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Improvement of viral recombinant protein-based immunoassays using nanostructured hybrids as solid support

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Abstract Protein adsorption onto solid surfaces is a complex process playing an important role in biological systems, being crucial to maintain the three-dimensional conformation during molecules interaction. Polymers hybrid network could be used to perform immunoassays with the required high level specificity. We have developed a novel route for incorporating recombinant protein from Bovine Herpesvirus into a network using as template Poly(vinyl alcohol) chemically tailored with five organosilanes followed by FTIR spectroscopy characterization. Protein expressed in *E. coli* was incorporated into hybrid network; all the PVA-hybrids interacted with this biomolecule and FTIR vibrational bands have endorsed the results. PVA hybrids modified with 3-(triethoxysilyl) propylisocyanate-TESPI presented the best result in the immunoassay, enhancing the sera specificity in comparison with the commercial microplate. We have successfully developed organic–inorganic hybrid based on PVA-silane to be used in serological tests with recombinant protein as a promising tool for the development and production new diagnostic assays.

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1 Introduction

A very attractive area of engineering surface is the immobilization process toward the material surface modification to be compatible with many biological molecules. The novel class of materials named organic–inorganic hybrids has a great potential to be used in implants, drug release materials, signaling molecules, and membranes to separate molecules [1]. These hybrids can interact with biological systems with high performance and has been proved its potential for immunodetection assays [2]. The organic–inorganic hybrids can be straightforwardly synthesized with improved physical and mechanicals properties. These characteristics and the lower production costs are advantageous for commercial application [3], especially to develop diagnostic tests. Protein immobilization in diagnostic tests are being developed for clinical assays using as solid support polyvinyl chloride (PVC) and polystyrene (PS) microplates which simplify the process automation. Poly(vinyl alcohol) (PVA) can be an alternative to use in solid support to perform antigen–antibody detection once this material, a synthetic water-soluble hydrophilic polymer, is highly versatile to be modified improving mechanical and chemical properties [4].

The bovine herpesvirus are important cattle pathogens distributed around the world that require high investments for eradication and control programs, besides semen and embryos commercialization. The *Bovine Herpesvirus 1* (Infectious bovine rhinotracheitis virus—BoHV-1) a major pathogen of cattle, is the agent of infectious bovine rhinotracheitis/infectious pustular vulvovaginitis virus. This virus causes great economic impact, needing hard disease control programs. Other herpesvirus pathogen, *Bovine Herpesvirus 5* (BoHV-5—Bovine encephalitis virus) co-circulate in cattle herds and share a high homology at genomic and antigenic

level with BoHV-1 [5]. Due to this great similarity, serological methodologies with enhanced specificity need to be developed, avoiding cross-reactivity in serological trials. This work aimed to develop a novel route for incorporating recombinant protein into a nanostructured organic–inorganic network, based on PVA polymer and different organosilanes to be potentially used in development of immunoassay kits for human and animal clinical laboratories use. This designed nanoplatform takes into account the capacity of the hybrid network on immobilizing biomolecules and retaining their biological properties, enhancing the sensibility, and specificity of serological tests. In summary, an ELISA was developed (Enzyme Linked ImmunoSorbent Assay) for bovine sera trial test of naturally infected animals, using in solid phase the recombinant glycoprotein G from Bovine herpesvirus 1 (recgG-BoHV-1).

2 Material and methods

2.1 Recombinant virus protein expression

Partial coding sequence of BoHV-1 glycoprotein G was obtained by amplification in PCR system (PROMEGA) from BoHV-1 Colorado strain (ATCC 864) and was previously cloned in pGEM-T easy plasmid vector (Invitrogen). The insert was excised by digestion with the enzymes *BamHI* and *HindIII*, and sub-cloned into expression vector plasmid pQE30 (QIAGEN), fused in a histidin tag in order to facilitate the purification (Fig. 1a, b). The resulting plasmid was sequenced for checking the insert. *Escherichia coli* strain M15 was used for cloning and expression experiments. The bacteria transformed was grown in LB medium containing ampicillin (100 mg/ml) and Kanamicyn (50 mg/ml) to an *A*600 of 0.3–0.4 at 37°C. Expression was induced by addition of IPTG (isopropyl-d-thiogalactopyranoside) (1 mM) followed by 5 h incubation at 37°C. Cells were collected by centrifugation and the protein was purified using Ni–NTA columns (QIAGEN) under denaturing conditions according to manufacture instruction.

