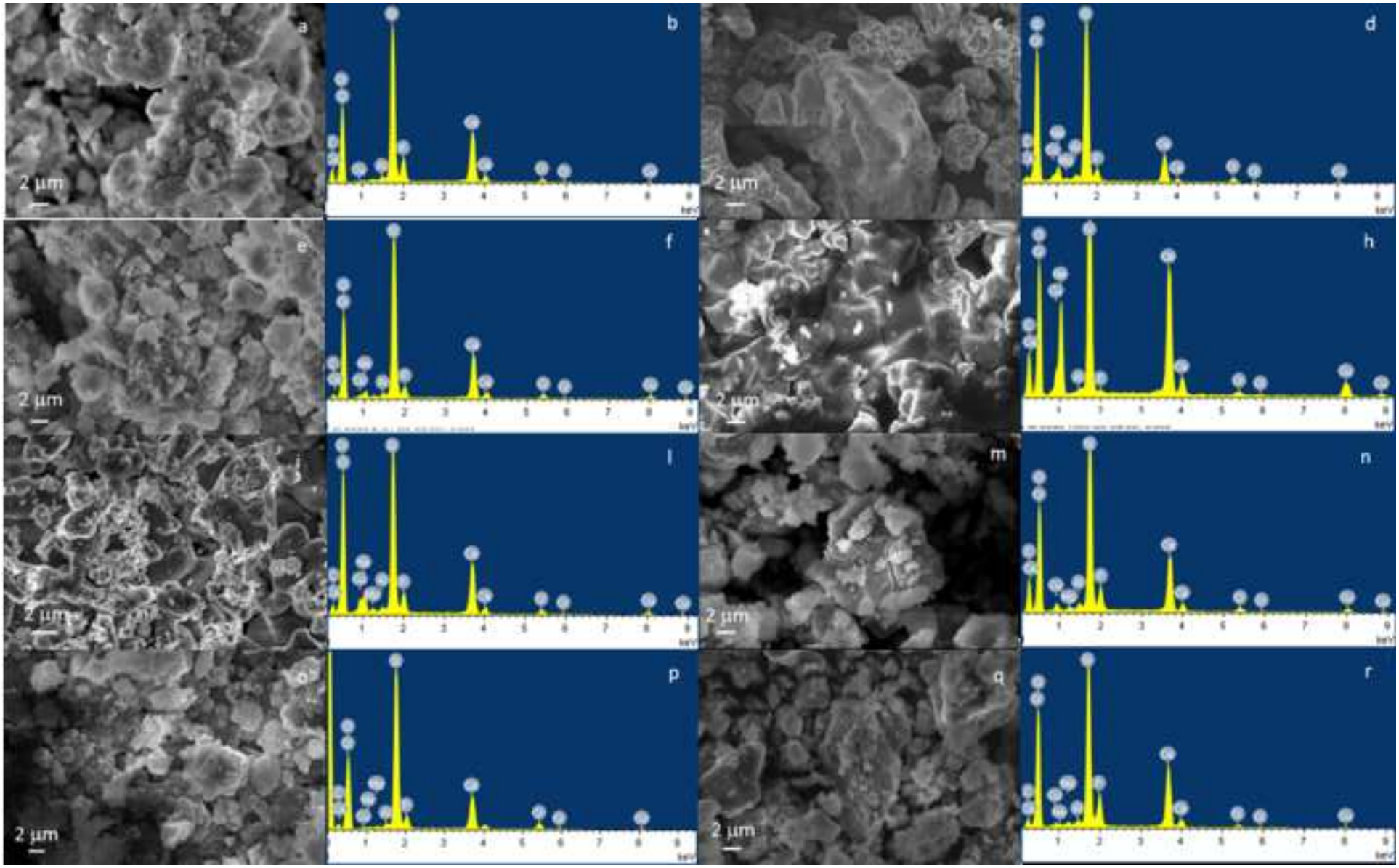

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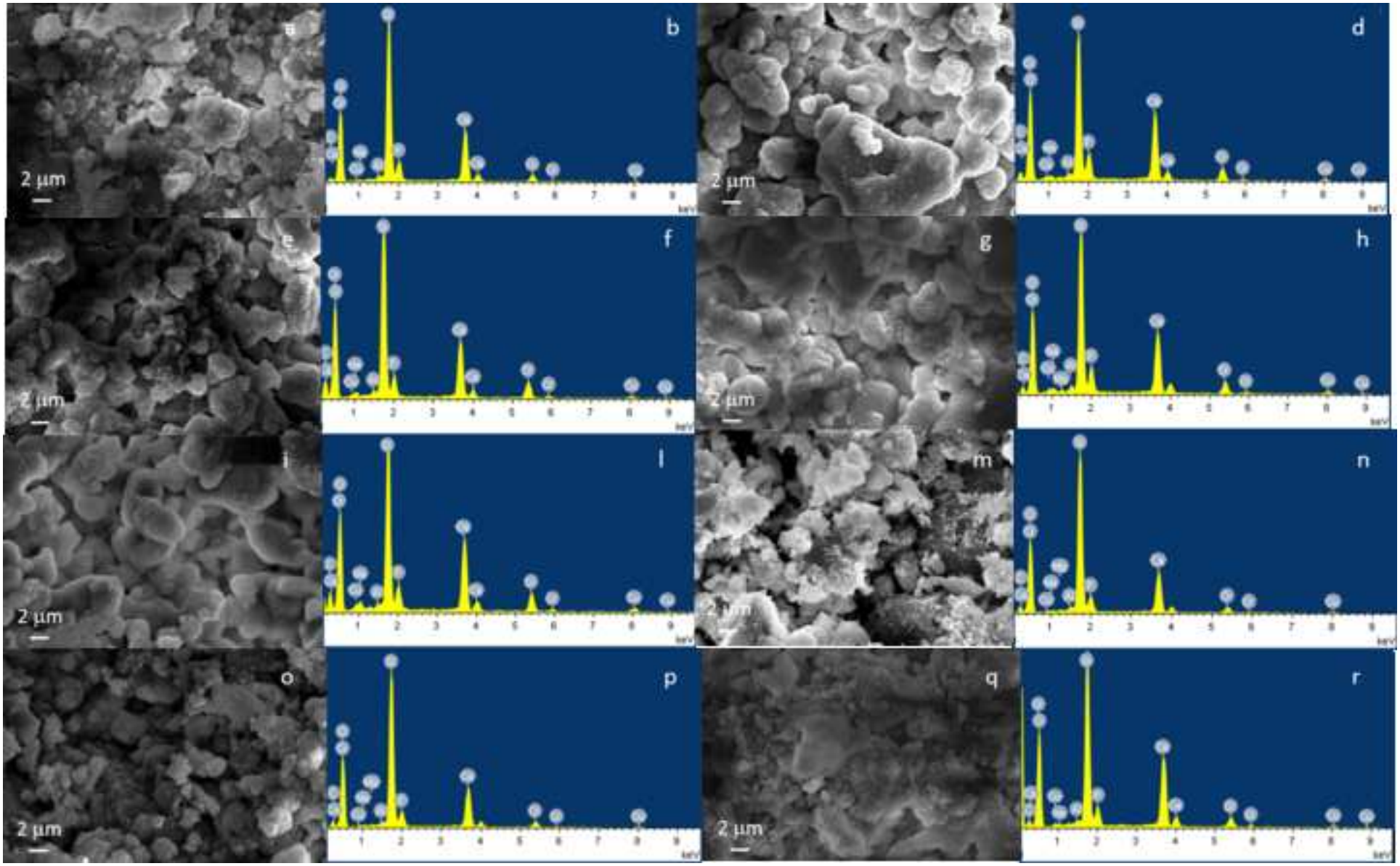

