PHYSICO-CHEMICAL ENHANCEMENTS OF NATURAL BIOPOLYMER MATRICES BY REINFORCING WITH COMPLEX CARBONACEOUS-OXIDIC FILLERS

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Hereby we highlight the studies on κ-carrageenan matrices reinforced with graphene oxide/calcium phosphate nano-fillers. Aiming to develop an accessible synthesis route for improved bone scaffold fabrication, we describe complex nano-filler synthesis and its incorporation into κ-carrageenan films to improve its physico-chemical properties. Graphene oxide/calcium phosphate composites were synthesized in various ratios and evaluated through Raman spectroscopy, X-ray diffraction and electronic microscopy. Afterwards, the composite polymer matrix / nanofiller was synthesized. The morpho-structural features of the composite matrices were depicted through microscopy and spectral techniques. Along these lines, rheology and bio-stability investigations in simulated physiological media were ensued, with remarkable results.

Keywords: graphene oxide, calcium phosphate, nanofillers, polymer composite

1. Introduction

In hard tissue tissue engineering, an ideal scaffold is one that mimics the extracellular matrix of the host tissue to act as a three-dimensional model to which cells attach, migrate, and proliferate to the extent of which injured tissue regains its features and functionality. The criteria for an ideal scaffold are numerous and are included in both chemical and biological register [1].

It is well known that calcium phosphates guide bone formation and develop a close bond with the new bone, being by definition osteoconductive. In addition to its osteoconductive properties [2], it has been found that these biomaterials induce bone formation in unspecific osseous sites and are therefore considered osteoinductive. Calcium phosphate ceramics are used as implantable materials due to their similarity to the inorganic constituents of the native hard

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tissues. Amongst the most important advantages of these biomaterials is the fact that the bone will form direct chemical bonds with the implant without the issue of an inert collagen interface as is the case with most implanted bioinert structures. In general, the ceramic materials used in bone regeneration are most often hydroxyapatite and tri-calcium phosphate. Hydroxyapatite, tricalcium phosphates and bioactive glasses have been successfully used as powder reinforcement materials in complex composites and hydroxyapatite based composite nanoparticles have been shown to improve the properties organic matrices in which they have been used as reinforcing agents [3]. They are used due to their good mechanical properties, low elasticity, high biocompatibility [4], and potential to promote the emergence of nucleation center of neoformation biomineralization.

Graphene oxide (GO) can be described as a monolayer of carbon atoms with aromatic regions, sp^2 hybridized carbon atoms, and oxygenated aliphatic domains, with sp^3 hybridized atoms, with a variable content of functional groups (hydroxy-, epoxy-, carbonyl and carboxy-) [5,6]. GO shows an amphiphilic character resulting from the content of functional groups on the surface, which allows to obtain dispersions even in aqueous solutions after undergoing an ultrasonic process. [7]. GO-based nanocomposites succeed in broadening the horizons of knowledge in interdisciplinary fields such as regenerative medicine. GO-reinforced two- and three-dimensional polymeric scaffolds have superior mechanical and thermal properties with respect to GO-free scaffolds [8]; moreover, the GO content has a beneficial effect on the porous architecture of the scaffold [9] and, implicitly, on meeting the conditions necessary for an environment conducive to cell adhesion and proliferation, essential attributes in tissue engineering [10].

Carrageenan is an anionic natural polysaccharide extracted from red algae presenting potential for a wider range of biomedical applications and in the pharmaceutical industry, prolonging the release of active substances and for obtaining controlled release systems sensitive to environmental factors (pH, temperature). Kappa-carrageenan (κ-CRG) consist of repetitive diglucidic units, composed of galactose and anhydrous 3,6-galactose, with a sulfate group at each diglucidic unit joined by alternative alpha-1,3 and beta-1,4 glycosidic bonds. κ-CRG forms very strong friable and thermoreversible gels [11]. The thermoreversible character of κ-CRG recommends it as the main component in injectable hydrogels used as a cellular support in the repair of cartilaginous tissue, as well as bone defects, especially spongy bone tissue [12,13] due to bioadhesive properties that promote the formation a tight interface with the host tissue.