2.2 Polyclonal antibodies

Bovine sera collected in naturally infected bovine herds were previously tested in a seroneutralization assay (SN) and in indirect ELISA (using virus BoHV-1 and BoHV-5 purified by sucrose ultracentrifugation as antigen) were

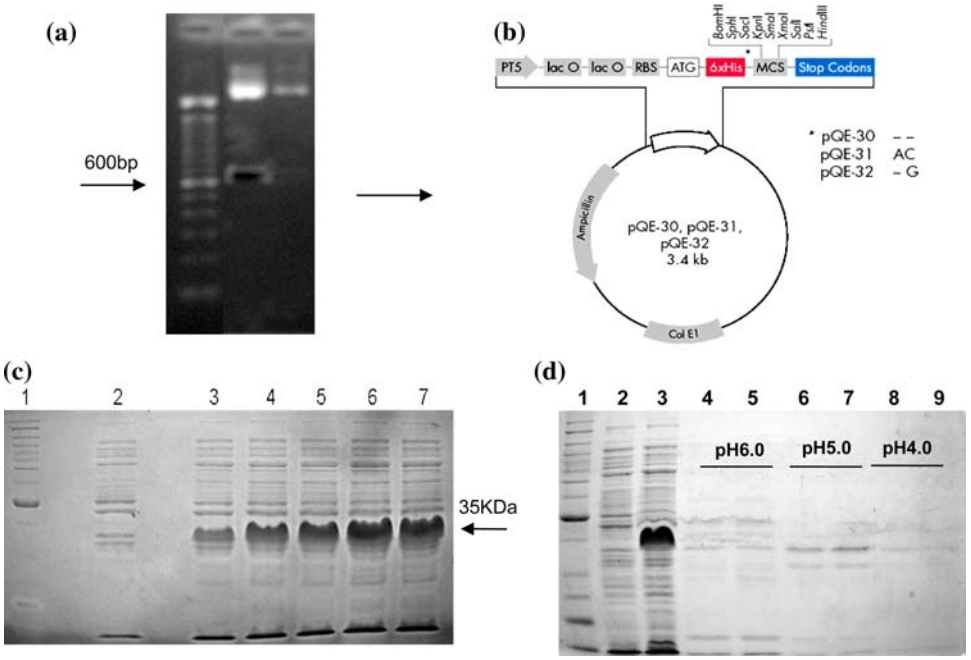

Fig. 1 Strategy used to produce histagged recombinant glycoprotein G of BoHV-1 in *E. coli*. **a** Partial coding sequence of BoHV-1 glycoprotein G (Colorado strain—ATCC 864) cloned in pGEM-Teasy vector was digested by *BamHI* and *HindIII* and was excised from agarosis gel. **b** The insert was sub-cloned into protein expression vector plasmid pQE30 (QIAGEN), fused in a histidin tag that. This vector was used to transform *Escherichia coli* M15 strain for protein production. **c** Comassie stained SDS-polyacrilamide gel 12% showing Rec-gG-BoHV-1 protein expressed in *E. coli* strain M15. Lane 1. BenchMark protein ladder; lane 2. M15 not induced; lanes 3–7 M15 induced with IPTG (1 mM) followed by 1–5 h incubation at 37°C. **d** Comassie stained SDS-polyacrilamide gel 12% showing Rec-gG-BoHV-1 purificated using Ni–NTA columns (QIAGEN) under denaturing conditions Lane 1. BenchMark protein ladder; lane 2. M15 not induced; lanes 3. M15 induced with IPTG (1 mM) for 5 h incubation at 37°C; lanes 4–9: Elution of recombinant protein in urea buffer pH 6.0 to pH 4.0

pooled comparing its stronger reactivity to BoHV-1 (anti-BoHV-1) or BoHV-5 virus (anti-BoHV-5). Chi-square was calculated for establishment of ELISA sensibility and sensitivity using as gold standard in the SN test. The optical density (OD) values that gave the higher test precision, as well as the addition of sensibility and sensitivity values were established as cut-off [6].

