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Small-angle X-ray scattering and FTIR characterization of nanostructured poly (vinyl alcohol)/silicate hybrids for immunoassay applications

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Abstract In the present work we aimed to develop and characterize hybrid organic–inorganic materials based on poly(vinyl alcohol) (PVA) polymer chemically modified by organosilanes and crosslinked network to be tested as solid support on immunoassay application. Hybrids were synthesized by reacting PVA with 5 different alkoxysilanes modifying chemical groups: tetraethoxysilane (TEOS), 3-mercaptopropyltriethoxysilane (MPTES), 3-glycidoxypropyltrimethoxysilane (GPTMS), 3-(triethoxysilyl)propylisocyanate (TESPI), and 3-aminopropyltriethoxysilane (APTES). PVA-derived hybrids were also modified by chemically crosslinking with glutaraldehyde (GA) during the synthesis reaction. In order to investigate the structure in the nanometer-scale, PVA-derived hybrids were characterized by using small-angle X-ray scattering synchrotron radiation (SAXS). Fourier transform infrared spectroscopy (FTIR) was used to investigate PVA hybrids chemical functionalities and their interaction with bovine herpesviruses. The morphology of silane modified PVA films were also analyzed by SEM coupled to EDX. The bioactivity assays were tested through Enzyme Linked Immunosorbent Assay (ELISA) with bovine herpesvirus (BoHV). SAXS results have indicated nano-ordered disperse domains for PVA hybrids with different X-ray scattering patterns for PVA polymer and PVA-derived hybrids. FTIR spectra have clearly showed that the proposed modifications of PVA by organosilanes were obtained. The chemical crosslinking of PVA polymer chain by GA was verified by FTIR. The immunoassay results have showed that PVA hybrids with chemically functionalized structures have played an important role on regulating to some extent the interaction of herpesvirus and solid substrate at the interface. These results have given strong evidence that PVA-derived hybrid nanocomposites were successfully formed with GA crosslinked network. Also, such PVA based material could be advantageously used in immunoassays with enhanced specificity for diagnosis.

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Introduction

Hybrid organic–inorganic (O–I) materials are promising systems for a variety of applications due to their extraordinary properties based on the combination of the different structure blocks. The combination of nanoscale inorganic moieties with organic polymers has a high potential for future applications in optical and biomedical devices, matrices for drug delivery systems and specially designed solid support for immunoassay [1–6] and has therefore driven a lot of attention during the last 2 decades [7–11]. The interactions between the organic and inorganic phases during synthesis can occur at the micro, nano or molecular scale, resulting in nanocomposites with small sizes and large interfaces [12, 13]. These different components can be mixed at length scales ranging from nanometer to micrometer, in virtually any ratio leading to the so-called hybrid organic–inorganic materials, which are normally

nanocomposites [4]. While in most of the reported studies, tetraalkoxysilanes (TEOS, TMOS) were used as precursors for the inorganic network, trialkoxysilanes with an organic functionality have also been employed. Often the organic group has a significant influence on the resulting material. Because of the nanometric and rather disordered nature of precursors, intermediate materials, and final products, their structural characterization is a challenge for materials scientists. As a consequence, the nanostructure of hydrogels and hybrids are very complex and up to now not properly understood. If the relevant structural features are at a super-atomic level, from 1 nm up to about 100 nm, small-angle X-ray scattering (SAXS) is the most broadly used technique [14–16]. SAXS provides statistical and overall information averaged in a volume on the order of 1 mm [3]. SAXS beamlines in synchrotron radiation laboratories provide very intense monochromatic X-ray beams that make studies of weak scatterer materials possible and, also, in situ analyses of structural transformations with a high time resolution. Besides providing a high photon flux, the nature of synchrotron radiation emission spectrum allows one to use the effect of anomalous scattering for many useful applications. FTIR spectroscopy can in many cases be performed because it is sensitive on altering the chemical environment, being an exceedingly useful complement for X-ray scattering investigations.

Conventional methods to immobilize biomolecules onto inorganic, organic or polymeric surfaces have usually been based on physical adsorption, covalent binding to surfaces, entrapment in semi-permeable membranes and microencapsulation into polymer microspheres and hydrogels [1–4]. Living organisms like cells and virus have several biomolecules on their surface, for instance proteins, that have very particular chain configurations and conformations, which promote high levels of specificity during chemical interactions. An emerging route for bioimmobilization involves the interaction of biological components into hybrid matrixes formed by a low temperature reaction route [5, 6]. A polymer-based hybrid network designed with flexible crosslinked hydrophilic polymeric chains could be an alternative to perform antigen-antibody detection with the required high level of specificity with great potential to be applied to immunoassays. Despite of PVA being already used in many industry applications, several challenges are yet to be overcome when dealing with nanotechnology and chemically modified networks.

Bovine herpesvirus is distributed around the world, causing a wide variety of clinical syndromes in cattle and has been described in Australia, Argentina, US, and Brazil [17–19]. Bovine herpesvirus is an alphaherpesvirus responsible for fatal meningoencephalitis reported in calves. As the Bovine herpesvirus types share great similarities at glycoprotein level, the current available commercial kits are based on Enzyme Linked Immunosorbent Assays (ELISA), but they have showed limited performance due to a lack of specificity. Therefore, the immunoassay application opens a window of opportunity for scientists to develop novel materials with specially designed properties in order to improve selectivity, specificity and effectiveness of these biological tests.

In the last 10 years, our research group has spent a great deal of effort investigating the interaction of biomacromolecules, such as proteins, with engineered materials [4, 16, 20–23]. Hydrogels, polymer blends, hybrids and surface modified materials have been synthesized by several research groups [7–11, 14–16] pioneered by Prof. J E Mark and co-workers [24–28] and characterized by using a number of techniques, for instance scanning electron microscopy, Fourier transform infrared spectroscopy, MAS NMR [10], ultraviolet–visible spectroscopy, wide and SAXS [9, 16, 26, 28] contact angle and surface area measurements, among others. Also, in vivo and biological assays have been carried out in order to understand the complex interaction mechanism involving synthetic materials with biomacromolecules and living organisms under physiological conditions [20–22].

In the present work, we aimed to develop PVA-derived hybrids based on the chemical modification of polymer network by organosilanes and characterize these systems through SAXS, FTIR spectroscopy, and SEM/EDX microscopy. In addition, a bioassay was conducted by using bovine herpesvirus as model for a better understanding of the interaction between living organisms with engineered materials. As far as we know, no similar research has yet been published where PVA-derived hybrids with such a broad range of organosilane modifier moieties were investigated.

Materials and methods

Tetraethoxysilane $Si(OC_2H_5)_4$ (TEOS >98%), 3-mercaptopropyltriethoxysilane (MPTES) , 3-aminopropyltriethoxysilane (APTES) were supplied by Sigma-Aldrich. 3-glycidoxypropyltrimethoxysilane (GPTMS) and 3-(triethoxysilyl)propylisocyanate (TESPI) were supplied by Merck. PBS solution (phosphate buffered solution) was prepared using the reagents: Na_2HPO_4 (>99.0%), NaH_2PO_4 (>99.0%), Na_2CO_3 (>99.5%) and NaCl (>99.0%) supplied by Sigma-Aldrich. Glutaraldehyde (GA) or 1,5-penta-dial (Sigma-Aldrich) was purchased as a 25% (w/w) aqueous solution. Poly(vinyl alcohol) was kindly donated by Celanese Chemicals (Celvol-PVA107) as a 98.5% hydrolyzed powder with a reported molecular weight of 31,000–50,000 g/mol. Flat bottom rigid plate 96-well standard polystyrene microplates (Sarstedt, USA) were used as plastic molds. Milli-Q deionized water was used in all aqueous solutions (18.0 MΩ).

PVA hybrids synthesis

PVA solution was prepared by fully dissolving 5.0 g of polymer powder without further purification in 100 mL of Milli-Q deionized water (18.0 MΩ), under magnetic stirring (avoiding foam formation), at temperature of 80 ± 2 °C. PVA solution (5.0 wt.%) was let to cool down to room temperature (25 °C) and the pH was corrected to 2.0 ± 0.1 with 1.0 M HCl.

