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GRAPHENE OXIDE SHEETS COVALENTLY GRAFTED WITH KERATIN OBTAINED FROM CHICKEN FEATHERS

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2D Carbon nanomaterials (graphene oxide sheets) were covalently grafted with keratin biostructures obtained from chicken feather under a redox reaction in acidic medium under two different conditions. The dispersion behaviour in solution of graphene oxide sheets modified with keratin was observed in water and hexane. The quantification of keratin grafting was done using the Bradford assay. Characterization of graphene oxide sheets and keratin grafted graphene sheets was achieved by FT-IR and Raman spectroscopy in order to corroborate the grafting; elemental analysis was realized in modified graphene to verify elements like Sulfur and Nitrogen typical in this protein. HRTEM, TEM and AFM complement the information showing that grafted graphene present different features and more thickness than graphene oxide. Keratin grafted graphene could be useful to diversify the properties of graphene. Compatibilization of graphene with polymers and the development of nanobiocomposites or biocompatibilization of these sheets are an opportunity field of modified graphene with natural polymers.

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1. Introduction

The graphene is a hexagonal arrangement of carbon atoms with sp^2 hybridization in a two dimensional structure, which proves to have very interesting. It is the builder block for carbon allotropes of other dimensionalities: fullerenes 0D, nanotubes 1D and graphite 3D [1]. The electronic confinement structure confers unique electrical [1,2] properties to graphene. On the other hand the structural arrangement of carbon atoms in the graphene sheet and the strong covalent bonds between them [3] provide mechanical [4] and thermal [5] unusual properties. These properties and their high theoretical superficial area [6] have been extensively explored for potential electronic applications [7], in materials like carbon paper [8], biological [9] and nanocomposites [10]. The chemical conversion of graphite into graphite oxide, allows it to be composed of highly oxygenated flakes with hydroxyl and epoxy functional groups above and below of each plane and carbonyl and carboxyl functional groups attached to the edges of the sheets [10,11]. The presence of these functional groups makes highly hydrophilic sheets,

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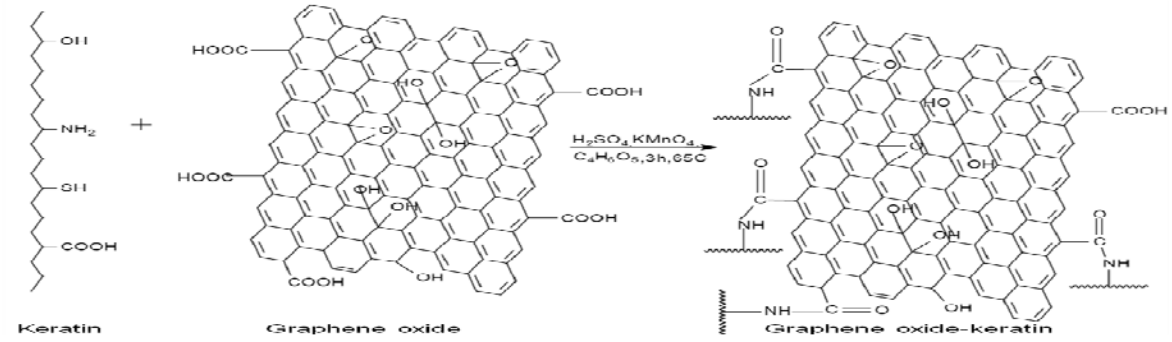
facilitating their dispersion in water; then, ultrasonic bath treatment allows their exfoliation in graphene oxide sheets [12]. Chemical modification of functional groups that exist in the graphene oxide sheets (which act as chemical anchoring sites) allow to improve their dispersion and processability [13]. The graphene and graphene oxide have recently been modified with various organic molecules and biomolecules providing them new properties. For example, graphene sheets were covalently functionalized with Poli-L-lysine resulting a water soluble material, biocompatible and later implemented in a biosensor [14]. Graphene sheets were noncovalent functionalized with the aromatic molecule 1-pyerenebutyrate obtaining materials such as films with improved conductivity [15]. Nano-graphene oxide sheets were covalently grafted with polyethylene glycol providing stability in aqueous and biological environments, as well as the ability to load and deliver drugs [16]. Graphene oxide sheets were chemically cross-linked with polyallylamine generating aqueous colloidal suspensions and samples like paper with improved mechanical properties [17].