Hydrogels used in tissue engineering require a crosslinking treatment to prevent the dissolution of the hydrophilic polymer chain in aqueous medium [14]. Synthetic chemical crosslinking agents, such as glutaraldehyde, are commonly

used, but they have a high degree of toxicity. As a crosslinking agent, genipin is non-toxic and biocompatible, ensuring very stable crosslinked hydrogel, either protein- or polysaccharide-based [15].

The purpose of this study was to address the physico-chemical gains of reinforcing crosslinked κ-CRG networks with nano-fillers recognized for their high potential to facilitate osteogenesis. We considered the reported osteogenic behavior of graphene oxide, as well as of the calcium phosphate's, as biomineralization nuclei. Another novel aspect of our study targets the investigation whether graphene oxide and calcium phosphate synergistically enhance the properties of the composite and provide a facile and cost-effective synthesis route for the inorganic composite filler. We detail comparatively the changes occurred as a result of additivating the κ-CRG matrix with GO, calcium phosphates and composites of GO and calcium phosphates synthesized *in situ via* precipitation method.

2. Experimental Data

2.1 Materials

Kappa-carrageenan and graphene oxide powder were purchased from Sigma-Aldrich. All the reagents used in the synthesis of the calcium phosphate, $Ca(NO_3)_2{\cdot}4H_2O$, $Ca(OH)_2$, Ammonia (anhydrous, ≥99.98%) were purchased from Sigma-Aldrich. Chemicals used in the experimental investigations, $CaCl_2$, Na_2HPO_4, Phosphate Buffer Saline (PBS) tablets and reagents needed to synthesize Kokubo solution (SBF) – NaCl, $NaHCO_3$, KCl, $K_2HPO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$, Na_2SO_4, $(HOCH_2)_3CNH_2$,HCl and KBr, were also supplied from Sigma-Aldrich and used without further purification.

2.2 Methods

2.2.1 Synthesis of calcium phosphate/graphene oxide nano-fillers

Graphene oxide dispersion are obtained by dispersing a certain amount of GO in distilled water. Dispersion occurs by sonication at 100% amplitude for 2 hours. The ultrasonic process was performed using VCX750 equipment for small and medium volumes, produced by Sonics & Materials, Inc. (53 Church Hill Road, Newton, CT 06470-1614 USA), equipped with a titanium alloy probe tip (Ti -6Al-4V) and a 750 W source operating at a frequency of 20kHz. The vibration amplitude of the probe was set to 100%.

To obtain the calcium phosphate with a Ca/P ratio of 1.67 by precipitation, corresponding amounts of Ca^{2+} precursors, $Ca(NO_3)_2{\cdot}4H2O$ and $Ca(OH)_2$ were dissolved in distilled water. After complete dissolution, a solution of H_3PO_4 was added dropwise (75°C) over the obtained mixture, until the system reached a theoretical Ca/P ratio of 1.67. The pH was adjusted to 11 by the addition of an ammonia solution. After the acid was added, the mixture was stirred for 12 hours.

Subsequently, the precipitate was washed with distilled water and centrifuged (3 cycles). The precipitate obtained was dried in an oven for 72 hours at 80 ° C.

The synthesis of composite calcium phosphate/graphene oxide was carried out similarly, with the only difference that $Ca(NO_3)_2 \cdot 4H_2O$ and $Ca(OH)_2$ were solubilized in graphene oxide aqueous dispersions instead of distilled water. Three calcium phosphate / graphene oxide composite systems were obtained, different by graphene oxide concentration (0.5, 1.5 and 2.5 wt. %). The composite nano-fillers obtained are listed in Table 1.

Table 1

Tabulation of the powder nano-filler abbreviation and the Ca/P ratio measured by XPS

Composite nano-filler	Abbreviation	Ca/P ratio
Calcium phosphate	H	1.89
Calcium phosphate 0.5 GO wt. %	HGO05	1.77
Calcium phosphate 1.5 GO wt. %	HGO15	1.92
Calcium phosphate 2.5 GO wt. %	HGO25	1.71

2.2.2 Synthesis of κ-carrageenan composited with HGO nano-fillers

The κ-CRG was solubilized in distilled water, at a temperature of 75 ° C, in order to obtain solutions with a concentration of 2% (w/v). To obtain the hydrogels, κ-CRG was crosslinked with genipin, a natural, non-toxic crosslinking agent. The amount of dry crosslinking agent was set at 0.5% of the amount of dry polymer used. For the purpose of a comparative study, κ-CRG was reinforced with calcium phosphates obtained by precipitation, graphene oxide and the most promising calcium phosphate / graphene oxide composite, HGO25 and crosslinked with genipin. To facilitate the preliminary assessment of the properties of the synthesized materials, they were modeled in the form of films by casting method followed by drying in air. Several compositions have been proposed, as detailed in Table 2.