Hybrids derived from poly(vinyl alcohol) (PVA) and organotrialkoxysilanes were synthesized via aqueous routes. Under steady stirring, 1.86 mL of the specific organosilane modifier reagent, TEOS (hydroxyl), MPTES (thiol), APTMS (amino), TESPI (isocyanate) or GPTMS (glycidyl) was gently added to 100 mL of previously prepared PVA acid solution (5 wt.%) at temperature of 25 ± 1 °C for hybrid network formation, resulting on [SiO_2/PVA] concentration of 10 wt.% (polymer modifier silane reagents structures are showed in Fig. 2). The pH is of crucial importance on coupling silanes to polymers. Hence, the pH was maintained constant pH = 2.0 ± 0.1 during the entire synthesis, driving both crosslinking and sol–gel processes to occur mostly though hydroxyls groups. The addition of organosilanes in small proportions (~1.8 mL) to PVA (100.0 mL) acid solution has not altered the pH.

Each PVA hybrid solution was poured into a 96-well polystyrene microplate, with volume of 200 μL/well, and allowed to solidify for 24–72 h. PVA hybrids crosslinked with glutaraldehyde (GA) were produced in a similar procedure, by mixing 20.0 mL of GA (25% aqueous solution) to 100 mL of PVA-silane solution (i.e., PVA/TEOS, PVA/MPTES, PVA/APTES, PVA/TESPI, or PVA/GPTMS) using steady stirring as described in the previous paragraph, with concentration of [GA/PVA] = of 1:1 (w/w). Then, PVA/GA hybrid solutions were cast into a 96-well polystyrene microplate, with 200 μL/well, where gelation and solidification have occurred (24–72 h) at room temperature (25 ± 2 °C).

After 5 days stored in closed dessicator, a volume of 10 μL of bovine herpesvirus solutions (BoHV) with virus concentration of 100 μg/mL were added to each polystyrene microplate well for herpesvirus immobilization to PVA hybrid network. Samples were incubated for 12 h at 25 °C and 24 h at 37 °C before immunoassay experiments and FTIR spectroscopy measurements.

PVA-derived hybrids and chemically crosslinked with GA have formed optically transparent disks that could be easily handled. All sample disks were accurately weighed before immunology assays. All tests were performed using at least triplicates. The complete process of PVA hybrid formation and chemical crosslinking is illustrated in Fig. 1. In Fig. 1a, the chemical structure of PVA polymer partially hydrolyzed with remaining acetate groups is showed followed by the crosslinking reaction with GA. In Fig. 1b are illustrated the major reactions of PVA with organotrialkoxysilanes for hybrid formation. Schematic drawings of chemically modified PVA hybrids produced are showed in Fig. 1b with their specific functional group, hydroxyl (–OH), glycidyl (GLY), amino (–NH_2), isocyanate (ISO), and thiol (–SH).

Characterization of PVA derived hybrids

Chemical characterization by FTIR spectroscopy

FTIR was used to characterize the presence of specific chemical groups in the PVA hybrids networks, reflecting the effectiveness of the developed procedure for producing different nanostructured materials. FTIR spectra were obtained within the range between 4,000 and 650 cm^{-1} (Perkin-Elmer, Paragon 1000), using Attenuated Total Reflectance method (ZnSe crystal, ATR-FTIR). Hybrids Samples were placed in a sampling handler and 64 scans were acquired at 2 cm^{-1} resolution with the subtraction of background. Transmittance FTIR spectrum was also obtained for PVA films cast in round glass molds used as reference. The incorporation of bovine herpesvirus (BoHV) within the PVA polymeric hybrid was also monitored by FTIR spectroscopy. Herpesvirus was incorporated by adding 100 μL of a solution with BoHV (antigen) concentration of 100 μg/mL in 5 mg of PVA hybrid sample (thin disk film) and characterized using ATR-FTIR. We would like to point out that FTIR spectra were not used as quantitative relationship but just as qualitative reference of virus adsorbed onto the hybrid network.

Synchrotron small-angle X-ray scattering characterization (SAXS)

The SAXS spectra of PVA films were performed using the SAS beam line of the National Synchrotron Light Laboratory (LNLS, Campinas, Brazil). This beam line is equipped with an asymmetrically cut and bent silicon (111) monochromator (λ = 1.608 Å), which yields a horizontally focused X-ray beam. A set of slits defines the beam vertically. A position sensitive X-ray detector (PSD) and a multichannel analyzer were used to determine the SAXS intensity, $I(q)$, as function of the modulus of the scattering vector q (Eq. (1)), being θ half the scattering angle.

$$q = \left(\frac{4\pi}{\lambda}\right) * \mathrm{sen}(\theta) \quad (1)$$

Each SAXS pattern corresponds to a data collection time of 900 s. From the experimental scattering intensity produced by all the studied samples the parasitic scattering intensity produced by the collimating slits was subtracted.

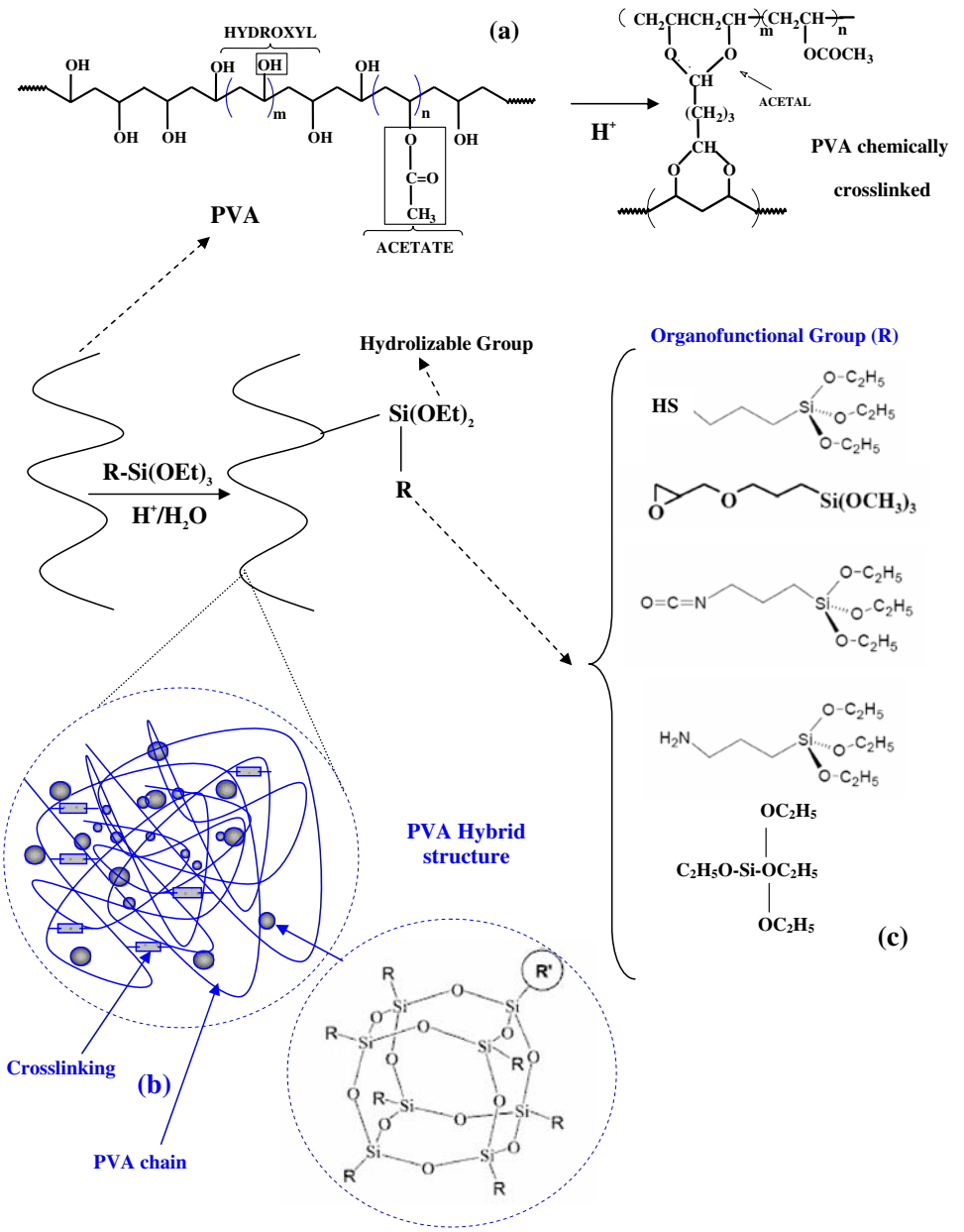

Fig. 1 Illustration of chemical reactions for the formation PVA hybrids; (**a**) PVA polymer partially hydrolyzed with remaining acetate groups with the chemical crosslinking reaction of PVA with GA; (**b**) hydrolysis reaction of silanes and schematic drawings of chemically modified PVA hybrids produced with their specific functional group, hydroxyl (–OH), glycidyl (GLY), isocyanate (O=C=N), amino ($–NH_2$) and thiol (–SH); (**c**) Chemical structures of organofunctional silanes (MPTES), (GMPTS), (TESPI), (APTES), (TEOS)

All SAXS patterns were corrected for the non-constant sensitivity of the PSD, for the time varying intensity of the direct synchrotron beam and for differences in sample thickness. Because of the mentioned normalization procedure, the SAXS intensity was determined for all samples in the same arbitrary units so that they can be directly compared [5, 16].