Here, it´s reported the production and characterization of graphene oxide (GO) sheets covalently grafted with keratin (K) obtained from chicken feather. Keratin is a natural biopolymer of α-amino acids, which can be found in hair, wool, horns, claws and feathers, being in this later an abundant natural source of the protein [18]. Considering the high level of structure, the keratin is classified as a fibrous protein with a large number of disulfide bonds and hydrophobic amino acids residues that confer insolubility in organic solvents and chemical stability [19]. It has a self-assembled arrangement under a hierarchical structure formed by helix, protofibrils, microfibrils and macrofibrils that provide high structural organization which confer to keratin outstanding mechanical properties of strength and elasticity [20]. Recent studies have shown that geckos have the ability to climb vertical surfaces thanks to basic device of adhesion called “setaes”, which are constituted as keratinous fibrils formed of β-keratins [21]. This kind of keratin is also found in feathers and it was suggested that geckos and birds concur on their keratinous fibrils by evolving low molecular weight β-keratin which are then polymerized into long filaments and cross-linked by disulfide bonds that can increase the stiffness of the material [22]. In addition, other report has mentioned that despite of this similarity, these kinds of keratins have different compositions [23]. Thus, its important to take advantage of keratin abilities related to the molecular size and adhesion properties which can be widely exploited to change the behaviour of different nanomaterials and in this way to obtain better interactions with biological molecules, to get self-assembled hybrid nanostructures or improve the interface between graphene and polymer matrices of novel nanocomposites. Also, the amino acids chains of keratin present hanging groups such as: amino, hydroxyl, thiol and carboxyl that under a redox system can generate free radicals and they can easily react with functional groups of the graphene oxide sheets (Scheme1). Thus, this report presents the synthesis of novel graphene sheets modified with a natural polymer, which is obtained from an interesting source that produces different behaviour and possible applications to the graphene oxide sheets.



Scheme 1. Reaction mechanism proposed in the synthesis of graphene oxide sheets grafting covalently with keratin.

2. Experimental

2.1 Materials

The graphite was supplied by Electron Microscopy Science in bars and then was pulverized and sieved in mesh 300. All reagents for each synthesis method were purchased in Sigma Aldrich.

2.2 Methods

Keratin grafted graphene oxide were synthesized in four stages:

1) Exfoliation of graphene oxide (GO) using graphite as precursor. First, graphite was oxidized to obtained GO by Hummers method [11]. Briefly, 2g graphite was mixed with 46 ml of concentrated sulfuric acid in a round flask and stirred continuously in an ice bath. Then, $KMnO_4$ (6g) was gradually added into the solution with stirring and cooling maintaining the temperature < 20 ºC. Next, the mixture was stirred for 2h at 35 ºC. After that, 92 ml distilled water was added and stirring maintained for 15 minutes. The reaction was finished by transferring the content of the flask into a container with 280 ml distilled water and adding 10 ml of hydrogen peroxide (30% solution in water) to remove $KMnO_4$ excess. Graphite oxide was isolated by centrifugation and washed with a solution of HCl in water (1/10 v/v dilution HCl) and then with DI water until pH close to neutrality.

Then, GO sheets were obtained in aqueous dispersion by the sonication for 3 h of graphite oxide in distilled water (125 mg 12.5 mg/ml).

2) Keratin Dissolution. 3g of keratin biofiber from chicken feathers were dissolved in a mixture of urea (8M 98%), EDTA (3mM 90%), 2-mercaptoethanol (125 Mm 98%) and hydroxymethyl aminomethane (200Mm 97%) in 75 ml of distilled water at room temperature and under controlled stirring for 24 h [24].

3) Keratin Dialyzed Solution. 10 ml of Keratin dissolution was deposited in a dialysis membrane (6 Spectra/Por®, MWCO: 8000 50 mm x 10 mm Vol/long) which is introduced into a flask with distilled water (it should be changed every 24 h) and stirred for 3 days in order to eliminate 2-mercaptoethanol and urea [24].

4) Keratin grafted graphene. Reaction of dialyzed keratin with the suspension of graphene oxide sheets was realized in a redox system of malic acid/$KMnO_4$ in H_2SO_4 at 65°C for 3h. In the last stages the reaction was developed under two different conditions of oxidation-reduction system in order to observe its influence on the grafting. In the first condition, the aqueous suspension of graphene oxide sheets were reacted with 7.5 ml of dialyzed keratin, 0.75 ml of H_2SO_4, 0.05g malic acid and 0.375g $KMnO_4$ (GKGO1). In the second condition we change only the amount of H_2SO_4 at 0.5 ml and malic acid 0.07g, (GKGO2). The remainder reactants stay unchanged.

Protein quantification by Bradford method. Aqueous suspensions of both grafts were prepared with a concentration of 100µg/ml and sonicated for two hours. The calibration curve was prepared with the standard solution of bovine serum albumin (50µg/ml) and in parallel were measure protein concentration by determining the absorbance at 550 nm.

2.3 Equipment

Fourier Transform Infrared (FT-IR) spectra were taken on a Bruker spectrophotometer alpha-p model with a laser wavelength of 1064 nm. The samples were prepared with KBr tablets. Raman spectra were run in a Dilor micro-Raman system at 514.4 nm, 20 mW and with a spectral resolution of 3 cm^{-1}, an integration time of 15 s and a temperature of 20°C; the samples were analyzed in powder. Transmission Electron Microscopy (TEM) was carried out in a JEOL model JEM-1010 operated at 80 kV using a carbon grid; the samples were prepared in an aqueous dispersion at a concentration of 0.5mg/ml sonicated and dropped previously on the grid. Elemental analysis was performed with an equipment of Energy Dispersive Spectroscopy (EDS): Oxford Inca wafer of detector doped with lithium and an equipment of Scanning Electron Microscopy (SEM) JEOL JSM-6060 LV. The analysis was performed on samples compressed into tablet form.