Figure10

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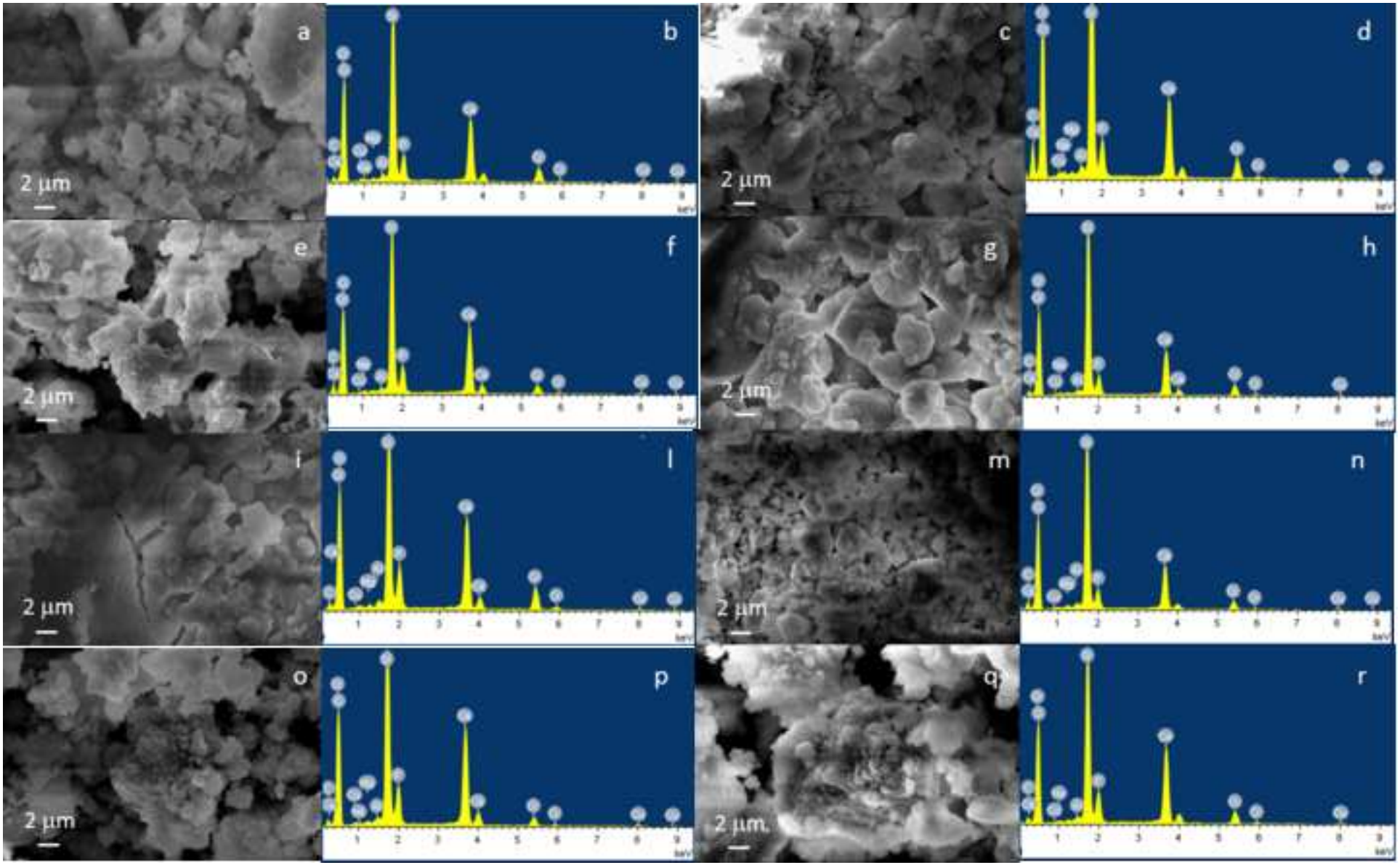

