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Synthesis and Characterization of Poly (Vinyl Alcohol) Hydrogels and Hybrids for rMPB70 Protein Adsorption

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Polyvinyl alcohol (PVA), PVA crosslinked with glutaraldehyde hydrogels (PVA/GA), PVA with tetraethylorthosilicate (PVA/TEOS) and PVA/GA/TEOS hybrids with recombinant MPB70 protein (rMPB70) incorporated were chemically characterized by Fourier transform infrared spectroscopy (FTIR). FTIR spectra of PVA hydrogel samples showed the absorption regions of the specific chemical groups associated with poly(vinyl alcohol) (–OH, –CO, $–CH_2$) and PVA/GA confirming the formation of crosslinked hydrogel (duplet –CH). It was observed C–H broad alkyl stretching band (ν = 2850-3000 cm^{-1}) and typical strong hydroxyl bands for free alcohol (nonbonded –OH stretching band at ν = 3600-3650 cm^{-1}), and hydrogen bonded band (ν = 3200-3570 cm^{-1}). The most important vibration bands related to silane alcoxides have been verified on FTIR spectra of PVA/TEOS and PVA/GA/TEOS hybrids (Si–O–Si, ν = 1080 and ν = 450 cm^{-1}; Si–OH, ν = 950 cm^{-1}). FTIR spectra of f PVA hydrogel with rMPB70 incorporated have indicated the specific groups usually found in protein structures, such as amides I, II and III, at 1680-1620 cm^{-1}, 1580-1480 cm^{-1} and 1246 cm^{-1}, respectively. These results have given strong evidence that recombinant protein rMPB70 was successfully adsorbed in the hydrogels and hybrids networks. These PVA based hydrogels and hybrids were further used in immunological assays (Enzyme-Linked Immunosorbent Assay – ELISA). Tests were performed to detect antibodies against rMPB70 protein in serum samples from bovines that were positive in the tuberculin test. Corresponding tests were carried out without PVA samples in microtiter plates as control. Similar results were found for commercially available microplates and PVA based hydrogels and hybrids developed in the present work regarding to immunoassay sensitivity and specificity response.

Keywords: *PVA hydrogel, protein, FTIR, spectroscopy characterization*

1. Introduction

The biomaterials and Bioengineering research field have broadened in the last 3 decades, including replacement of diseased or damaged parts, assist in healing, correct and improve functional abnormality, drug delivery systems, immunological kits and biosensors[1-7]. Hence, there is a potential to generate a series of products from biological reactors to diagnostic assays. New biosensors have been constructed and techniques of immobilization have been developed in parallel with the intention to stabilize and incorporate biomolecules to their surface[8].

Synthetic polymers have been widely used in biosensors including poly (acrylamide) and hydrogels in polyurethane and poly(vinyl alcohol) solid supports. The hydrogels most commonly known are poly(2-hydroxyethyl metacrylate) (PHEMA), PVA, poly(N-vinyl-2-pyrrolidone (PNVP), poly (ethylene glycol) (PEG), and their copolymers and their structures can be controlled by physical and chemical crosslinking of chains[9].

PVA is a synthetic water-soluble hydrophilic polymer. The basic properties of PVA are dependent on the degree of polymerization or on the degree of hydrolysis. It has been widely used in adhesives, emulsificants, in the textile and paper industry applications and in the attainment of amphiphilic membranes for enzyme immobilization[6]. Most recently, PVA has been used in pharmaceutical and biomedical applications for controlled drug release tests due to its degradable and non-toxic properties[9]. Chemical crosslinking is a highly versatile method to create and modify polymers, where properties can be improved, such as mechanical, thermal and chemical stability[6,7]. Also, a novel class of materials called organic-inorganic hybrids would combine properties of organic polymers with ceramics. Hybrids would combine properties of organic polymers with ceramics. 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. They are also

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termed as 'ceramers' and 'ormosils' (organically modified silicates) or 'ormocers' (organically modified ceramics), which are normally nanocomposites[4,6,7].

New materials are being developed as supports for protein immobilization with the aim of increasing sensitivity, as well as lowering costs and offering different alternatives for diagnostic test. The direct adsorption of proteins in polymer (polyvinyl chloride and polystyrene) microplates is widely used in imunoenzymatic assays such as Enzyme-Linked Immunosorbent Assay (ELISA), whose simplicity and automatization make it an important procedure widely used clinical assays. The intradermic tuberculin test is considered to be the international standard method for the diagnostic of bovine tuberculosis. This test is based on a delayed-type hypersensitivity reaction using bovine tuberculin – PPD (purified protein derivatives of *Mycobacterium bovis*)[10]. Occurrence of false-negative reactions mainly due to anergic animals, the need of a second visit to the farm 72 hours later, a high number of doubtful reactions and a delay from 60 to 90 day between tests to confirm the diagnosis are the most significant problems related to this method. Thus, considerable efforts have been directed to develop serologic assays as alternative tests for the diagnosis of bovine tuberculosis[11].