Characterization by scanning electron microscopy (SEM/EDX)

SEM images were taken from PVA-hybrid film surfaces with a JSM 6360LV (JEOL/NORAN) microscope coupled to Energy Dispersive Spectrometer (EDX) for semi-quantitative chemical analysis. Before examination, samples were coated with a thin gold film by sputtering using low deposition rate, cooling of substrate and maximum distance between target and sample in order to avoid sample damage. Images of secondary electrons (SE) and backscattered electrons (BSE) were obtained using an accelerating voltage of 10–15 kV.

Characterization of PVA-hybrids by immunoassay

The herpesviruses samples were kindly donated by Dr. P. M. Roehe (FEPAGRO/IPVDF, RS, Brazil). The effect of chemical modification of the designed hybrids on adhesion of herpesvirus proteins was measured comparing

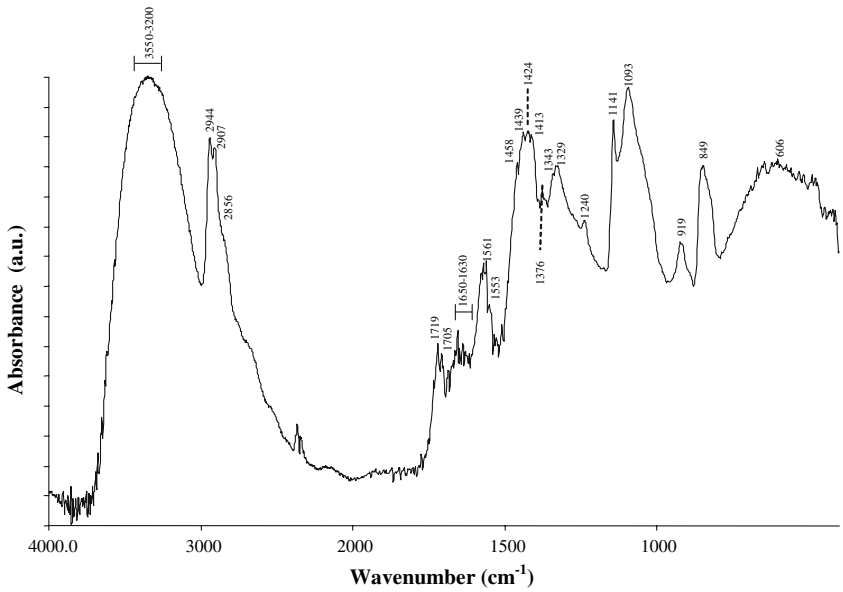

Fig. 2 FTIR spectrum of pure PVA polymer sample used as reference with major vibration bands assigned

ELISA performance of BoHV antigen detection by polyclonal antibodies (anti-BoHV-1) using commercial solid-phase. The immunoassay was conducted as reported in our previous work [23]. Briefly, commercially available polymeric microplates were used as reference in the immunoassay and compared with the new PVA-derived hybrids produced in this research. Hence, the bioassay was used to make possible the evaluation of specificity of interaction at the interface among the several organosilane modifiers used in the PVA network. All tests were performed using replicates (n = 4–20).

Results and discussion

Characterization of PVA derived hybrids

Chemical characterization by FTIR of PVA hybrids

In Fig. 2 is showed the FTIR spectrum of pure PVA with high degree of hydrolysis used as reference. In fact, PVA can be called a copolymer of vinyl alcohol and acetate, poly(vinyl alcohol-co-vinyl acetate) was schematically represented by a proportion in mol% "m" and "n," respectively (Fig. 1a). It must be said that it is an average of alcohol groups found in the polymer chain and the exact position and distribution of these moieties known as tacticity will deeply depend on the production process. PVA is usually a random copolymer (atactic) because it is made by free radical vinyl polymerization of the monomer vinyl acetate as PVA monomer is unstable. During this saponification, acid acetic radicals of poly(vinyl acetate) are gradually replaced by hydroxyl groups in alkaline medium. The hydrolysis reaction does not go to completion resulting in a copolymer poly (vinyl alcohol-co-vinyl acetate). It is important to note that degree of hydrolysis has an overall effect on PVA properties. Therefore, in the studied system, 98.5% hydrolyzed grade PVA would be "m = 98.5%" and n = 1.5%" also referenced as degree of hydrolysis = 98.5%. That would give a choice of amphiphilic interaction with biomacromolecules such as proteins. That means, the alcohol groups are hydrophilic and the acetate groups are hydrophobic. Thus, FTIR spectrum (Fig. 2) clearly reveals the major peaks associated with PVA. For instance, it can be observed C–H broad alkyl stretching band (v = 2,850–3,000 cm^{-1}) and typical strong hydroxyl bands for free alcohol (non-bonded –OH stretching band at v = 3,600–3,650 cm^{-1}), and hydrogen bonded band (v = 3,200–3,570 cm^{-1}) [16, 22]. Intramolecular and intermolecular hydrogen bondings are expected to occur among PVA chains due to high hydrophilic forces. An important absorption peak was verified at v = 1,141 cm^{-1}. This band has been used as an assessment tool of PVA structure because it is a semi-crystalline synthetic polymer able to form some domains depending on several process parameters [16, 22]. FTIR spectroscopy was used to characterize the PVA hybrid formation when reacting with different polymer modifier organotrialkoxysilanes, for instance TEOS (hydroxyl), MPTES (thiol), APTES (amino), TESPI (isocyanate), and GPTMS (glycidyl). The vibrational spectra offer a great number of information regarding to the chemical bonds, interactions due to weak and strong forces acting intra and inter-molecules. In Fig. 3 is showed the typical FTIR spectrum of PVA-derived hybrid that MPTES was used as one polymer modifier silane reagent. Among several peaks (–OH, $–CH_2$), this