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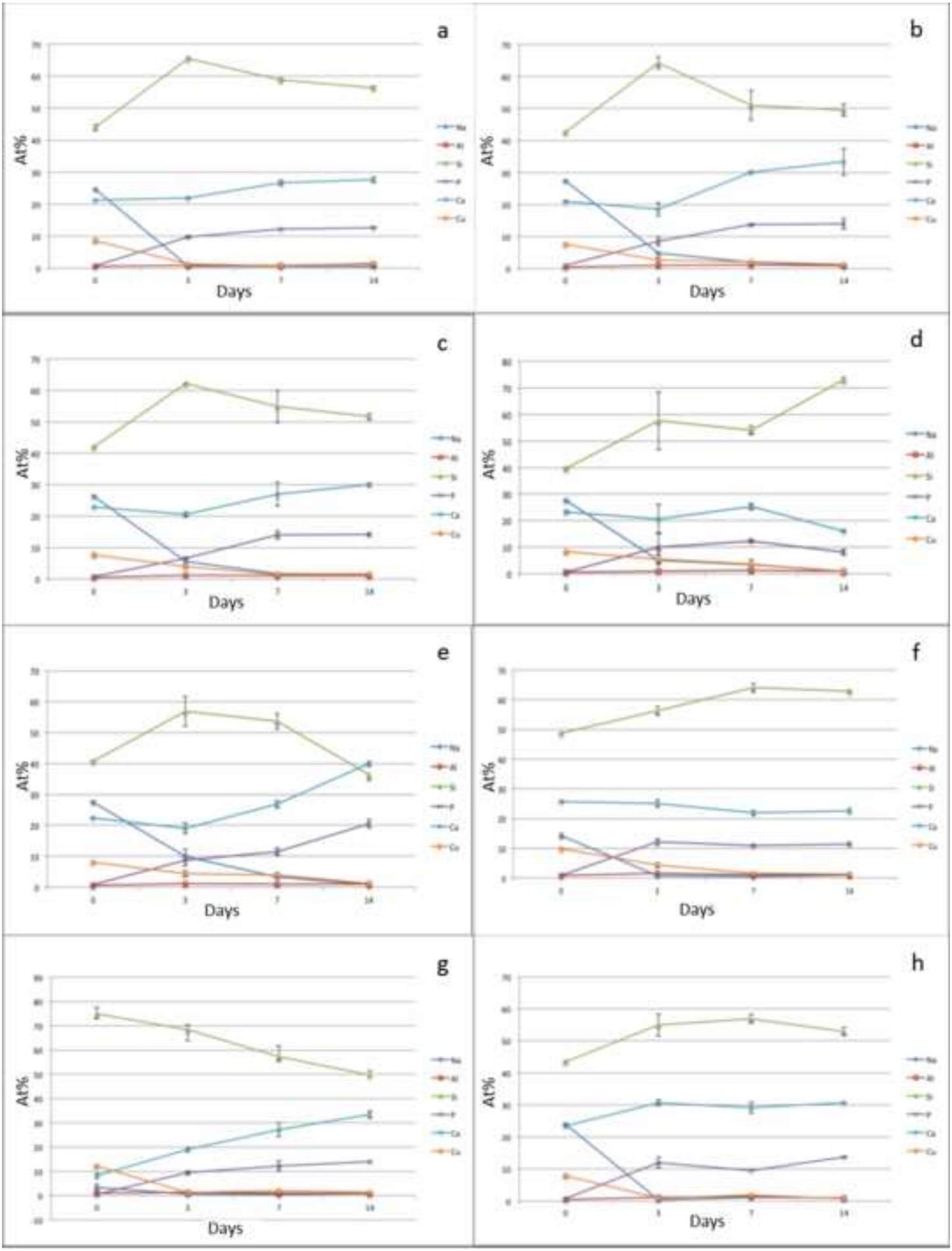

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