The advent of genetic engineering made available recombinant antigens easily purified by chromatographic systems. Several recombinant antigens have been investigated in the search for species-specific antigens to be used in TB serologic tests. MPB70 protein is a promising antigen to be used in the diagnosis of bovine tuberculosis. Recombinant MPB70 or just rMPB70 is an immunodominant antigen of *M. bovis* that contains epitopes with a high degree of species- specificity[12-15]. Figures 1a and 1b show *M. bovis* bacteria colony and the MPB70 protein structure model, respectively.

The aim of this study was the development and characterization of novel hydrogels and hybrid materials based on PVA to test their application in a serological test like ELISA immunoassay and future potential for manufacturing biosensors.

2. Material and Methods

2.1. Synthesis of hydrogels and hybrids with a polymeric base of PVA

Tetraethoxysilane $Si(OC_2H_5)_4$ (TEOS, > 99%) and glutaraldehyde (GA, 25% aqueous solution) were supplied by Sigma-Aldrich. 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.

For the synthesis of the polymeric hydrogels and hybrids, PVA with an average molecular weight of 72.000 g/mol (hydrolysis > 90%, CRQ, Brazil) was used. PVA polymer solution was prepared by dissolving 5% PVA (wt. (%)) in Milli-Q water (> 18.MΩ) and vigorously stirred at 60 °C, using a magnetic stirrer. After total dissolution of the polymer, pH was corrected to 2.0 ± 0.2 with HCl 1N solution. PVA solution was used for the synthesis of PVA/GA hydrogel, PVA-TEOS and PVA-GA-TEOS hybrids and part was reserved as stock solution. PVA samples were crosslinked by using glutaraldehyde with concentration of 1.0 mol% to PVA. For each sample, 100 µL of their respective solution was poured into 96-well polystyrene cell culture microplates (SARSTEDT, USA). Chemical crosslinking reaction was achieved by conditioning PVA hydrogels with GA for 24 hours at room temperature followed by 1 week in vacuum desiccator, and subsequently dried in oven for 24 hours at 60 °C. Hybrids derived from PVA and TEOS were synthesized via aqueous route. Under steady stirring, 5.0 mL of TEOS was gently added to previously prepared PVA acid solution at temperature of 25 °C ± 1 °C. PVA/TEOS solution was poured into a 96-well microplate and allowed to solidify for 24-72 hours. Crosslinked hybrids were prepared by mixing 20.0 mL of PVA/TEOS aqueous solution with 5.0 mL of GA. The procedure was conducted under moderated stirring at temperature of 25 °C ± 1 °C.

PVA hydrogels and PVA-TEOS hybrids had the appearance of optically transparent films. PVA-GA hydrogels and PVA-GA-TEOS

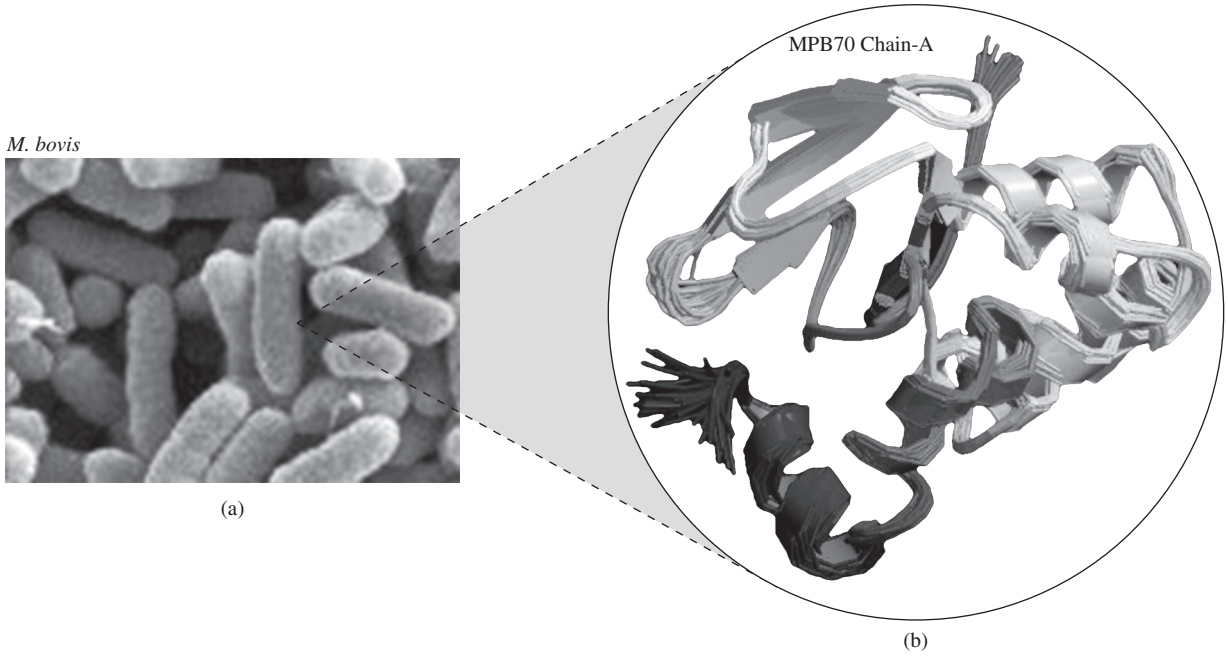

Figure 1. *Mycobacterium tuberculosis*; Solution Structure of the Antigenic Tb Protein MPT70/MPB70; Authors: M. J. Bloemink, E. Dentten, R. G. Hewinson, R. A. Williamson, M. D. Carr; Exp. Method: NMR, Polymer Chains: A; Residues: 163; Source: The RCSB Protein Data Bank.