2.3 Equipments

The XPS analysis was performed with a K-Alpha apparatus from Thermo Scientific equipped with a monochrome Al K sur source (1486.6 eV) at a pressure of 2x10-9 mbar. KBr pellet Fourier-transform infrared spectroscopy (FTIR) was performed using a BRUKER VERTEX 70 spectrometer to identify the functional groups content; ground calcium phosphate samples were mixed with KBr powder in a weight ratio of 1:150. The mixture was pressed at 5 bar to form a transparent pellet, further scanned at a resolution of 4 cm^{-1} within the range 4000-400 cm^{-1} (32 accumulations). X-ray diffraction (XRD) analyzes were performed at room temperature, using Panalytical XPert Pro MPD equipment, equipped with CuKα radiation source with a wavelength of 1.54 Å. The reported diffractograms were recorded in the range 2θ from 5 to 60° and the signal strength is displayed in random units. The morphology of the composite powders was investigated with a

QUANTA INSPECT F SEM scanning electron microscope (SEM) equipped with EDX spectrometer (OXFORD Instruments) for qualitative and quantitative X-ray microanalysis. The samples subjected to SEM analysis were covered with a thin layer of gold. The rheology tests (frequency sweep) were performed with a Kinexus Pro rotary rheometer, equipped with plate-plate geometry and Peltier system. The 20 mm diameter samples were placed on the bottom plate of the rheometer and analyzed at a temperature of 37 ^{o}C.

3. Results and Discussion

3.1 Characterization of calcium phosphate/graphene oxide nano-fillers

3.1.1 X-ray photoelectron spectroscopy

XPS revealed the elemental composition of calcium phosphate-graphene oxide composites, as well as their Ca / P ratio. Thus, it was possible to determine that in the presence of an increased amount of GO (HGO25), calcium phosphates were formed with the Ca/P ratio (1.71) closest to that of stoichiometric hydroxyapatite, 1.67 (Table 1). For these composites, a linear increase in the amount of oxygen with an increase in the mass percentage of GO is detected, which confirms the differences in the carbonaceous material ratio of the composites (Fig. 1a). As for the 1.89 Ca/P ratio in the GO-free H sample, we suspect that the precipitation was carried out with the embedment of a considerable amount of Ca^{2+} ions within the phosphate particles. Since entrapped, they were not removed during the rinsing process.

3.1.2 Fourier-transform infrared spectroscopy

The FTIR spectrum of calcium phosphates and GO composites is illustrated in Fig. 1b. The spectrum of calcium phosphates contains the characteristic bands of natural hydroxyapatite at 473, 569 and 603 cm^{-1} attributed to the P-O extent of the phosphate group. Low intensity bands corresponding to the phosphate group are distinguished at 1092 and 1038 cm^{-1}. The characteristic wide band between 3520 and 3136 cm^{-1} and the weak band from 764 cm^{-1} can be attributed to the intensification and vibration of the OH- group in the hydroxyapatite structure. The 1632 cm^{-1} band corresponds to the asymmetric stretching vibration of CO_3^{2-} ions which indicates the formation of carbonated hydroxyapatite, recognized for its excellent bioactive and osteoinductive properties. The vibration associated with the band located at 1640 cm^{-1} corresponds to the vibration of sp^2 hybridized graphene due to the stretching vibrations of C = C [16].

3.1.3 X-Ray diffraction

XRD spectra of the powders obtained by precipitation indicate the formation of three distinct types of calcium phosphates. The sharp peak from 2θ = 11.8º is associated with tricalcium β-phosphate [17,18]; conversely, the formation