Atomic Force Microscopy (AFM) images were obtained preparing aqueous dispersion of GO and GKGO1 on freshly cleaved mica substrates. The scans were performed in a digital instrument CPII in non-contact mode operated at a frequency of 200 kHz and a scan rate of 0.5 Hz. The absorption spectrum to quantify protein was obtained in a 680 Bio Rad Microplate reader at 550 nm. High Resolution Scanning Electron Microscopy (HRSEM) analyses were performed on a FEG electron microscopy Hitachi S-5500 operated at 30 kV. High Resolution Transmission Electron Microscopy (HRTEM) images were obtained using a JEOL JEM 2010 F operated at 200 kV.

3. Results and discussion

The hanging groups of keratin are sensitive to react under a redox system of malic acid/$KMnO_4$ in H_2SO_4 and generate free radicals involved in the formation of the graft. In this study we performed the graft reaction of keratin and graphene oxide sheets under two different conditions (GKGO1 and GKGO2) by changing the amounts of malic acid and H_2SO_4 in the redox system. These parameters were changed because both are important factors in the grafting of polymers which influence directly the graft in the material [18].

The quantification of grafted keratin in graphene oxide under both reaction conditions was performed by colorimetric method of Bradford. The Bradford assay depends on the reaction of the dye Coomassie brilliant blue G-250 to protein which causes a shift in the absorption maximum from 465 nm to 595 nm. The assay requires a calibration curve using known concentrations of a standard protein such as bovine serum albumin. The calibration curve carried out in parallel with the test and under the same conditions allowed to measure the protein concentrations in the micrograms range [25].

The Figure 1 shows the results obtained for the quantification of grafted keratin of four absorption spectra by sample and calibration curve obtained. The first graft (GKGO1) shows an average absorbance of 0.448 corresponding to 0.726 µg of protein and the second (GKGO2) an average absorbance of 0.403 corresponding to 0.487µg of protein. The ratio of µg protein/µg sample shown that GKGO1 have 12.1% of grafted keratin and 7.9 % to GKGO2.

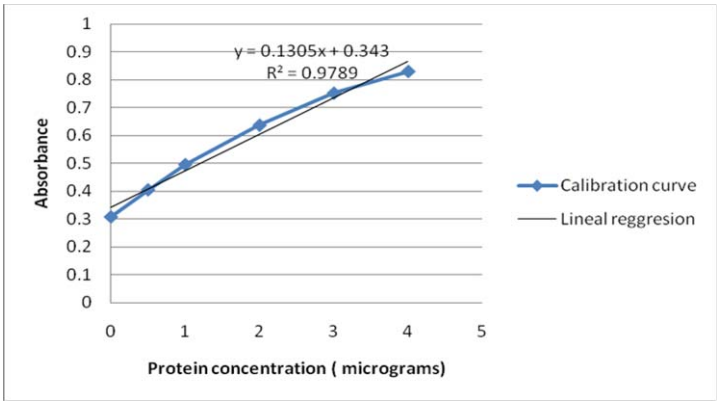


Fig. 1. Calibration curve for Bradford assay to quantify keratin grafted in graphene oxide sheets.

The dispersive behaviour of graphene modified with keratin was observed in water (polar) and hexane (non-polar) at a concentration of 0.2 mg/ml in comparison with graphite (G) and GO. The solutions are subjected to a generous sonication. The graphene grafted with keratin in both conditions (GKGO1 and GKGO2) forms a stable suspension in water but not in hexane (Figure 2a and 2b) and the dispersion behaviour in both solvent is not the same than G and GO. This later indicates that keratin grafted change dispersion behaviour in these suspensions in comparison with GO. It´s evident that modified carbon materials form hydrogen bonds with polar solvent that improve dispersion. Contrary, good dispersion in non polar solvent is not achieved due to the nature of solvent. The aqueous suspension of GO and graphene grafted with keratin forms a small precipitate after 24 h, as can be observed in Figures 2c and 2d, which can be easily re-suspended.