hybrids formed disks of 10-20 mg with an average diameter of 5 mm that could be easily handled. Chemical crosslinking reaction of PVA and TEOS alcoxide hydrolysis reaction are showed in Figures 2a and 2b, respectively.

2.2. Chemical characterization of PVA hydrogels and hybrids by FTIR

Fourier transform infrared spectroscopy (FTIR) was used to characterize the presence of specific chemical groups in the materials. PVA hydrogels, PVA crosslinked with GA (PVA/GA) and PVA derived hybrids (PVA/GA/TEOS, PVA/TEOS) were milled and mixed in a ratio of 1.0% (wt. (%)) to KBr powder dried for 24 hours at 120 °C. FTIR spectra were obtained in the range of 4000-400 cm^{-1} during 64 scans, with 2 cm^{-1} resolution, using diffuse reflectance mode (Paragon 1000, Perkin-Elmer, USA). Transmittance FTIR spectrum was also obtained for PVA films cast in round glass molds. The incorporation of rMPB70 protein within the PVA polymeric hydrogels was also monitored by FTIR spectroscopy. We would like to point out that FTIR spectra were used as a qualitative reference of protein adsorbed into the hydrogel network.

2.3. Adsorption of rMPB70 in synthesized PVA hydrogels and hybrids

The adsorption of rMPB70 protein was carried out after all the samples were chemically characterized. The polymeric base, the PVA/GA hydrogels and the PVA/GA/TEOS and PVA/TEOS hybrids were individually immersed in a 100 µL solution containing 5 µg of rMPB70 in "Tris buffer" (Tris(hydroxymethyl) methylamine, pH 9.0, Merck, Germany) for 1 hour at room temperature and then for 2 hours at 42 °C. After that, FTIR analysis was carried out as previously described in section 2.2.

2.4. Detection of anti-MPB70 antibodies in immunological ELISA assay

96-well cell culture microplates (Corning Incorporation Life Sciences, USA) were used as solid support and mold. The microplates were previously treated with HNO_3 aqueous solution (20% v/v) for 72 hours, washed 10 times in running water, three times in Milli-Q water (> 18.0.MΩ), and subsequently dried for 2 hours at 37 °C. These surface prepared microplates were used as support to the samples during the ELISA immunoassay. PVA/GA and PVA/GA/TEOS hybrids disks have been moved from microplate well at each step of the immunoassay. On the other hand, PVA hydrogel and hybrids PVA/TEOS were kept in the same microplate as thin adherent films difficult to move without damaging throughout the ELISA procedure.

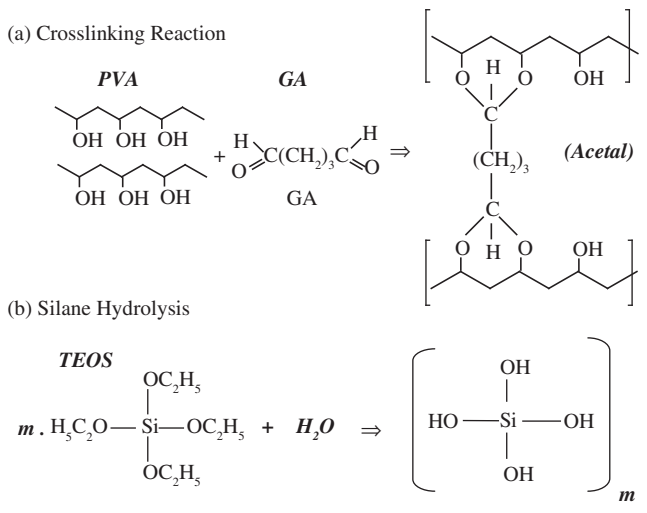

Figure 2. Chemical structure of reagents and schematic representation of reactions involved; a) PVA and glutaraldehyde crosslinking; and b) TEOS alcoxide hydrolysis reaction.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