of a small amount of hydrated calcium phosphate (2θ = 20.82º) but also of hydroxyapatite crystals (2θ = 23.5º and 29.32º related to planes (101) and (120)) is highlighted [18]. Fig. 1c illustrates the spectra of HGO05, HGO15 and HGO25 powder composites. The characteristic peak of graphite can be observed at 2θ = 26.62º (002). In the three spectra appear better defined peaks at 2θ values of 25.14º, 29.52º, 30.48º, 31.58º, 31.94º, 32.66º, 34.6º, 39.92º, 46.7º assigned to the sets of planes (002), (002) (211), (300), (202), (310), (222) and (321) characteristic of hydroxyapatite [16]. Peaks at 27.6º and 28.86º are also attributed to hydroxyapatite [19]. The peak splitting and the appearance of some intermediates (between 28.86º- 29.52º) are indications of a mechanism of nucleation-aggregation-agglomeration crystal growth. According to this pathway, apatite particles are formed in three steps: i. nucleation and growth of crystallites, ii. aggregation of nanocrystals by physical attraction and iii. continued growth in order to form stable agglomerations [20]. Compared to the spectrum of pristine calcium phosphate, in the presence of GO, specific peaks of hydroxyapatite may indicate that, the formation of apatite crystals is favored detrimental to tricalcium phosphate and hydrated calcium phosphate. GO specific signals are not detected in the composite powder spectra. This can be attributed to the overlapping of GO layers and the formation of disordered structures; thus, the specific peak would be diminished and eventually disappear. In addition, hydroxyapatite nanoparticles can distance the graphene layers, weakening the diffraction of carbon monolayers.

3.1.4 Scanning Electron Microscopy

The morphology investigation of pristine calcium phosphate powders revealed that the particles are in the form of sharp-edged platelets, with a narrow thickness distribution (Fig 2a). Their lateral size is less than 120 nm and 72.5% of the investigated particles have a thickness of less than 100 nm.

The morphology of calcium phosphate and GO composite powders is radically different from that of calcium phosphate powder. In Fig.2b,c,d it is observed that as the GO content increases in the precipitation media, the tendency of calcium phosphates to aggregate in the form of fine powder emerges; also, powder-like particles tend to agglomerate in the form of sharp clusters, partially coating the larger plane particles. The increase of the GO concentration does not cause major changes; hence, the morphology and particle size are heterogeneous for all GO concentrations. It can be concluded that the precipitation itself in GO solutions causes changes in the appearance of the samples and the GO concentration has a less important influence. However, the porous appearance of the fine phase of the composite powder seems to have increased with the carbon material ratio, a strong argument in support of the idea that GO causes the specific surface area of the composite material to increase.

The diversity of particle shapes obtained could have a beneficial effect in their use as a reinforcing agent for polymeric matrices, as they could have superior reinforcing properties due to their potential even distribution within the matrix.