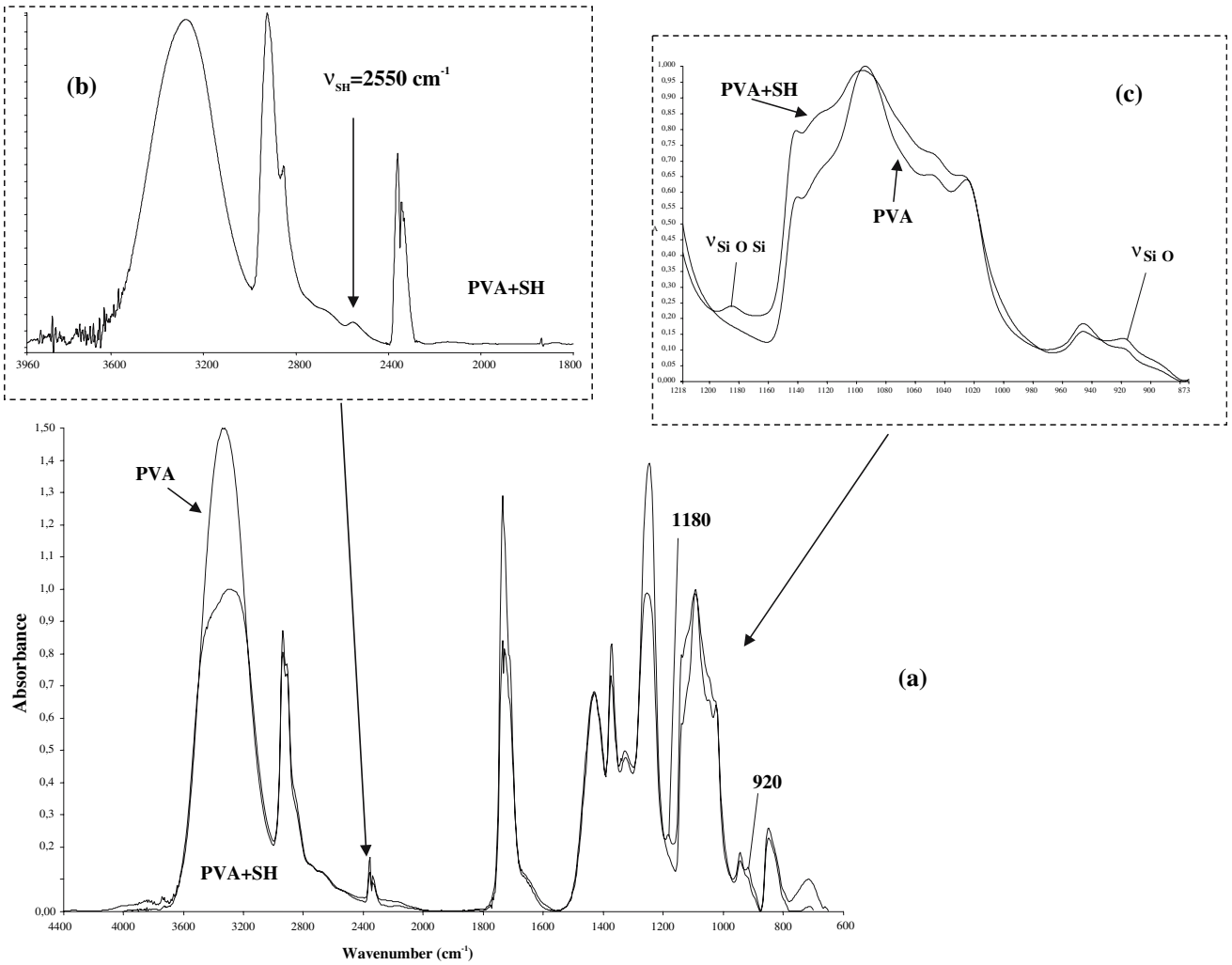

Fig. 3 (**a**) FTIR spectrum of pure PVA and PVA-derived hybrid with MPTES used as silane modifier by thiol group; (**b**) detailed region of main vibrational band associated with –SH group; (**c**) IR absorption region related to silicon bonding Si–O

spectrum (Fig. 3b) shows an important peak at 2,550 cm^{-1} that is associated to the thiol (S–H) vibration band [4, 20, 23, 29]. This functional group (–SH) is referred to as thiol, mercaptan, or as sulfhydryl group. It can be observed that major vibration bands (Si–O–Si, ν = 1,080 and 450 cm^{-1}; Si–OH, ν = 950 cm^{-1}) associated with polysiloxane (R–Si–O–) reactions of hydrolysis and condensation added to PVA polymer solution (Fig. 3c). Also, absorption peaks in the region from ν = 1,260 to 1,200 cm^{-1} can be attributed to silicon-alkyl bonds, indicating some hybrid organic–inorganic structure formation [25]. Furthermore, in the frequency range from 3,000 to 3,650 cm^{-1}, mainly related to hydroxyl groups [16, 30, 31] a broader band was noted for PVA/MPTES hybrid spectrum compared to pure PVA (Fig. 3). Such result is thought to be due to the organotrialkoxysilane sol–gel reactions (schemes in Fig. 1) that have altered PVA chains tri-dimensional structure. PVA molecular entanglements and crystallinity depend on hydrophilic/hydrophobic force balance. Hydrogen bonds play a crucial role in such conformational arrangements, creating hydrophically associated domains [16, 31–33]. Therefore, introducing of Si–OH and Si–O–Si through hydrolysis and condensation reactions of MPTES has modified PVA semi-crystalline structure. Similar results were obtained for PVA hybrids functionalized with other organosilane polymer network modifiers (Fig. 4). Most vibration bands associated with samples chemical functionalities such as PVA/APTES with amino at 3,300–3,500 cm^{-1} (N–H, C–N), PVA/TESPI with isocyanate at 2,100–2,270 cm^{-1} (–N=C=O), PVA/ GPTMS with glycidyl at 1,055–1,060 cm^{-1} (νC–O–C broad band) and at 1,250 cm^{-1} (epoxy ring [34]) were evidenced by FTIR spectra showed in Fig. 4a and b (detailed). The strong in-plane NH_2 scissoring absorptions at 1,550–1,650 cm^{-1}, and out-of-plane wagging at 650–900 cm^{-1} (broad) are usually characteristic of 1°-amines (PVA/APTES). In

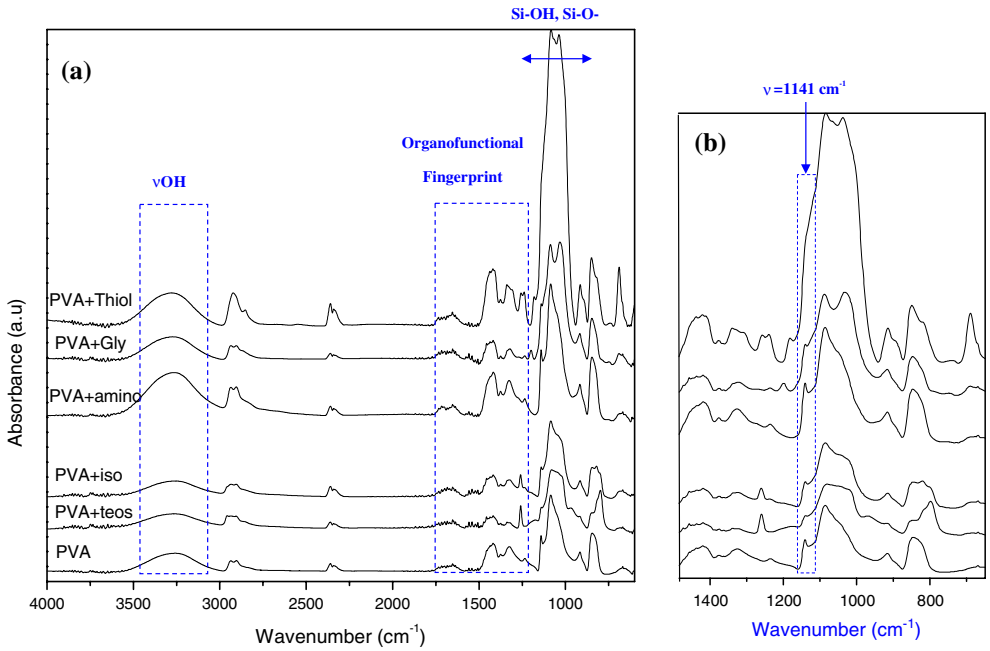

Fig. 4 FTIR spectra of PVA and PVA-derived hybrids modified by organosilanes functionalization; (**a**) Spectra of hybrids with functional groups: PVA + TEOS (hydroxyl), PVA + iso (isocyanate), PVA + Amino, PVA + Gly (glycidyl), PVA + thiol; (**b**) detailed region of vibrational spectra

addition, it can be observed the contribution of silicon groups in the region ranging mostly from 900 to 1,100 cm^{-1} (Si–O–Si, Si–OH). The interpretation is not direct because these bands are affected by chemical environment and other groups. For PVA-derived hybrids peak due to C–O stretching movements in PVA at about 1,040 cm^{-1} interferes with asymmetric Si–O–Si stretching and causes shift towards the higher value [35]. Also, it has been reported that the silanol absorption band at 950–920 cm^{-1} in PVA hybrids interferes with C–O–C symmetric stretching [35]. Aiming to proceed with further modifications on the PVA and PVA-derived hybrids networks we have used glutaraldehyde (1,5-pentadial, GA). GA has acted as inter- and intra-band crosslinker among PVA chains and an organic–inorganic covalent binder as showed in a typical FTIR spectrum of PVA/MPTES/GA in Fig. 5. Major proposed chemical reactions were summarized in Fig. 1a and b (schematic) involving both hydroxyl functional groups from silanes and from PVA. It should be said that GA was chosen as crosslinker due to its well-known excellent activity as chemical reagent that is very effective on crosslinking through hydroxyls and amine groups, mostly