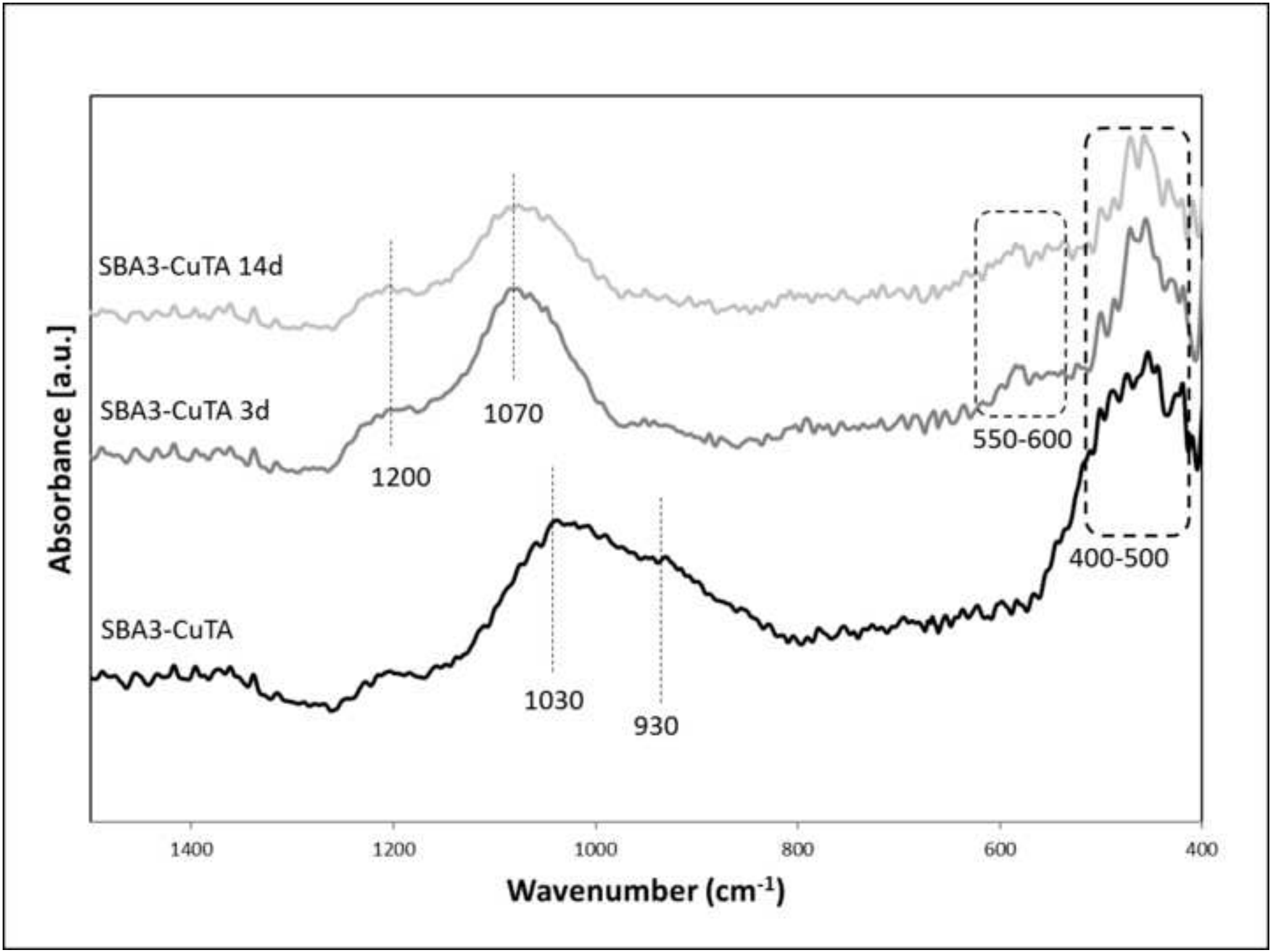

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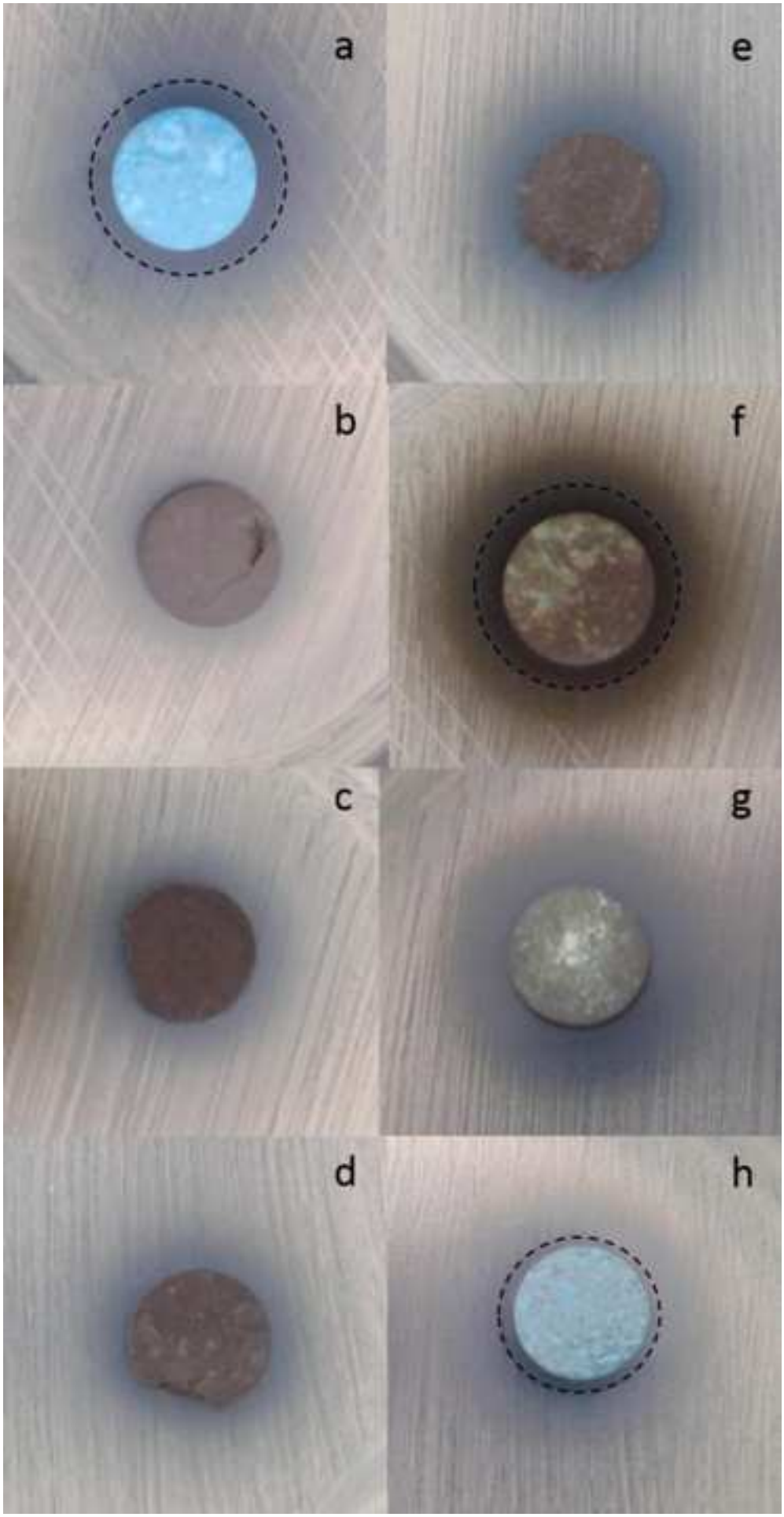