PVA hydrogels and hybrids were placed in the 96-well microplates, followed by addition of 50 µL of a solution containing 0.5 µg of rMPB70 in Tris-buffer pH 9.0 and incubated for 1 hour at 37 °C. Phosphate-buffered saline (PBS, pH = 7.4) and PBS-T (PBS containing 0.05% Tween-20, Merck, Germany) solutions were used as washing buffers for ELISA assay. Skimmed milk in PBS-T solution (4% w/v) was prepared and used as blocking buffer. After that, it was added to each microplate well and samples were incubated for 3 hours at 37 °C. After washing, either sera rabbit anti-rMPB70 or anti-*M. avium*, and bovine serum diluted 1/100 in blocking buffer was added (50 µL) to each well. Microplates were incubated for 1 hour at 37 °C, subsequently washed six times with PBST and incubated for 1 hour at 37 °C with 50 µL of protein G peroxidase conjugate (Sigma, USA) diluted 1/3 000 in blocking buffer. Samples were again washed six times with PBST. OPD, Ortho-Phenylenediamine, is generally used as a chromogen substrate in ELISA procedures. OPD produces a yellow color during the enzymatic degradation of hydrogen peroxide by horseradish peroxidase (HRP) with an absorption maximum at λ = 450 nm. After addition of sulfuric acid, a very stable orange end solution is obtained (λ = 492 nm). Therefore, 100 µL of OPD substrate solution (10 mg of OPD dissolved in 20 mL of 3 M citrate buffer pH 5.0 and 5 µL of 30% hydrogen peroxide) was added to each microplate well for ELISA assay. After incubation for 45 minutes at 37 °C, the reaction was interrupted with the addition of 50 µL of H_2SO_4 1.0 M (Merck, Germany). Samples were removed from the wells and the color intensity of the solutions was read at wavelength λ = 492 nm by an ELISA Microplate Reader (model 550, BioRad Laboratories, USA). A reference ELISA was carried out simultaneously in 96-well ELISA plates (Maxisorp®, Nalgene Nunc International, USA), using the same parameters defined for the hydrogel evaluation but without the PVA hydrogel and hybrid samples. This plate was used as a positive control. All reaction mixtures used in ELISA assays were set up in duplicate, with the average value used for calculations.

3. Results and Discussion

3.1. Chemical characterization by FTIR of the PVA hydrogels and hybrids

In Figure 3a, FTIR spectrum of pure PVA sample is showed. It clearly reveals the major peaks associated with poly(vinyl alcohol). For instance, it can be observed C–H broad alkyl stretching band (ν = 2850-3000 cm^{-1}) and typical strong hydroxyl bands for free alcohol (nonbonded –OH stretching band at ν = 3600-3650 cm^{-1}), and hydrogen bonded band (ν = 3200-3570 cm^{-1})[16-18]. Intramolecular and intermolecular hydrogen bondings are expected to occur among PVA chains due to high hydrophilic forces. An important absorption peak was verified at ν = 1142 cm^{-1}. This band has been used as an assessment tool of poly(vinyl alcohol) structure because it is a semicrystalline synthetic polymer able to form some domains depending on several process parameters[16-18]. FTIR spectrum in Figure 3b is associated with PVA crosslinked by glutaraldehyde (PVA/GA). It can be observed that two important peaks at ν = 2860 and 2730 cm^{-1} of C–H stretching are related to aldehydes, a duplet absorption with peaks attributed to the alkyl chain[7,16,17]. By crosslinking PVA with GA