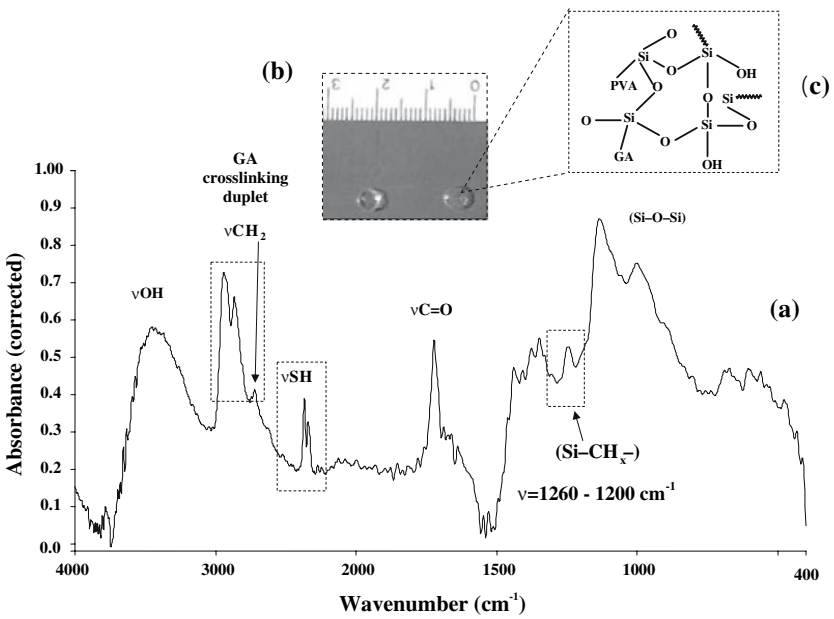

Fig. 5 (**a**) FTIR spectrum of PVA hybrid modified by thiol functionalization followed by chemical crosslinking with GA (PVA/MPTES/GA); (**b**) photograph of some hybrids produced; (**c**) Insert: schematic representation of hybrid nanostructure based on PVA, silane and GA crosslinking

depending on pH [20–23]. The chemical crosslinking which occurs via covalent bonds in the large majority of the cases reduces the polymer chain mobility and flexibility. Hence, the "hardening" effect is caused by the reduction on possible chain conformation and restrained movement associated with these new covalent bonds. That is the case in the present research, where hydroxyls groups from PVA were chemically crosslinked with GA, where hemi-acetals and acetals bridges were formed by the mechanism of intra and inter-chain bonds in the PVA (Fig. 1). It is expected that the relative amounts of hydroxyls are reduced by crosslinking. However, the interaction between protein and solid phase, in other words, the PVA-based hybrids, is rather complex and has to be addressed as a result of the overall balance of forces acting at the same time, i.e., hydrophilic, hydrophobic, electrostatic, and steric hindrance [20–23]. In order to establish crosslinking, some physical–chemical conditions have to be applied, for instance reactions occurring in low pH solution, where Schiff bases are formed [16, 36]. PVA crosslinking reaction with GA can be observed by two important peaks at ν = 2,860 and 2,730 cm^{-1} of C–H stretching are related to aldehydes, a duplet absorption with peaks attributed to the alkyl chain [16]. PVA-derived hybrids i.e., PVA/TEO, PVA/MPTES, PVA/APTES, PVA/TESPI and PVA/GPTMS, and chemically crosslinked with GA have formed optically transparent disks (slight yellowish color) of 10 ± 2 mg with an average diameter of 5 mm as showed in Fig. 5b. The representation of (O–I) hybrid structure is illustrated in Fig. 6c. Hence, FTIR spectroscopy has given strong evidence that the experimental procedure developed in this work was successful in obtaining and altering the organic–inorganic materials based on PVA and chemically crosslinked with aldehyde promoting the formation of nanocomposites with hybrid (O–I) structures.

The typical FTIR spectrum of lyophilized sample of bovine herpesvirus (type BoHV-1) is showed in Fig. 6a. The peaks associated with amide-I (1,620–1,680 cm^{-1}) were observed on the spectrum of pure BoHV-1.1 used as reference (Fig. 6a). The herpesvirus, like most of living organisms, has several proteins as building block of its structure as schematically illustrated in Fig. 6b. As a consequence, proteins vibrational bands were expected to give strong contribution to the FTIR spectroscopy analysis. In fact, based on the literature [37–39] the peptide group, the structural repeat unit of proteins, has 9 characteristic bands named amide (A, B, I, II, ... VII). Amide I and amide II bands are two major bands of the protein infrared spectrum. The amide I band (ranging from 1,600 to 1,700 cm^{-1}) is mainly associated with the C–O stretching vibration (70–85%) and is directly related to the backbone conformation. Amide II results from the N–H bending vibration (40–60%) and from the C–N stretching vibration (18–40%) [37]. The amide III band is usually weak in the FTIR spectroscopy but can be found in the region from 1,250 to 1,350 cm^{-1}. The FTIR spectrum in Fig. 7 shows the results of the PVA network after herpesvirus (BoHV, protein) incorporation, where all major important amide stretching vibration bands were present typically from 1,640 to 1,200 cm^{-1}. It should be emphasized that the vibrational band associated with the strong absorption of amide-I is clearly verified in Fig. 7 (insert). Hydrogen bonded shifts some of the protein absorptions, as well as the prominent N–H stretching absorptions (ν = 3,170–3,500 cm^{-1}). In addition to that, the broadening effect noted in the infrared

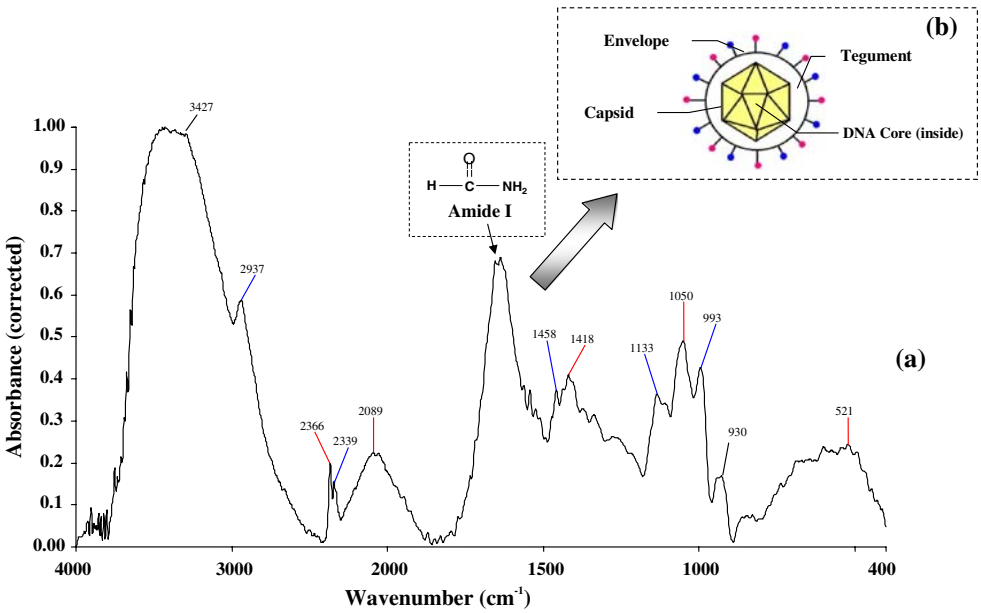

Fig. 6 (**a**) FTIR spectrum of Herpesvirus purified and characterized. (**b**) Schematic representation herpesvirus structure

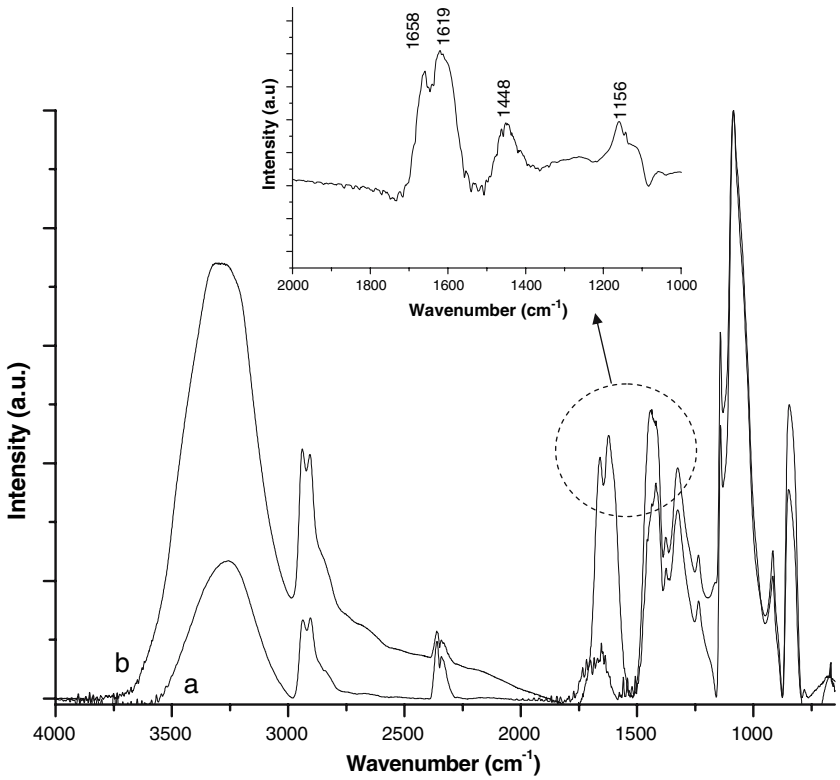

Fig. 7 FTIR spectra of PVA (**a**) and PVA with herpesvirus BoHV incorporated (**b**). Insert: Major absorption region associated with amides present in proteins