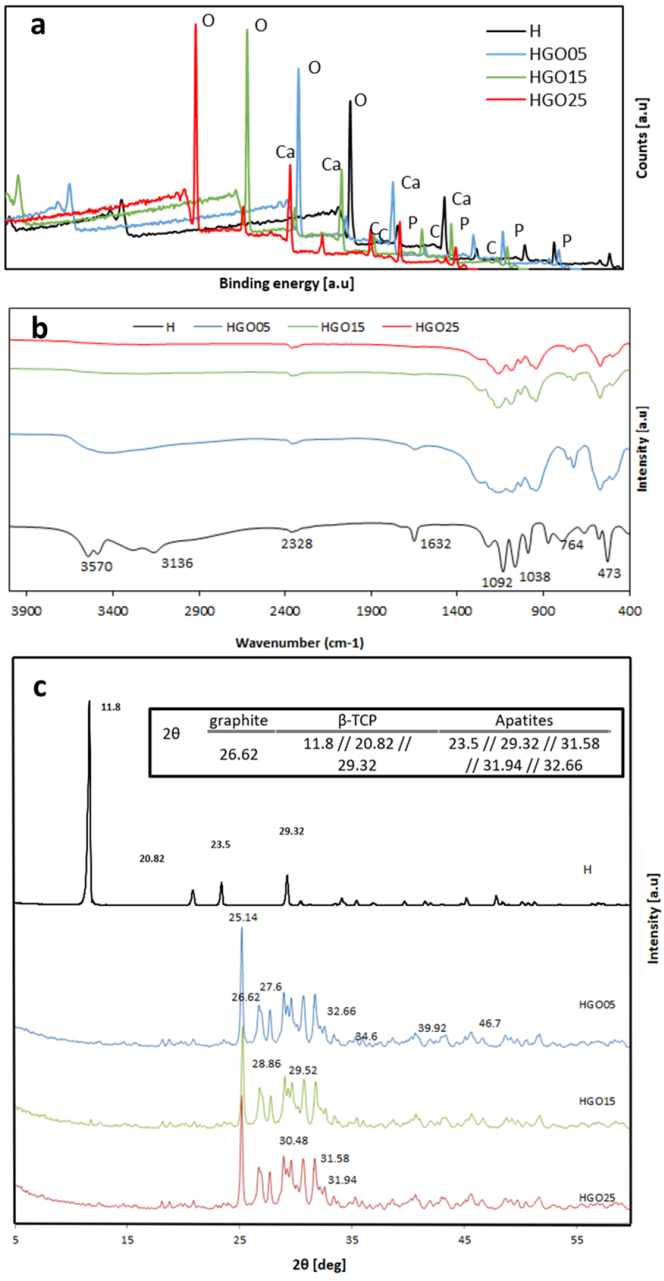

Fig. 1. Structural characterization of calcium phosphate/GO nano-fillers by means of a) XPS, b) FTIR and c) XRD

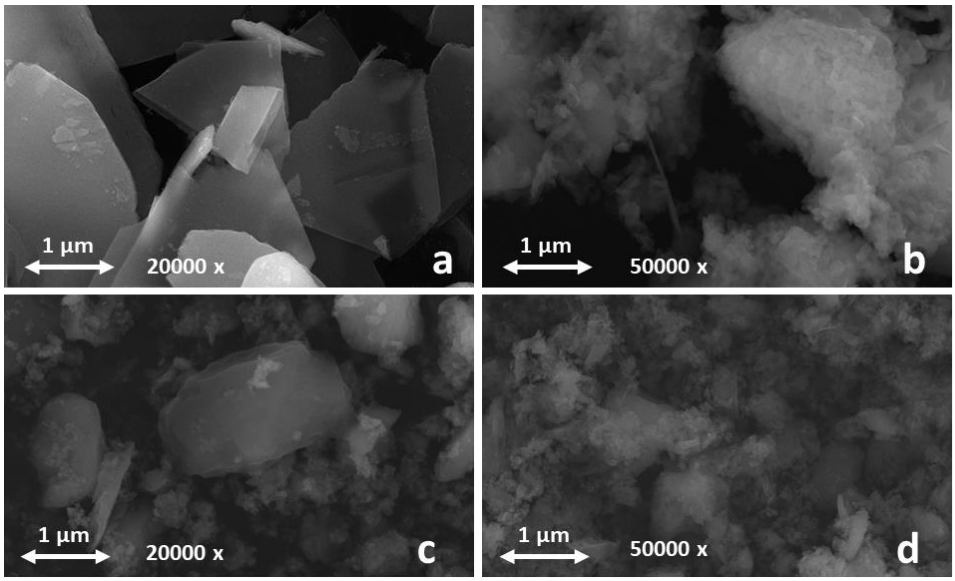

Fig. 2. SEM images of a) H, b) HGO05, c) HGO15 and d) HGO25 nano-fillers

3.2. Characterization of κ-carrageenan composited with HGO nano-fillers

Table 2

Indexing of the synthesized polymer composite films

Film formulation	Abbreviation
K-carrageenan, uncrosslinked	K
K-carrageenan crosslinked with genipin (0.5 wt. %)	K_g
K-carrageenan crosslinked with genipin (0.5 wt. %) + calcium phosphate (1 wt. %)	K_H1
K-carrageenan crosslinked with genipin (0.5 wt. %) + calcium phosphate (2 wt. %)	K_H2
K-carrageenan crosslinked with genipin (0.5 wt. %) + calcium phosphate (3 wt. %)	K_H3
K-carrageenan crosslinked with genipin (0.5 wt. %) + graphene oxide (1 wt. %)	K_GO1
K-carrageenan crosslinked with genipin (0.5 wt. %) + graphene oxide (2 wt. %)	K_GO2
K-carrageenan crosslinked with genipin (0.5 wt. %) + graphene oxide (3 wt. %)	K_GO3
K-carrageenan crosslinked with genipin (0.5 wt. %) + HGO05 nano-filler (1 wt. %)	K_C1
K-carrageenan crosslinked with genipin (0.5 wt. %) + HGO15 nano-filler (2 wt. %)	K_C2
K-carrageenan crosslinked with genipin (0.5 wt. %) + HGO25 nano-filler (3 wt. %)	K_C3

3.2.1 Water affinity assessment (%SD)

The swelling kinetics and the maximum degree of swelling of the κ-CRG / calcium phosphate / GO films were investigated in PBS (pH 7.4, 37 ° C), according to a well know protocol [21]. For this, dry samples were weighed (M_d - dry weight) and immersed in 10 ml of PBS (triplicate). The degree of swelling was determined by immersing the samples in PBS and maintaining them for different periods of time followed by weight measuring (Mw – wet weight).