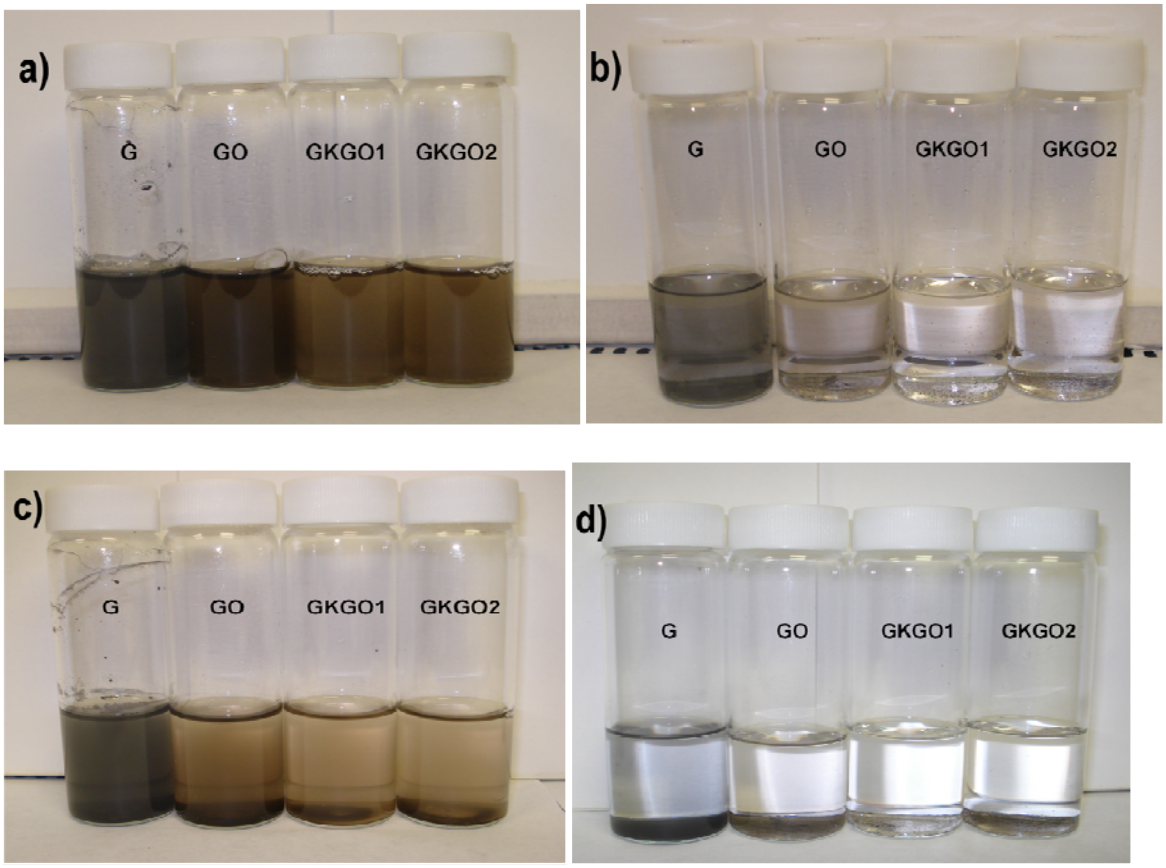


Fig. 2. Carbon materials dispersed in water and acetone (left to right) graphite (G), graphene oxide (GO), graphene oxide sheets covalently grafted with keratin at condition 1 (GKGO1) and grafting at condition 2 (GKGO2). a) Aqueous dispersions just sonicated; b) dispersions in hexane just sonicated; c) aqueous dispersions 24 hours after sonication; d) dispersions in hexane 24 hours after sonication.

Fig. 3 shows IR spectra of grafted graphene in comparison with GO and keratin. The IR spectrum of graphene oxide sheets (GO) illustrates the presence of O-H(ν_{OH} 3382 cm^{-1}), C=O($\nu_{C=O}$ 1700 cm^{-1}), C=C($\nu_{C=C}$ 1624-1587 cm^{-1}), C-O carboxy(ν_{C-O} 1402 cm^{-1}) C-O epoxy(ν_{C-O} 1228 cm^{-1}), C-O alkoxy(ν_{C-O} 1059 cm^{-1}) which agrees with previous reports[26]. In both spectra of grafted graphene (GKGO1 and GKGO2) the double band which corresponds to vibrations C=O and C=C present in the graphene oxide sheets spectrum are absents; instead of this pair of signals, only one band at 1645 cm^{-1} is found, this peak is also related with C=O; however, it is observed that the same signal appear in keratin spectrum, but in this occasion related with carbonyl vibration of amide I of this protein. Other two bands related with amine group that confirm keratin grafting in graphene sheet are observed in the three spectra (GKGO1, GKGO2 and Keratin); one signal found at 1532 cm^{-1} and corresponds to the deformation of the amide II δ(N-H) of the keratin. The other one is found at 1230 cm^{-1} from the C-N link (amide III) [19].
In addition, a significant decrease in the intensity of the peaks related with C-O vibration at 1402 cm^{-1} and 1228 cm^{-1} (epoxy) is observed in grafted graphene with respect to graphene oxide. This later indicates that C-O moieties in graphene surface could be sensible sites to graft keratin.

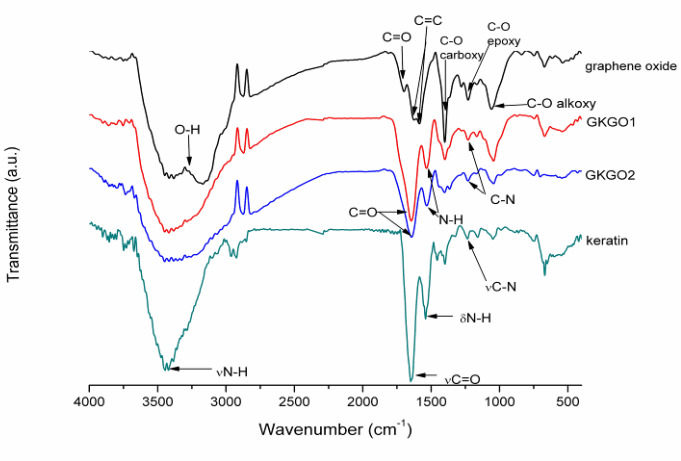


Fig. 3. FT-IR spectra of graphene oxide, graphene oxide sheets covalently grafted with keratin (GKGO1 and GKGO2) and keratin.