region from v = 2,800 to 3,500 cm^{-1} are thought to the correlated to the presence of exposed herpesvirus glycoproteins which have given strong contribution from amines (–NH) and organic cyclic rings, some aromatic residues, adding to the absorption of the alkyl (–CH_x) peaks generally found on saturated carbon chain (v = 2,850–3,000 cm^{-1}) as previously observed in PVA spectrum (Fig. 2). Also, an important increase on the IR absorption for PVA with herpesvirus (Fig. 7b) could be verified above 2,550 cm^{-1} associated with the cysteine groups present on the virus proteins (–SH). Similar FTIR spectra were obtained for PVA-derived silanes with different chemical functionalities (data not showed). Consequently, we have confirmed the incorporation of herpesvirus in the PVA and PVA hybrid network by using FTIR spectroscopy.

Characterization of PVA hybrids by X-ray scattering

In general, X-ray scattering has been extensively used as a technique for the investigation of crystallinity of materials. Wide-angle X-ray scattering (WAXS, also called X-ray diffraction) is powerful tool on understanding crystalline phases, crystal orientations, planes, unit cell structure On the other hand, SAXS is very important to get information from materials structure at the nanometer scale, typically varying from few nanometers to 100 nm. Synchrotron SAXS curves for pure PVA hydrogel and PVA chemically crosslinked are presented in Fig. 8. SAXS results from PVA films have showed a single peak with a maximum located at scattering vector (q) 0.054 up to 0.057 Å^{-1}. Such trend can be explained by assuming a semi-crystalline structure of PVA polymer sample previously described. For the model of isolated domains (crystallites) embedded in a continuous matrix, the average distance between domains, δ, can be estimated by using the equation $\delta = 2\pi/q_{max}$, where q_{max} is the modulus of the scattering vector corresponding to the maximum of the SAXS intensity function [5, 16]. Based on this equation, we would have average distance of 12 nm among PVA nanocrystallites. Considering the conditions of films preparation, that is,

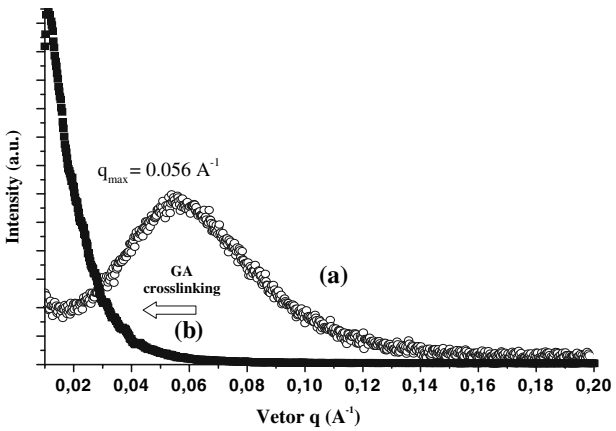

Fig. 8 SAXS curves from (**a**) pure PVA polymer and (**b**) PVA/GA crosslinked network

PVA crystallized from a dilute solution (5.0 wt.%), one can expected that polymer will form lamellar crystals. These lamellar structures are plate-like with the polymer molecules folded upon themselves resulting in parallel chains perpendicular to the face of the crystal. These crystals are connected to the amorphous regions by polymer chains [16]. On the other hand, SAXS curve for PVA/GA crosslinked hydrogels (Fig. 8b) reveals the absence of the observed peak for pure PVA. That is associated with the chemical crosslinking of polymer chain by GA, as verified by FTIR spectroscopy. Thus, it is reasonable to assume that a significant reduction on crystallization due the presence of the crosslinking bridges restraining PVA chains mobility. Moreover, as the dialdehyde crosslinking mechanism is related to the reaction with hydroxyl groups of PVA forming acetal bridges among PVA chains, it is most likely to reduce the formation of hydrogen bonds and therefore, the driving force for crystallization is also weakened [23].

SAXS curves of pure PVA and PVA hybrids modified by organosilanes are showed in Fig. 9. In Fig. 9a, the scattering results for pure PVA, and hybrids PVA/MPTES, PVA/GPTMS and PVA/TEOS are represented by curves (1)–(4), respectively. All curves have indicated the formation of a semi-crystalline structure, with a typical maximum on vector q and small shifts from that maximum was verified. Despite they all shared the same trend on the scattering vector one exception was clearly noted on the curve-3 (Fig. 9a) related to PVA/GPTMS. It has showed a much broader peak and also a quite different scattering pattern. Such effect can be attributed to the presence of the glycidyl chemical moieties on the hybrid, which may have caused steric hindrance on the PVA chain 3-D conformation and therefore altering the formation semi-crystalline domains.

In Fig. 9b (curve-2 in insert), it can be clearly observed a shift to lower vector q values (Δq~0.008 A^{-1}) when MPTES was added to PVA matrix. In other words, the nanostructure of domains was altered by increasing the value of "d" spacing of about 10% (1.5 nm) among PVA crystalline domains. That means, the formation of PVA hybrid with the presence of thiol ended propyl group regions has distorted the uniform distribution among amorphous and crystalline regions verified on pure PVA samples (Fig. 9b, curve-1). A similar effect was observed for the addition of other silane modifier reagents to the PVA network. Thus, SAXS results have supported the proposed system of obtaining PVA structure modified at the nanoscale order by hydroxyl, thiol, amino and glycidyl organosilane reagents and chemically crosslinked with GA.

Characterization by scanning electron microscopy (SEM/EDX)

SEM/EDX analysis was conducted in order to evaluate the microstructure, morphology, and chemical composition of PVA hybrids films. In Fig. 10 is showed some examples of photomicrographs of PVA (Fig. 10a), PVA/TEOS (Fig. 10b) and PVA/GPMS (Fig. 10c) hybrids. It can be noted that besides some "fringes" on the surface morphology the produced samples, which we think is related to a shrinking of the film during the drying procedure, no

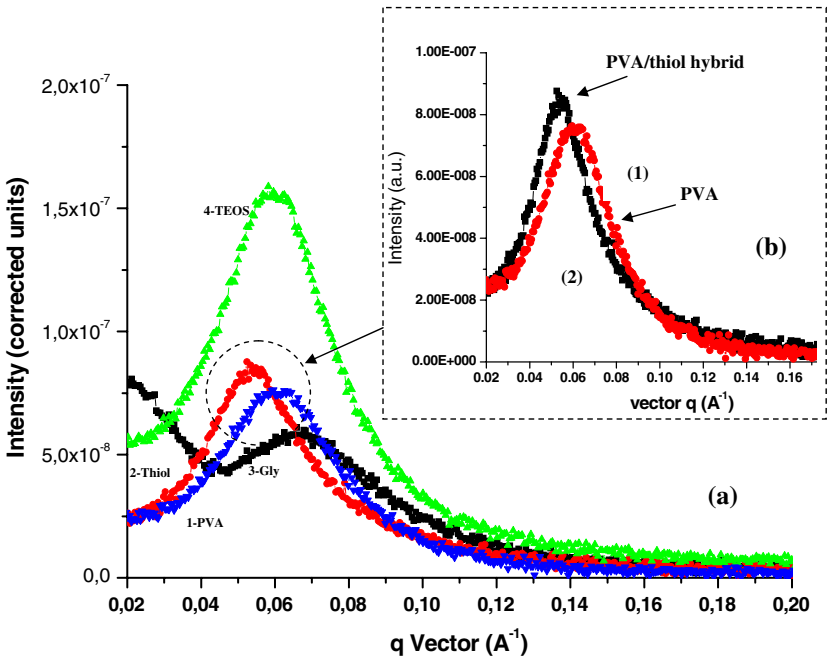

Fig. 9 SAXS patterns of PVA chemically modified by organosilanes; reference of pure PVA (1), and PVA hybrid PVA/MPTES (2-thiol), PVA/GPTMS (3-Gly), PVA/TEOS (4-Teos); Detail: vector q shift of PVA-thiol compared to pure PVA