Sample monitoring was carried out up to 48 hours as their mass remaining constant after about 2 hours of immersion. The maximum water absorption capacity of the samples represents the highest value of absorption at a time (t), after which it remains constant. After each immersion period, the samples were removed from the container, dried by wiping with filter paper and weighed within one minute of their removal from the water.

Water absorption, expressed as a percentage, is calculated by the formula:

$$\%SD = 100*(M_w - M_d)/ (M_d) \quad (1)$$

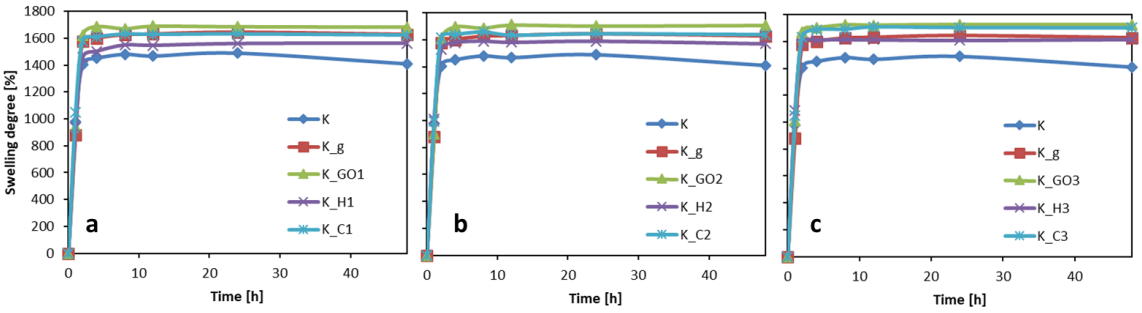

Fig. 3. Water uptake profiles of the polymer composites with a) 1 b) 2 and c) 3 wt. % nano-filler

The profile of water uptake is plotted Fig. 3. In the case of all samples, their mass remains approximately constant after 2 hours of immersion. Unsurprisingly, at longer immersion times, there is a downward trend in the mass of uncrosslinked κ-CRG films. Crosslinked films have a more stable behavior, showing the smallest mass fluctuations. Despite this, reinforcing agents have a minor effect on the swelling of the films. κ-CRG films with the addition of GO or calcium phosphate composite / GO crosslinked with genipin have the ability to absorb more water.

3.2.2 Rheology tests

During the rheological evaluation whereby storage modulus G' and loss modulus G" were measured, dehydration of the films was prevented by the use of a sealing jacket. In a first stage, the samples were subjected to a constant frequency amplitude variation test (1 Hz), determining the linear viscoelastic region (LVR). The second stage consisted of a frequency sweep test (10-0.1 Hz), keeping the effort constantly below the LVR limit.

The G' increase indicates the decrease of the elasticity of the material. Therefore, it can be shown that the elasticity decreases in the following order for the investigated materials: K_GO3 > K > K_g > K_C3 > K_H3. It can be concluded that GO improves the elasticity of κ-CRG (friable gel).