The Raman spectra of graphene oxide sheets and graphene oxide covalently grafted with keratin (GKGO1, GKGO2) are shown in Figure 4. The graphene oxide displays typical bands of graphitic carbon structure; the D band at 1351 cm^{-1}, a second broad peak to 1598 cm^{-1} for the G band. First band is associated to out of plane breathing mode of the sp^2 atoms and is active in the presence of defects [27-29]. G band can be assigned to the dispersion of first-order phonon E_{2g} [12,29] and the 2D overtone 2839 cm^{-1} band of small intensity compared to bands D and G. The intensity and broad of 2D band in graphene is a function of number of layers. The band is a second-order process related to a phonon near the K point in graphene, activated by double resonance processes, which are responsible for its dispersive nature and cause a strong dependence on any perturbation to the electronic and/or phonon structure of graphene. Thus, the 2D band is very susceptible for characterizing specific sp^2 nanocarbons [27-29]. After grafting in graphene oxide, the Raman spectrum shows a slight shift in the positions of the bands D and G and a significant variation in the intensities of these and the 2D band. In the graft keratin graphene oxide condition 1 (GKGO1) shows a D band at 1353 cm^{-1} and G band a 1594 cm^{-1}. There is also a significant increase in the intensity ratio of D/G with respect to the graphene oxide, which indicates increase in disorder in the sample interpreted as a decrease of sp^2 bonds. In the graft keratin graphene oxide condition 2 (GKGO2) the peak at 1347 cm^{-1} corresponds to the D band and 1586 cm^{-1} to G band. There is a minor variation of the intensity ratio of D/G with respect to the graphene oxide compared with the GKGO1. The shift in the position of the bands and the variation in intensities can be interpreted as evidence of graft. Also, the most evident changes in the Figure 3 in the grafted samples are observed in 2D band. As it was mentioned, this band is very susceptible to changes in graphene sheets [27-29]. The non common broad peaks are related with the changes produced in the graphene oxide layers by the grafted keratin. Also the Raman spectra of graphite oxide and graphene oxide are shown in the supplementary information where the differences in the bands D, G and 2D are verified. Likewise high resolution SEM images of both are presented and they reveal the different morphology of the sheets.

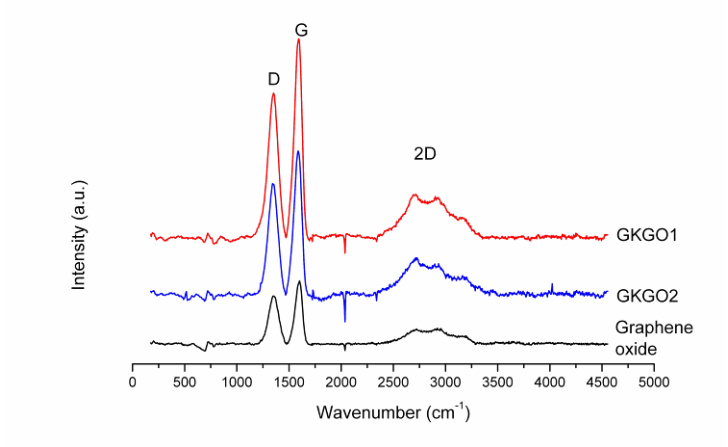


Fig. 4. Raman spectra of graphene oxide and graphene oxide sheets covalently grafted with keratin (GKGO1 and GKGO2).

In the elemental analysis results obtained by EDS, the samples present a C/O atomic ratio of 2 in GO, while the graphene oxide sheets grafted with keratin under conditions 1 and 2 (GKGO1 and GKGO2) show a C/O atomic ratio of 2.1 and 2.3 respectively, due to the presence of keratin. Also, both grafted graphene samples show nitrogen and sulphur. Both elements only appear in grafted graphene samples. GKGO1 shows an atomic ratio C/N of 15.1 and C/S of 282.5 and the GKGO2 have an atomic ratio C/N of 12.5 and C/S of 221.7. Both elements are typical of proteins groups such as: amide and thiol groups or S-S bond of different amino acids of keratin [18,19].

Figure 5a shows AFM images. These samples exhibit isolated graphene oxide sheet, with an irregular morphology and whose average thickness is around 0.6 nm. This average thickness is below of some reported thickness of the graphene oxide sheets completely exfoliated(1-1.4 nm) [11,30]. However, some irregular sheets with almost similar thickness have been also found in graphene materials [31]. Figure 5b shows a sheet of graphene oxide grafted with keratin (GKGO1) which exhibits the presence of material accumulated in small groups. The average thickness of the sheet is 4.5 nm and the increase in thickness is due to deposit of keratin on the surface of the graphene oxide sheet.