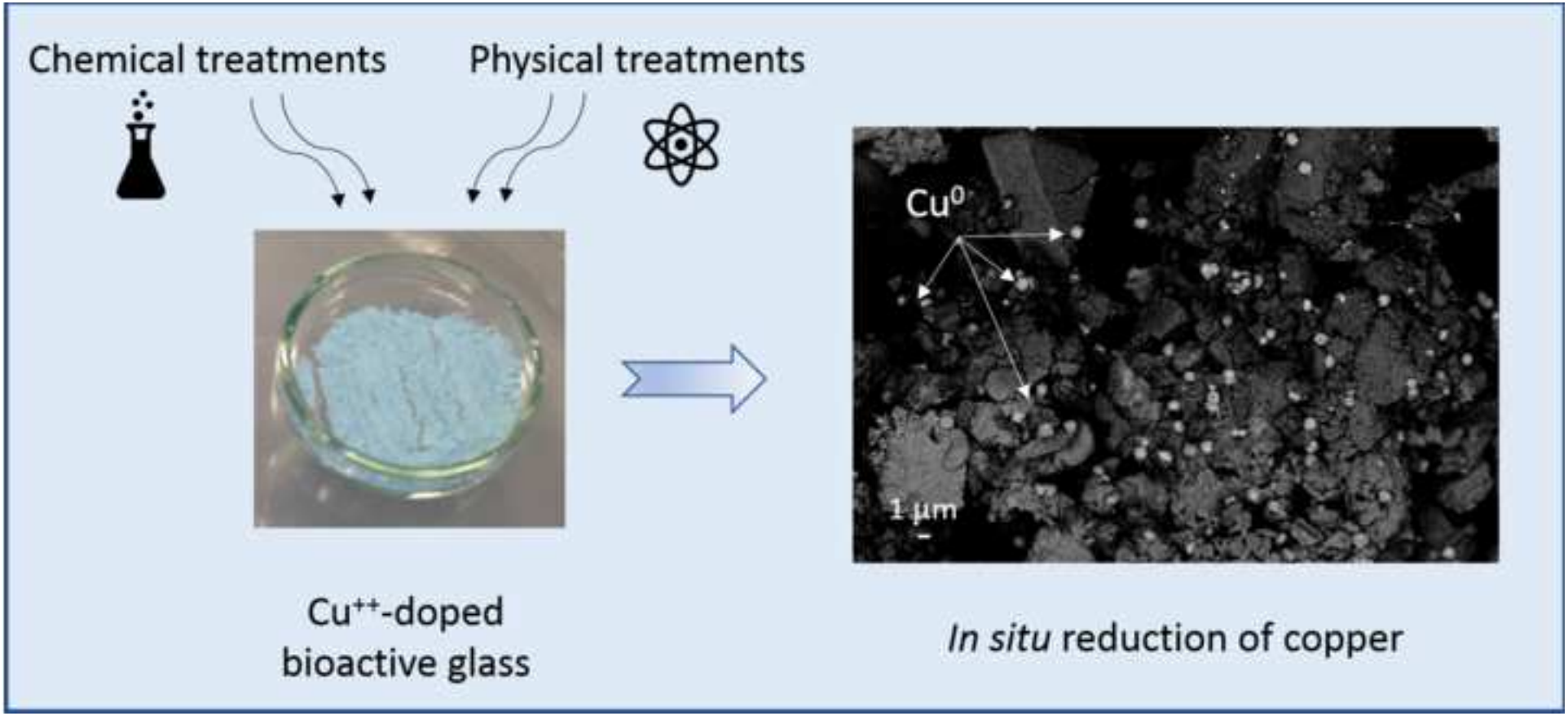


Graphical Abstract (for review)



Highlights

- Study of different physical/chemical treatments to induce the Cu *in situ* reduction
- Chemical treatments induce the formation of metallic copper
- All the investigated glasses maintained a bioactive behavior
- The best antibacterial effect is achieved by Cu^{++} and Cu^{0} NPs-containing glasses