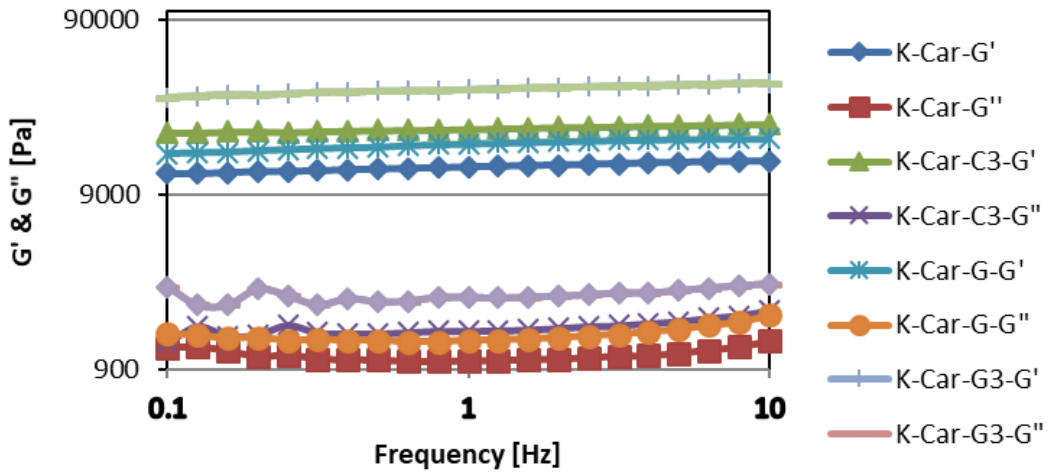

Fig. 4. G' and G" variations vs. frequency

3.2.3 Scanning Electron Microscopy

SEM images (Fig. 5a) reveal classical topical morphology of polymer films (smooth, homogeneous surface). Upon adding GO, wrinkled formation appear on the surface of the composite films, resembling the carbon layers random orientation (Fig. 5b). In Fig. 5 c,d, crystalline agglomerations on the surface of the films emerge associated to the nano-fillers embedded in the polymer matrix. This roughness increase of the composite's superficial features can be beneficial for future biocompatibility *in vitro* studies. Fig. 5e,f show the internal structure of the K_GO1 and K_GO3 films.

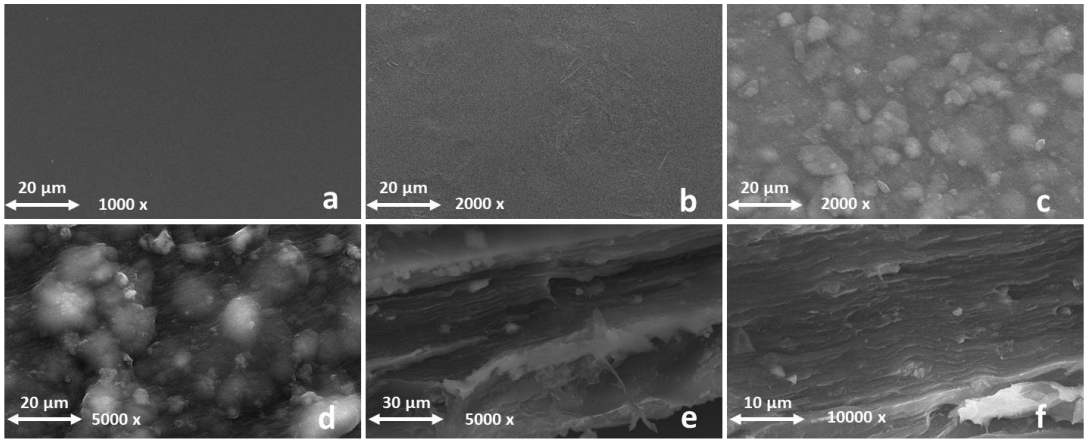

Fig. 5. SEM images captures on the surface of a) K, b) K_GO3, c) K_H3, d) K_C3 and in the cross-section of e) K_GO1 and K_GO3 composite films

It can be seen that the internal structure is quasi-lamellar, probably dictated by the orientation of some GO layers. At the same time, several areas

with distinct morphologies can be distinguished, which represents the confirmation that the reinforcing agents and the amount in which they are incorporated determine changes in the internal architecture of the composite matrices. We argue that by finely-tuning the GO ration within, multilayered architectures with controlled porosity gradient can be fabricated.

3.2.4 *In vitro* tests

In the present paper, two *in vitro* studies were proposed to help predict the film's behavior in more complex investigations involving biocompatibility assessment: degradation in simulated physiological environment (SBF) and biomineralization in alternating Ca / P baths.

As it is mandatory to check any biomaterial before use, its purpose in this case was to investigate gel fraction. For this purpose, specimens were taken from the synthesized films and immersed in SBF. The experiment was carried out for two days, the medium being replaced after 24 hours to maintain a constant electrolyte concentration. After every 12 hours, specimens from the samples were dried and weighed to determine the mass percentage of polymer leaving the system. The study was performed on polymeric films (simple and crosslinked) and on composite films with maximum reinforcing agent content. Improved stability can be observed for GO or calcium phosphate / GO composite films (Fig. 6).