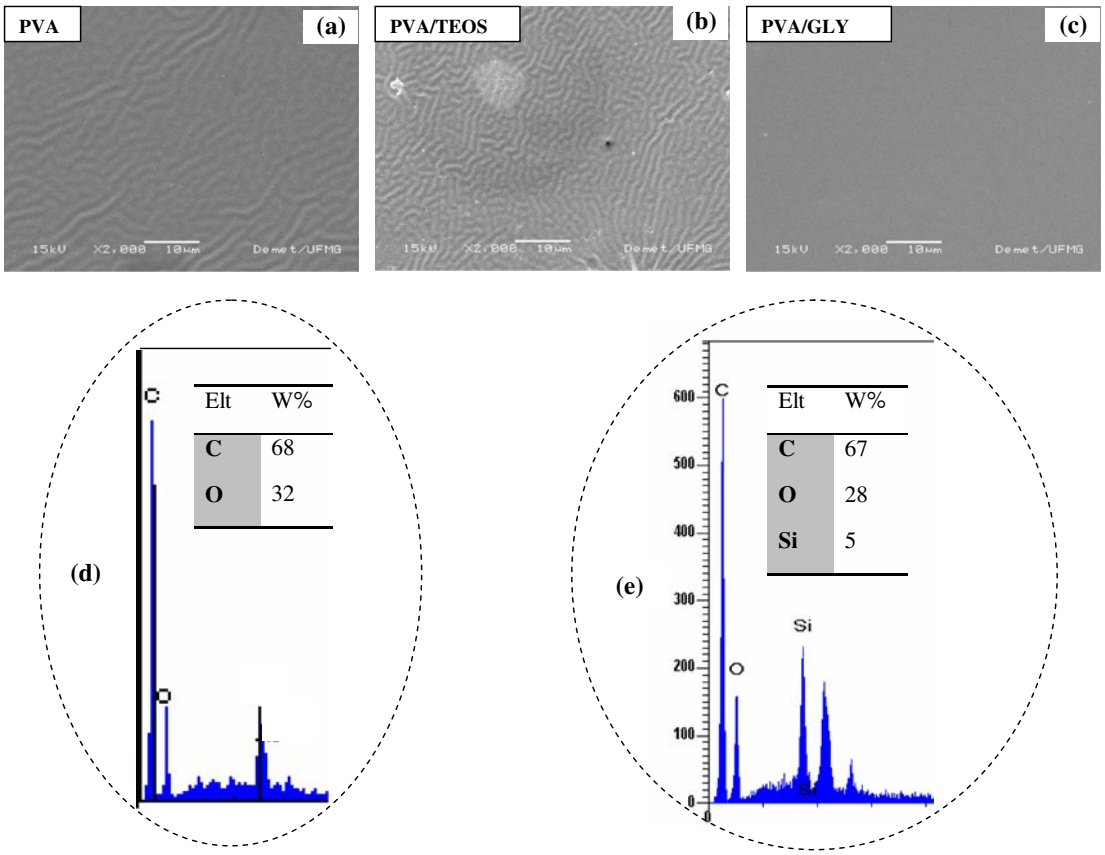

Fig. 10 SEM/EDX analysis of PVA hybrids films; Photomicrographs of PVA (**a**), PVA/TEOS (**b**), and PVA/GPMS (**c**) hybrids; EDX chemical analysis of PVA (**d**) and PVA-hybrids (**e**)

phase separation or segregations was detected. In fact, that was endorsed by EDX chemical composition, which has indicated uniform composition in all randomly selected areas under evaluation. The typical results of EDX chemical analysis are showed in Fig. 10d (PVA) and Fig. 10e (PVA-hybrids) where there was an important difference between them associated with the silicon concentration that was not present in pure PVA samples. It should be pointed out that beyond SEM resolution used in this study, for instance in the nanometer range, the PVA-derived hybrid composites may have some undetected degree of phase segregation forming domains. Although, it is unlikely that has occurred in our system. That would require complementary investigations such as TEM and AFM to be conducted. On the other hand, PVA hybrids derived from amino organosilane has indeed presented strong evidence of phase separation as it can be seen in Fig. 11. Dark dots scattered in the light gray matrix were observed for PVA/APTES samples. The EDX chemical analyses have indicated one phase richer in silicon content (Fig. 11c) associated with the "dark dots" than the other phase (Fig. 11b) related to the matrix. This result can be explained by the high kinetics of sol–gel reaction with amino group present, which may have caused the creation of silicate domains before that the actual reaction with hydroxyls groups from PVA has taken place. No similar segregation or any phase separation could be observed for the other organosilane modifiers of PVA network.

Immunoassay of PVA modified hybrids

The effect of chemical modification of the plates on adhesion of proteins was measured comparing ELISA performance of herpesvirus antigens detection by polyclonal antibodies using Falcon commercial solid support and PVA based hybrids. The solid-phase sample as pure PVA was not suitable for protein immobilization due to its solubility in water based solutions used in all biological experiments and as a consequence they must be necessarily chemically crosslinked to produce a stable network and undergo immunological assays. Tests were done with at least 4 replicates (n = 4–20). Figures 12 and 13 showed the treatments evaluated. The results in the histogram (Fig. 12) clearly showed that thiol-modified PVA hybrids have presented the best selectivity and specificity mechanism of interaction with bovine herpesvirus-1 (BoHV-1) when compared to all other chemically functionalized solid phases. Also, it has performed much better when compared

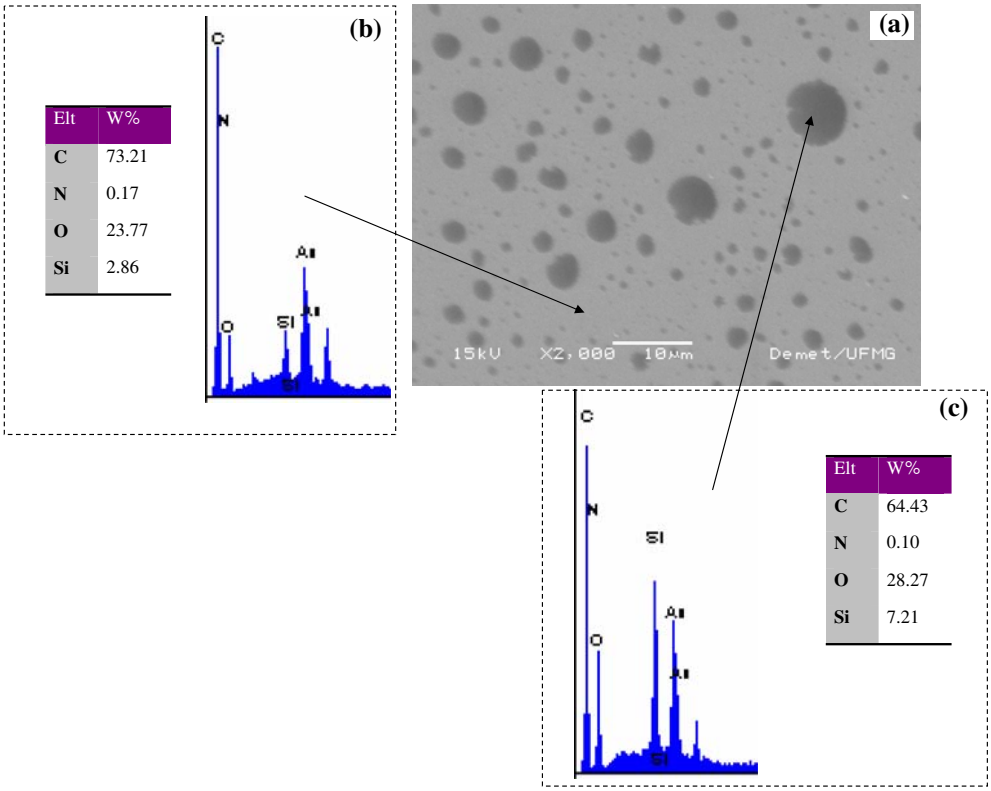

Fig. 11 Photomicrograph of PVA hybrids derived from amino organosilane (PVA/APTES) (**a**); EDX chemical analyses of "light gray" related to the matrix (**b**) and "dark dots" phase (**c**)

to the commercially available material (Fig. 12, Reference). The interaction between protein and solid phase is a rather complex mechanism and has to be addressed as a result of the overall balance of forces acting at the same time, i.e., hydrophilic, hydrophobic, electrostatic, and steric hindrance. That means, by reducing the number of hydroxyls (hydrophilic) the interaction of PVA hybrid is actually enhanced by hydrophobic stabilization (acetates) with proteins. Nevertheless, it is important to point out that despite of the unquestionable high effectiveness observed for thiol-modified hybrids the large majority of the chemical moieties have also performed as suitable solid-phase