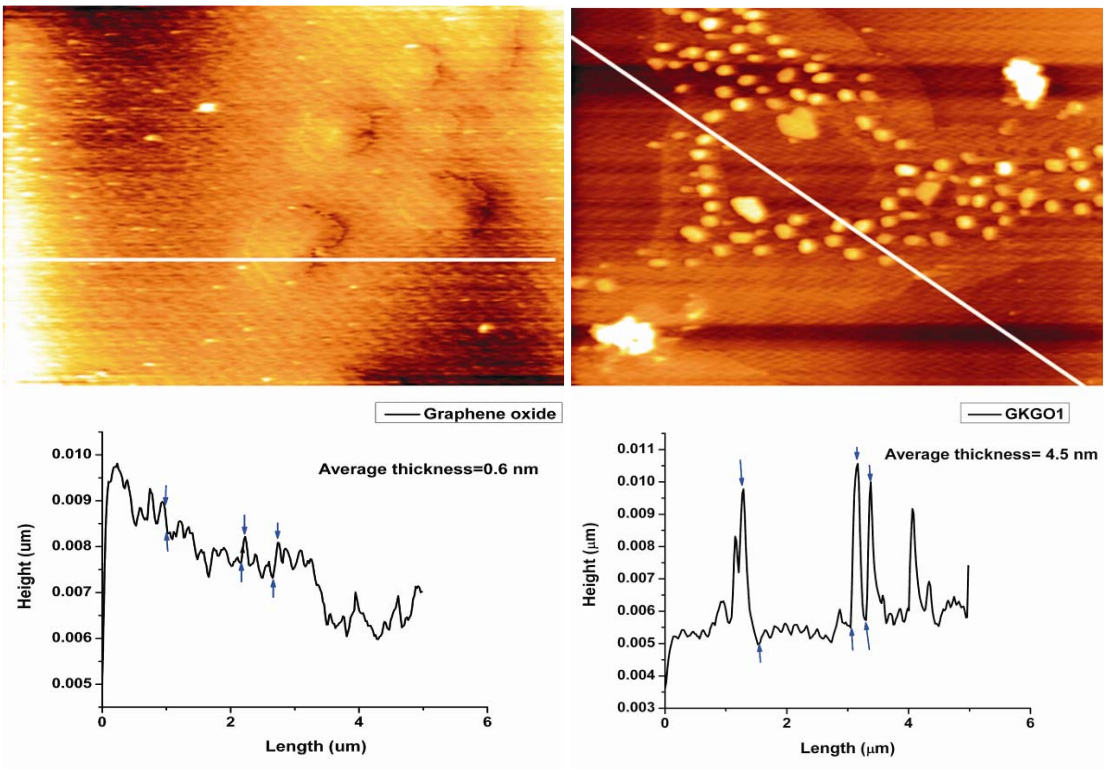


Fig. 5. AFM images and line profiles a) graphene oxide sheets onto mica substrates and; b) graphene oxide sheets covalently grafted with keratin (GKGO1) onto mica substrates.

The morphology of graphene oxide sheets and graphene oxide covalently grafted with keratin is examined by TEM. Figures 6a shows a sheet of graphene oxide, which is shown as a sheet of large lateral dimensions, rippled and transparent. Figure 6b shows the graphene oxide grafted with keratin (GKGO1). In these images can be observed the presence of sheets with a darker tonality due to the accumulation of keratin. Other image related with GKGO1 (Figure 6c) shows the presence of a clearly formed keratin granule attached to the side of the graphene oxide sheet. A similar behaviour is reported by Bourlins et. al. using the albumin biopolymer [32]. Figure 6d shows the TEM image related with grafting number 2 (GKGO2). This picture presents keratin sheets clearly soaked up in the graphene oxide sheet which can be seen as a transparent and wrinkled flake.