Next, the phospho-calcium mineral loading capacity of the composite films was checked. Solutions of $CaCl_2$ (0.5 M) and Na_2HPO_4 (0.3 M) in distilled water were prepared at room temperature. The samples were immersed, in turn, in equal volumes of $CaCl_2$ and Na_2HPO_4 according to the protocol described by Serafim et al [22]. The duration of an immersion was 10 minutes; after each immersion, the meshes were washed lightly with distilled water. Each sample was subjected to three immersion cycles in Ca and P solutions. After each immersion cycle, the samples were dried and weighed to highlight the amount of phospho-calcium mineral deposited. Fig. 6 depicts the percentage gain of biomineralized samples. The study was performed on polymeric films (simple and crosslinked) and on composite films with maximum reinforcing agent content. The phenomenon of mineralization could be observed in the case of all samples; a more pronounced mineralization was determined in the case of GO and hydroxyapatite composite films.

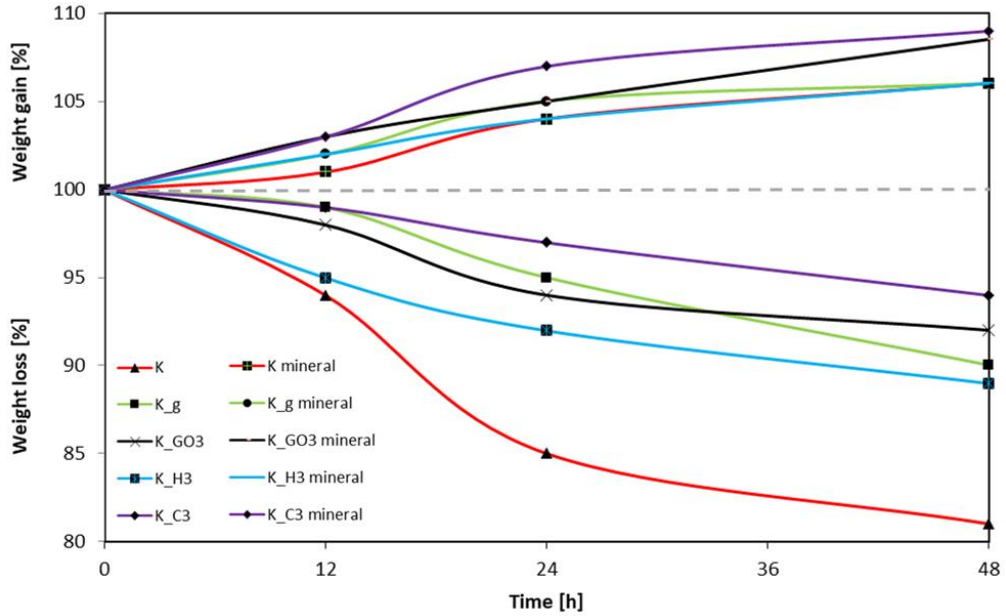

Fig. 6. Weigh variations after *in vitro* mineralization and degradation profile of K, K_g, K_GO3, K_H3 and K_C3 films

4. Conclusions

The present paper aimed to quantify and describe the means graphene based nano-fillers influence the properties of a polymer composite material, with potential applications in tissue engineering. According to the literature, GO and calcium phosphates are known to favor osteoregeneration. However, at first, we targeted the assessment of the physico-chemical changes these nano-fillers induce in polymer networks, mostly focusing on GO. Therefore, GO, calcium phosphates and GO/calcium phosphate composites were used to strengthen a biopolymer, κ-CRG. GO / calcium phosphate composite powders were facile synthesized *in situ* by a modified precipitation synthesis protocol. In order to determine the chemical composition, structural characteristics, processes that take place in the samples, the composite powders were studied by techniques such as: SEM, FTIR, XRD, XPS, while the polymer / nano-filler systems were investigated from a rheological point of view, with respect to water absorption, gel fraction and *in vitro* mineralization capacity. Subsequently, stability tests in physiological media performed on samples of the composite material obtained had promising results; the films have been shown to have good stability in synthetic plasma and the ability to support the emergence of mineralization centers. The next directions of research will be to establish the *in vitro* behavior of the composite material, as well as the potential osteoregenerative effects.

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