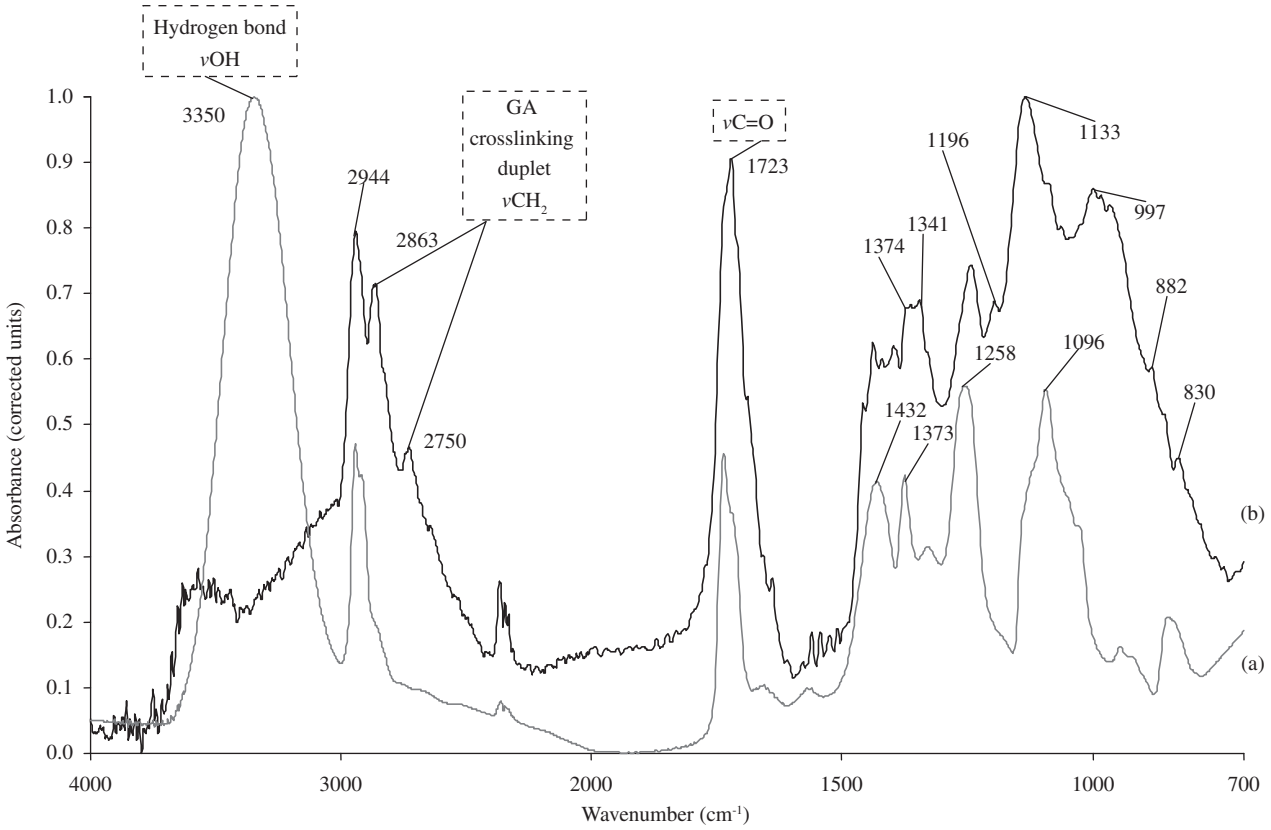

Figure 3. FTIR spectrum of PVA hydrogel a) pure PVA; and b) PVA hydrogel crosslinked with GA or PVA/ GA.

(Figure 3b), the O-H stretching vibration peak (ν = 3330-3350 cm^{-1}) was decreased when compared to pure PVA (Figure 3a). This result suggests that the hydrogen bonding becomes weaker in crosslinked PVA than in pure PVA because of the diminution in the number of OH groups and acetal formation[7] (scheme Figure 2a). The relative increase of the C=O band at approximately ν = 1720 cm^{-1} indicates that the aldehyde groups of GA did not completely react with O-H groups of PVA chain. In addition, the C-O stretching at approximately 1100 cm^{-1} in pure PVA is replaced by a broader absorption band (from ν = 1000 to 1140 cm^{-1}), which can be attributed to the ether (C-O) and the acetal ring (C-O-C) bands formed by the crosslinking reaction of PVA with GA (reaction scheme.1)[17-19]. Therefore, it can be assumed that GA has acted as chemical crosslinker among PVA polymer chains. FTIR spectrum of hybrid made of PVA/TEOS is showed in Figure 4b. It can be observed that major vibration bands (Si–O–Si, ν = 1080 and 450 cm^{-1}; Si–OH, ν = 950 cm^{-1}) associated with polysiloxane (TEOS) reactions of hydrolysis and condensation added to PVA polymer solution. Also, in the frequency range from 3000 to 3650 cm^{-1}, mainly related to hydroxyl groups[7,17-18], a broader band was noted for PVA/TEOS hybrid spectrum (Figure 4b) compared to pure PVA (Figure 3a). Such result is believed to be due to the TEOS sol–gel reactions (Scheme 1 and 2) that have altered PVA chains tridimensional structure (Figure 4c). 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[17]. Therefore, introducing of Si–OH and Si–O–Si through hydrolysis and condensation reactions of TEOS has modified PVA semi-crystalline structure. FTIR spectra showed in Figure 4a have confirmed the formation of PVA/TEOS/GA hybrids with network crosslinking. Glutaraldehyde (1,5-pentadial) has acted as a crosslinker among polymer chains of PVA and an organic-inorganic covalent binder (Figure 4c). Major proposed chemical reactions are summarized in Figure 2a and Figure 2b (schematic) involving both hydroxyl functional groups from silanol and from poly(vinyl alcohol). In order to establish crosslinking, some physical–chemical conditions have to be applied, for instance reactions occurring in low pH solution, where so called Schiff bases are formed[7,17]. Hence, FTIR spectra showed in Figures 3 and 4 have given strong evidence that the experimental procedure developed in this work was successful in obtaining and altering the organic-inorganic structure of PVA, PVA/TEOS and PVA/TEOS/GA. The peaks associated with amide-I (1620-1680 cm^{-1}) and amide-II (1480-1580 cm^{-1}) were observed on the spectrum of pure rMPB70 used as reference (Figure 5). Based on the literature[20-22], 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 1600 to 1700 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%)[20]. The amide III band is usually weak in the FTIR spectroscopy but can be found in the region from 1250 to 1350 cm^{-1}. FTIR spectra of the reference protein rMPB70 and PVA/GA hydrogel are showed in Figures 6a and 6b, respectively. The FTIR spectrum in Figure 6c shows the results of the PVA/GA network after protein (rMPB70) incorporation, where all major important amide stretching vibration bands are present. The typical protein bonds in the absorption regions of amides I, II, III are indicated (Figure 6c). Hydrogen bonded shifts some of these absorptions, as well as the prominent N–H stretching