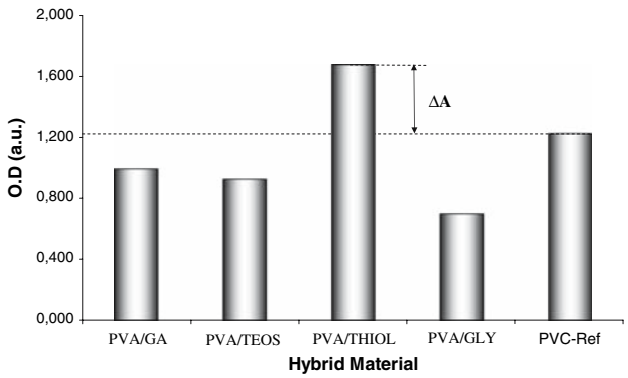

Fig. 12 Histogram of ELISA immunoassay for herpesvirus with different chemically modified hybrids of PVA/silanes; Thiol, hydroxyl, glycidyl, PVA + GA crosslinked and PVC-Ref (control); UV–Vis spectroscopy absorbance at $\lambda = 492$ nm

for the protein immobilization. However, they were less effective when compared to standard commercial kits because these are high binding surfaces made of polystyrene, which have outstanding behavior on protein immobilization but lack of specificity (Fig. 12). We may assume that such predominant effect of thiol-modified hybrid materials is related to the important reduction of the non-specific adsorption of antigens onto the solid-phase. We propose a mechanism of interaction between the hybrid PVA modified by thiol with the glycoproteins on the herpesvirus envelope. More specifically, it is likely to be related to the cysteine amino acid which has a thiol group and is found in most proteins, such as herpesvirus glycoproteins. Cysteine is a naturally occurring hydrophobic amino acid which contains a highly nucleophilic thiol group, and one of its primary purposes is to act as a nucleophilic catalyst. A disulfide bond (SS-bond), also called a disulfide bridge, is a strong covalent bond between two sulfhydryl (–SH) groups. It is reasonable to accept that thiol–thiol interaction would have taken place among PVA based hybrid thiol-modified samples with the cysteine residues from the herpesvirus glycoproteins, reducing random and non-specific adsorption, resulting on an significant increase of sensitivity of the ELISA immunoassay for BoHV (Fig. 12). The origins of the interactions between protein and substrate are found to be due to Coulomb forces, van der Waals forces, Lewis acid-base forces, and more entropically based effects such as hydrophobic interactions, conformational entropy and restricted mobility. It is difficult

to determine exactly the extent of these interactions and even more difficult to predict them. At the molecular level, proteins are chemically and physically extremely complex molecules, and the nature of the physical–chemical interactions taking place is implicit widespread. However, based on the immunoassay results, we may assume that the thiol-functionalized hybrid has favored the orientation of herpesvirus proteins when compared to other chemically modified PVA-hybrids. Despite the vast array of potential interactions that usually exist between proteins and surfaces, a general rule has been formulated to predict the outcome. Proteins will have a higher affinity for a surface with thiol or amino groups due to potential aminoacid strong interactions. This simplified view often works well for well-defined model proteins and surfaces. Nevertheless, for more complex systems where the heterogeneous nature of surfaces complicates interpretation, some other important aspects have to taken into account.

In order to move a step further on investigating the mechanism proposed for the thiol–thiol interaction, we have produced hybrids of PVA with thiol-modified groups and also chemically crosslinking with GA during the hybrids preparation. These results are presented in Fig. 13. It can be seen the high reduction of antigen binding to the solid phase caused by the chemical crosslinking with GA. As a consequence, such effect was attributed to the limited degree of mobility of PVA chains due to covalent bond among them, altering the tree-dimensional conformation and, as a consequence, reducing the number of thiol sites for the disulfide bridging with virus envelope. Thus, a decrease of the exposure of binding epitopes from the virus envelope is expected suggesting lower specificity for ELISA recognition assay.

In summary, the thiol-functionalized PVA hybrid has given some evidence that it interacts preferably with the cysteine residues of virus envelope, reducing the non-specific adsorption of antigen, most likely to drive the formation of stable disulfide bridging (scheme Fig. 13b). Therefore, if one aims at suppressing or minimizing non-specific adsorption of herpesvirus and solid-phases cysteine residues may play a decisive role on the interacting system [40, 41]. Further investigation regarding to the specificity of such interactions must be conducted. In fact, recombinant protein, specially designed for that purpose and FT-Raman spectroscopy for the evaluation of disulfide bond formation are currently under development.

Conclusion

FTIR spectroscopy was found to be an important technique on characterizing the PVA-derived hybrid formation when reacting with different polymer modifier organotrialkoxysilanes. In addition, SAXS and SEM/EDX results have given support for the proposed formation of hybrid system based on PVA network structure modified at the nanoscale order by both silane coupling agents and chemical crosslinking. We have successfully designed and developed novel hybrid organic–inorganic materials based on PVA and silane modifiers to be used in bovine herpesvirus immunoassay. In summary, the distribution of chemical entities in the obtained hybrid, for instance hydroxyl, thiol, glycidyl, and amino would give unlimited possibilities to tailor the nanostructure of producing hybrid based on PVA-Silane for biomolecules interactions. Also, the characterization techniques used complementary in this study have

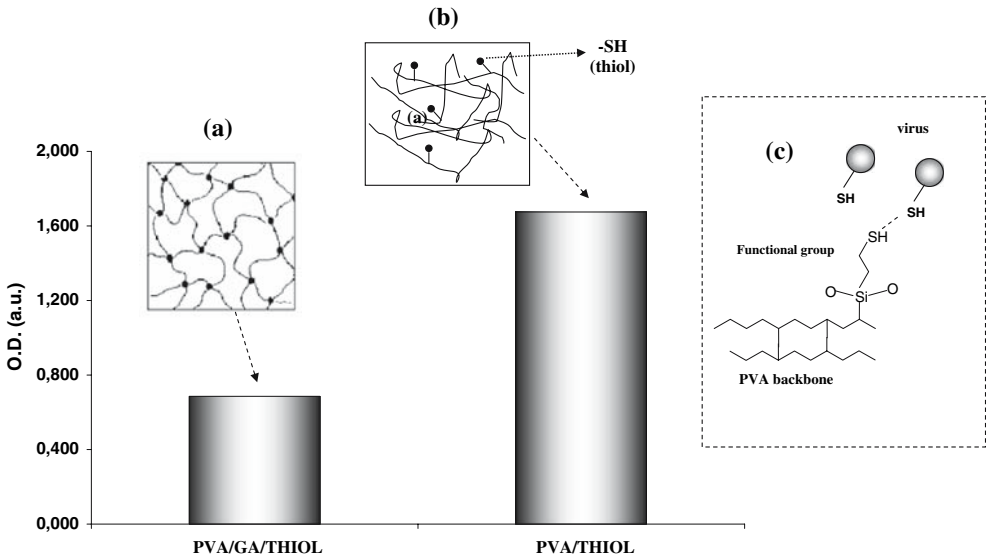

Fig. 13 Histogram of ELISA immunoassay for herpesvirus with thiol-modified hybrids of PVA/MPTES compared to PVA/MPTES/GA chemically crosslinked; insert: (**a**) no GA crosslinking; (**b**) reduction of mobility for PVA polymer chains with GA crosslinking due to covalent bonds. UV–Vis spectroscopy absorbance at λ = 492 nm. (**c**) Schematic representation of the overall system associated with hybrid PVA modified by silane moieties and herpesvirus

proven to be powerfully tools on examining micro- and nanostructures and interaction of hybrids (O–I) with biomolecules at interfaces.

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