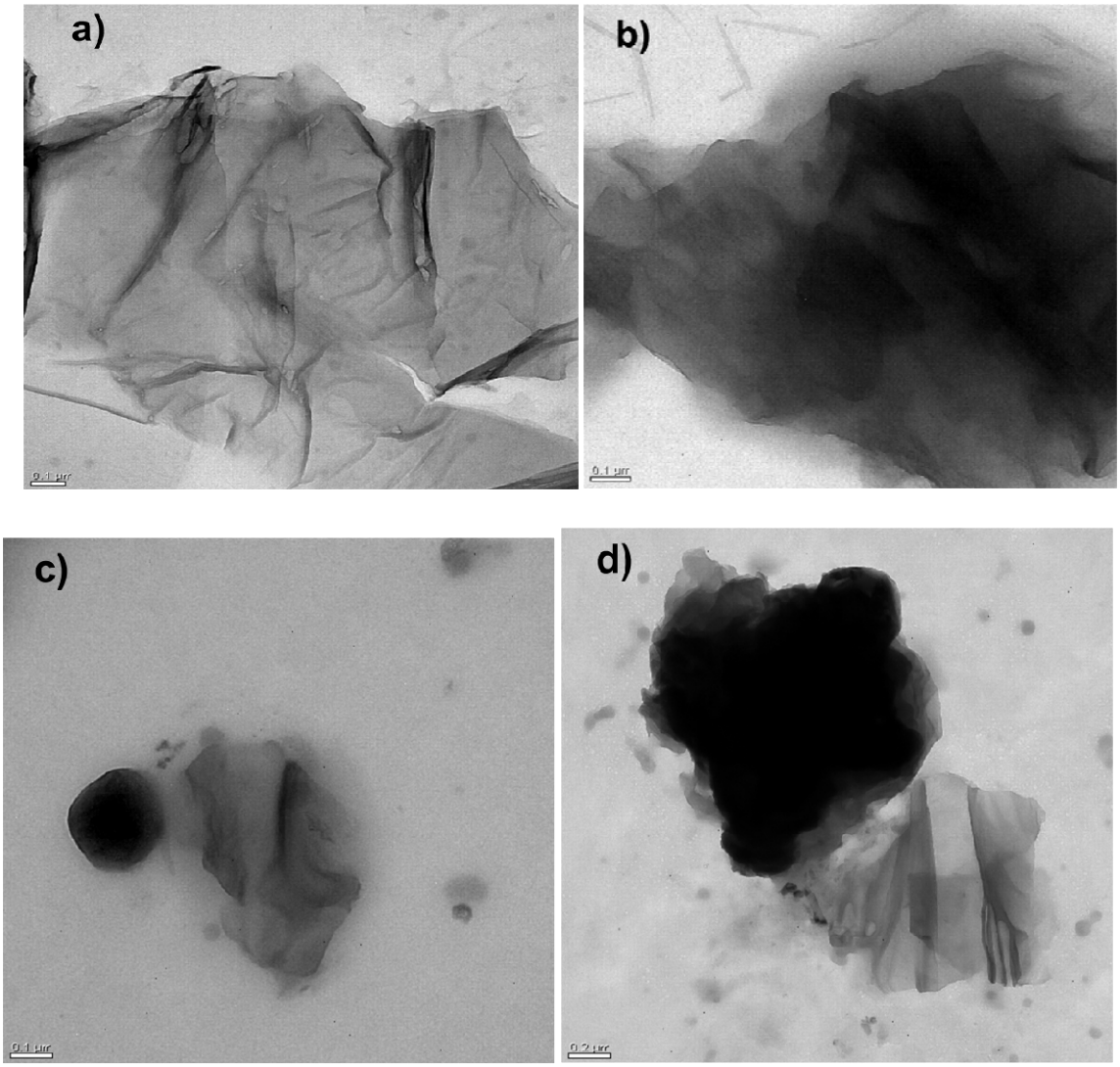


Fig. 6. TEM images a) graphene oxide sheets; b)-c) graphene oxide sheets covalently grafted with keratin (GKGO1); d) graphene oxide sheets covalently grafted with keratin (GKGO2).

In order to give more specific details of these nanomaterials, morphological characterization provided by HRTEM was realized. The images are illustrated in Figure 7. The HRTEM image of graphene oxide (Figures 7 a-b) shows some stacked and clearly ordered graphitic layers. In the Figures 7 c-d are shown some images related with graphene oxide grafted with keratin (GKGO1); in these figures is observed that the graphene oxide sheets maintain their layered structure after the grafting reaction; however, undergo corrugation, folds and wrapping on themselves. The Figures 7e-g) shown some HRTEM images for the graphene oxide grafted after condition 2 (GKGO2). It is clearly the small keratin particles with diameters around 1-10 nm; some of these are indicated by arrows in the Figures 8e-g. The particles lean to adhere along the border of the sheets; mainly, in the Figure 7g can be seen typical graphitic layers of graphene oxide sheets surround the keratin particles embedded in them. Figure 7h shown an image with high magnification; the picture presents a keratin particle on graphene oxide surface. The presence of keratin linkage and the folding of the graphene oxide sheets on themselves caused by the interactions with keratin confirm a successful and novel grafting produced by natural polymer chain in graphene sheets.