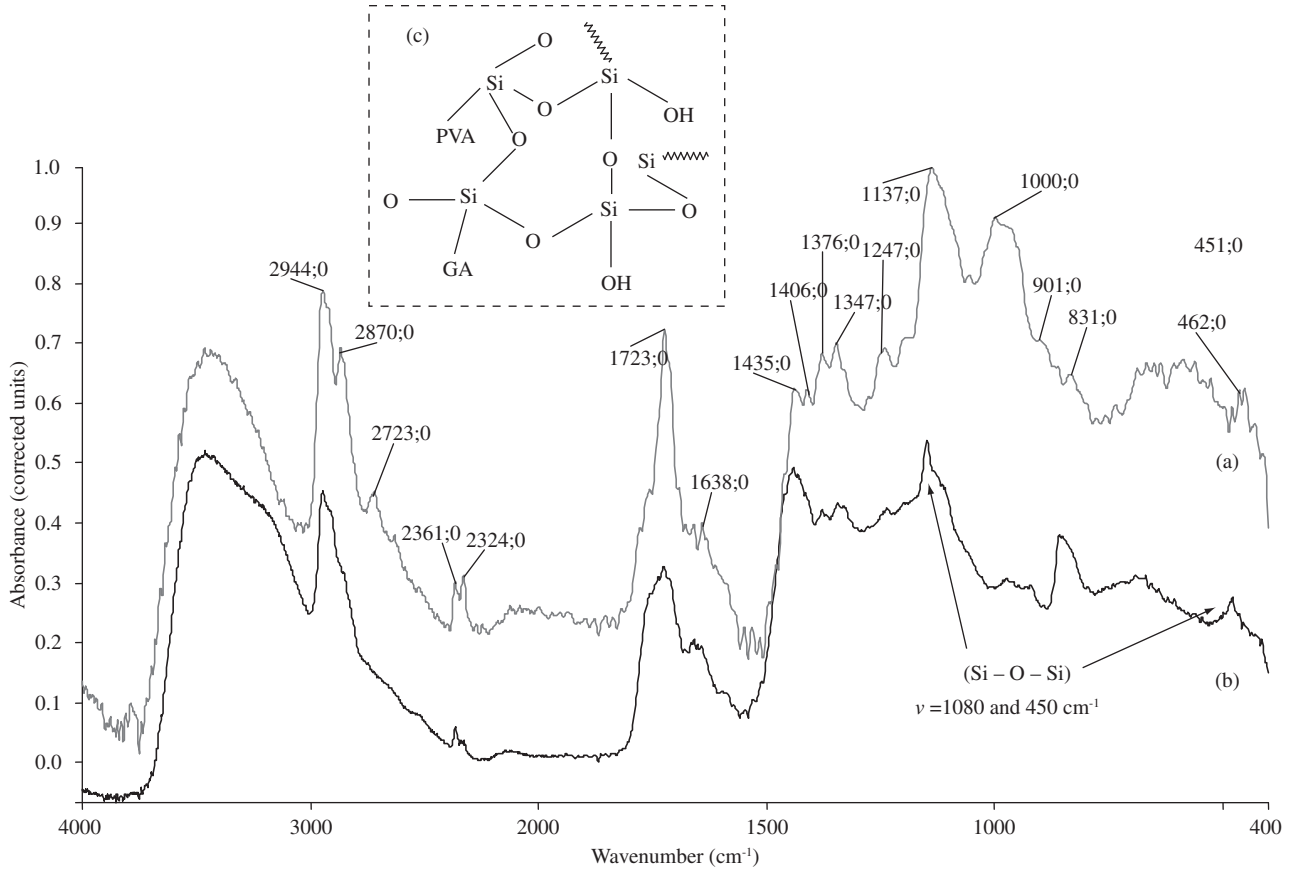

Figure 4. FTIR spectrum of a) PVA/TEOS/GA hybrid; b) PVA/TEOS/GA hybrid; and c) schematic representation of PVA/TEOS/GA hybrid nanostructure.

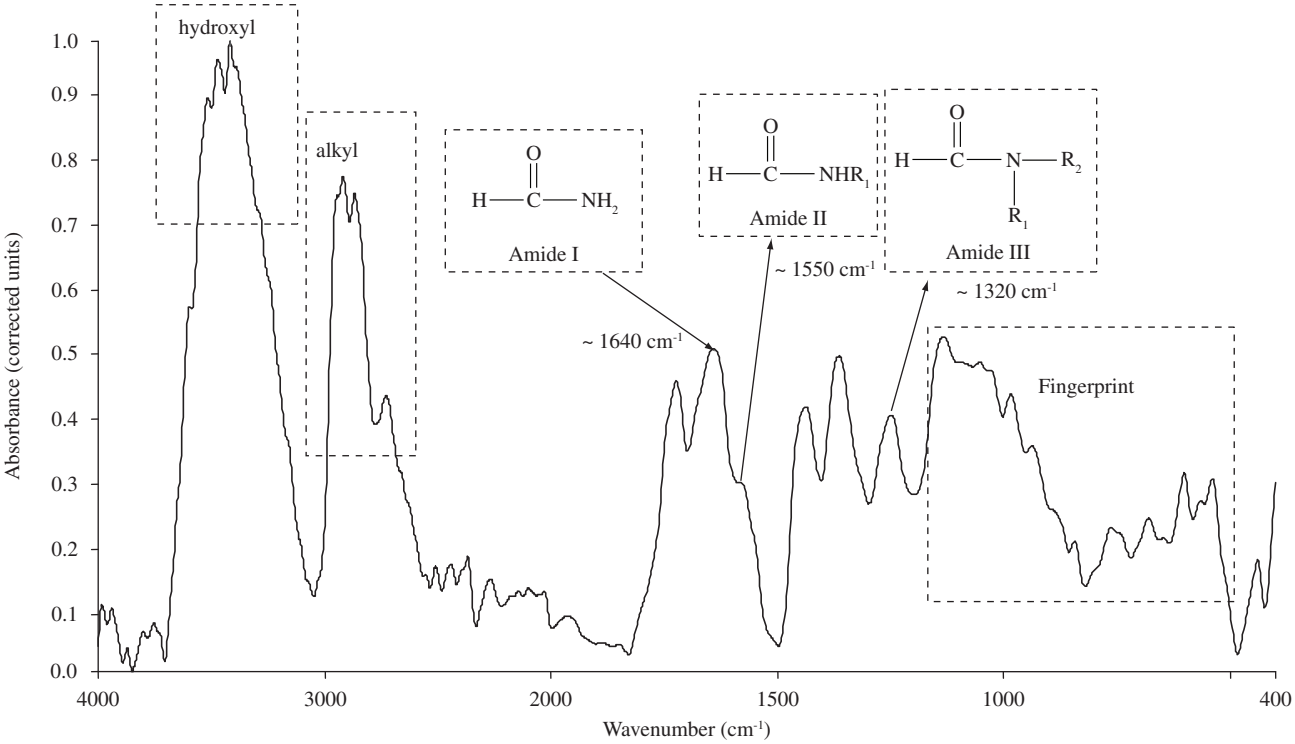

Figure 5. FTIR spectrum of pure MPB70 recombinant protein produced, purified and characterized.

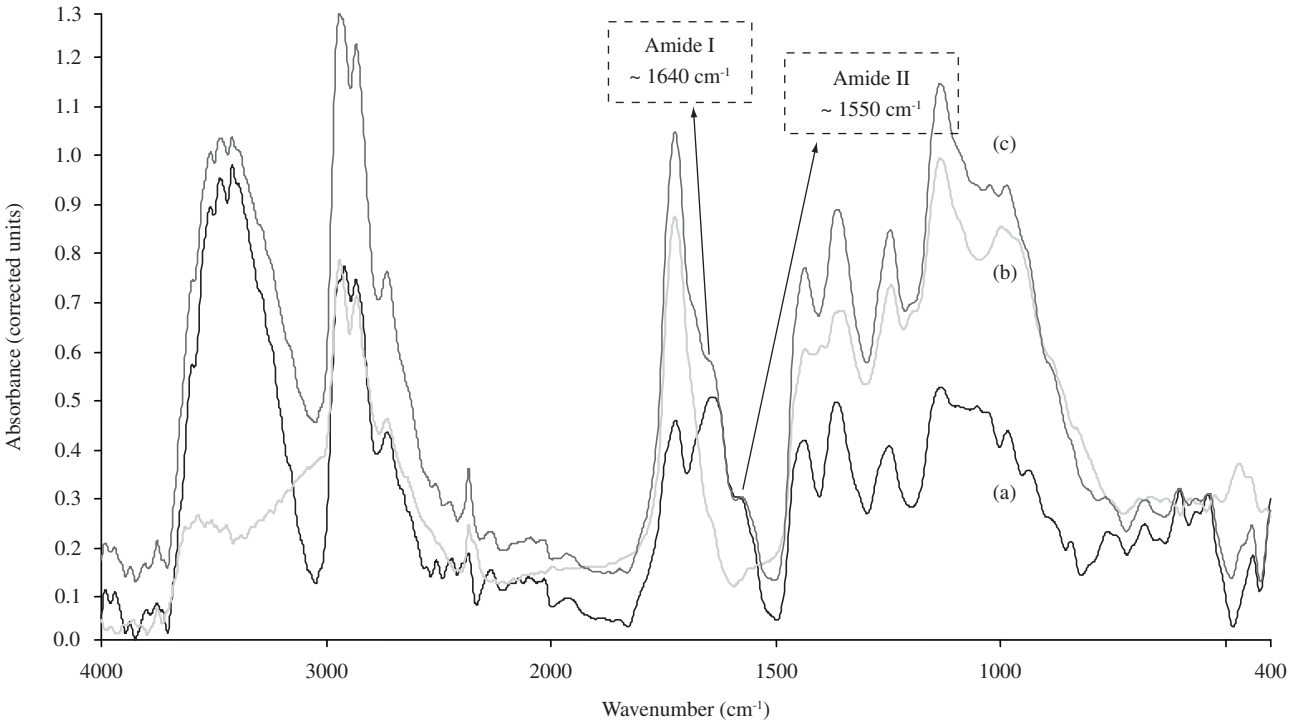

Figure 6. FTIR spectrum of a) pure MPB70; b) PVA/GA hydrogel; and c) rMPB70 incorporated in PVA/GA hydrogel, PVA/GA/rMPB70.