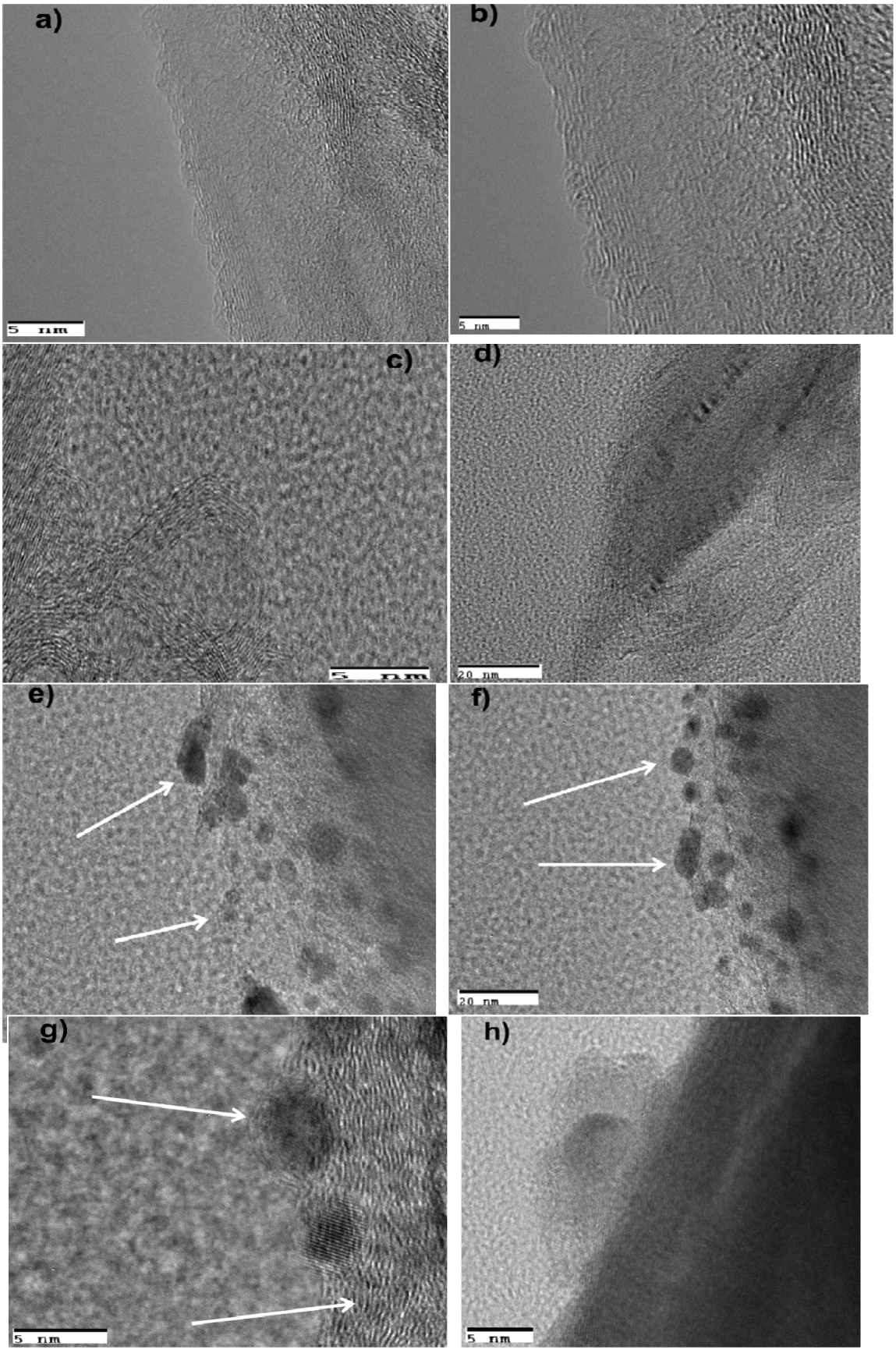


Fig. 7. HRTEM images. a)-b) graphene oxide sheets; c)-d) graphene oxide sheets after the grafting reaction (GKGO1); e)-h) graphene oxide sheets grafted with keratin (GKGO2).

4. Conclusions

Graphene oxide sheets covalently grafted with keratin extracted from a novel source, chicken feathers, were synthesized and characterized by different techniques. The IR spectra support the links between keratin and graphene oxide by diverse bands. Raman spectroscopy completes the evidence of keratin grafting due to the changes in typical graphitic bands related to sp^2 hybridization in carbon materials. The changes in 2D band in the spectra of grafted graphene sheets in comparison with graphene oxide give a clear evidence of modification of graphene material structure. Also the elemental analysis and Microscopy techniques complete the evidence of successful keratin grafting.

Keratin proteins in graphene sheets produce clear changes in morphology. The sheets obtained after grafting are curved and have a propensity to fold. It is possible that the clear tendency of proteins to self-assembled could produce the fold in graphene sheets. This effect could also be related to Raman spectra changes in the D and G bands and mainly in 2D signal to the grafted materials in comparison with graphene oxide spectrum.

Keratin quantification in modified graphene is a useful method to verify protein chains in carbon structure. The redox reaction system in base of malic acid to generate free radicals in the keratin is effective. The little changes in malic acid and acid medium, in which redox reaction operates, produce little changes in grafted keratin in graphene oxide.

In addition microscopies present evidence related with the keratin embedded in the graphene layers and the increment in the sheet thickness to the grafted nanomaterials. The graphene oxide grafted with keratin shows good dispersion in aqueous medium which facilities processing. The adhesion properties of keratin and the availability of reactive groups in the polypeptide chain grafted into the graphene oxide can be used for further applications such as nanocomposites based in chemically modified graphene, biosensors, biomaterials or in the biomedical field.

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