absorptions (3170 to 3500 cm^{-1}). Therefore, we could confirm the immobilization of rMPB70 protein in hydrogel network by using FTIR spectroscopy.

3.2. Enzyme-Linked Immunosorbent Assay (ELISA)

MPB70 recombinant protein incorporated into polymeric hydrogel and hybrids samples were evaluated by ELISA immunoassay. In order to evaluate the specificity and selectivity of these samples, reference control was used simultaneously to determine the presence of anti-MPB70 in bovine serum reactive to the tuberculin test. Also, anti-rMPB70 antibodies were detected in rabbit immune serum and in bovine serum reactive to the tuberculin test. As expected, in the reference negative control sample, no antibodies were detected in rabbit immune serum anti-*M. avium*, and bovine serum non reactive to the tuberculin test. A summary of such ELISA results are presented in Table 1. Therefore, the specificity of the immunoassay was validated. ELISA assays conducted with rMPB70 incorporated onto PVA, PVA/TEOS and PVA/TEOS/GA, did not present any detectable antigen-antibody. That means, either no reaction has occurred or the low concentration of rMPB70 incorporated was not detectable. However, antibodies were detected for the PVA/GA hydrogel, with similar results to those obtained for ELISA reference sample used, 96-well ELISA microplates (Maxisorp®, Nalgene Nunc International, USA). In immunoassay tests performed by changing hydrogels samples from one well to another at each step of the ELISA, anti–rMPB70 antibodies did not reach a detectable range. Consequently, anti-rMPB70 antibodies were detected only when hydrogel samples were maintained in the same microplate well throughout the entire ELISA assay (Table 1). Based on these results, one may assume that weak interactions have taken place of rMPB70 with hydrogels and hybrids networks, mostly van der Waals, hydrophobic, hydrophilic and electrostatic forces. Such interactions are not strong enough to maintain the bond between solid support and the biomolecule during the ELISA immunoassay several washing steps and buffer solutions[19]. The adsorption of protein from solution onto solid surfaces is a complex process playing a major role in biological systems[6]. The high efficiency presented by biological macromolecules in selecting chemical species has motivated the development of devices that combine synthetic materials with biological entities. Proteins can de immobilized in many different ways, but it is crucial that they retain their active conformation after the incorporation procedure. There are three major methods for immobilizing biomolecules and cells. Two of them are physically based, physical adsorption and physical entrapment. The third method is based on covalent (chemical) attachment. Thus, it is important to note that the term immobilization can refer either to a temporary or to a permanent localization of the biomolecule on or within a support[6]. For that reason, several immunological assays based on ELISA and "Western Blot" reported in the literature[19-26] have used an antigen covalently fixed onto solid supports via chemical crosslinking to guarantee the stability during the immunological procedure. In addition to that, similar systems reported in the literature[23-26] using polymer-polisiloxane supports have maintained the hybrid sample in the same microplate well during the whole ELISA assay and have used antigen covalent binding to these surfaces with crosslinking reagents. In summary, based on FTIR and immunological ELISA results, it can be concluded that a temporary incorporation of the rMPB70 protein into the novel PVA hydrogel and hybrids was accomplished.

4. Conclusion

FTIR spectroscopy was successfully used to characterize hydrogels of PVA, crosslinked PVA/GA and hybrids containing tetraethylorthosilicate (TEOS). In addition to that, the results have supported that recombinant protein rMPB70 was successfully adsorbed into PVA hydrogels and hybrids networks. Some PVA hydrogels and hybrids

Table 1. Comparison of ELISA using hydrogel PVA/GA, commercial ELISA plates as support and rMPB70 as solid phases.

	PVA/GA[1] Abs (λ = 492 nm)	PVA/GA[2] Abs (λ = 492 nm)	ELISA plates Abs (λ = 492 nm)
Rabbit anti-rMBP70 serum	0.510	0.027	0.611
Positive tuberculin test cattle sera	0.250	0.049	0.511
Rabbit anti-*M. avium* serum	0.022	0.011	0.074
Negative tuberculin test cattle sera	0.058	0.024	0.046

1: Hydrogel remained in the same microplate well at each step of ELISA assay.
2: Hydrogel removed from the microplate well at each step of ELISA assay.
All values were averaged.

developed in this study were found to be equivalent to commercially available microplates regarding to ELISA immunoassay sensitivity and specificity response.

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