2.3 PVA hybrids matrix synthesis

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. Poly(vinyl alcohol) was kindly donated by Celanese Chemicals/USA (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 solution was prepared by fully dissolving 5.0 g of polymer powder in 100 ml of Milli-Q water under magnetic stirring 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) and organotrialkoxysilane were synthesized via aqueous route as previously reported by our group [7]. Briefly, 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%. The PVA hybrid solution was poured into a 96-well polystyrene microplate, with volume of 200 ml/well, and allowed to dry and structure consolidation for 24–72 h. After 5 days stored in closed dessicator, 250 ng/well of purified recgG-BoHV-1 (stock solution 5 μg/ml—PBS pH 7.2) was added to each polystyrene microplate well for virus protein immobilization to PVA hybrid network. Sample was incubated for 12 h at 25°C, after it was washed once with PBS pH 7.2 in order to eliminate non-adsorbed protein. Then, it was incubated 24 h at 37°C before immunoassay experiments and FTIR spectroscopy measurements.

2.4 Chemical characterization of PVA derived hybrids by FTIR spectroscopy

FTIR was used to characterize the presence of specific chemical groups in the PVA hybrids networks. FTIR spectra were obtained within the range between 4,000 cm^{-1} and 400 cm^{-1} (Perkin-Elmer, Paragon 1000), using diffuse reflectance spectroscopy method (DRIFT–FTIR). Hybrids samples were milled and mixed with dried KBr powder, then placed in a sampling cup and 64 scans were acquired at 2 cm^{-1} resolution with the subtraction of KBr background. Transmittance FTIR spectrum was also obtained for PVA films cast in round glass molds used as reference. Herpesvirus recombinant protein (antigen) was incorporated by adding 100 μl of a solution with recgG-BoHV-1 (concentration of 100 μg/ml) in 5 mg of fine crushed powder from PVA hybrid and characterized using FTIR.

2.5 Indirect ELISA

The effect of chemical modification of the designed hybrids on recognizing specificity and adhesion of recombinant viral protein was measured in an ELISA assay using the polyclonal bovine antibodies pools (anti-BoHV-1 and anti-BoHV-5). Polyvinyl chloride (PVC) plates (Falcon 3911, Microtest III flexible assay plate; Becton Dickinson, San Diego, California, USA) were coated with 250 ng/well of recgG-BoHV-1 diluted in 0.05 M carbonate–bicarbonate buffer (pH = 9.6) overnight at 4°C. PVA/silane hybrid was formerly coated with recgG-BoHV-1 during the matrix preparation (as described previously). The microplates were then washed in PBS–T (0.1% nonfat milk powder, 0.05% tween-20 in Phosphate Buffered Saline pH 7.2) and blocked with 150 μl/well of 5% non-fat milk powder in PBS for 1 h at 37°C. The wells were further washed in PBS–T and then 100 μl of 1:100 diluted polyclonal sera was added (anti-BoHV-1 or anti-BoHV-5) and the plates were incubated at 37°C for 1 h in humid camera. The plates were then washed in PBS–T and 100 μl of anti-IgG bovine peroxidase conjugate (SIGMA—1:5,000 dilution) was added. They were incubated further for 1 h at 37°C in humid camera, washed thoroughly and 100 μl of substrate solution was added/well—5 mg of o-phenylenediamine and 5 μl of H_2O_2 (38% v/v) in 5 ml of citrate buffer (pH 5.0). The plates were incubated at room temperature (25°C) for 5–10 min in a dark chamber and after adding the stop solution (H_2S0_4 1:20) the optical density (OD) was recorded at $\lambda = 492$ nm in a microplate reader (Expert Plus microplate Reader—G020 150—ASYS HITECH—AUSTRIA). Tests were performed using minimum four replicates.

3 Results and discussion

3.1 Recombinant protein production

BoHV-1 gG clone successfully expressed the recombinant protein in the estimated molecular mass (~35KDa) (Fig. 1c). The protein was purified using Ni–NTA columns and denaturant conditions from pH 6.0 to pH 4.0 (Fig. 1d). The eluate recovery using Urea buffer pH 5.0 was used in the ELISA experiments, due to better purification and higher production in comparison to eluates from pH 6.0 and pH 4.0, respectively.

3.2 Characterization of PVA/silane hybrids by FTIR

FTIR spectroscopy was used to characterize the PVA hybrid formation when reacting with different polymer modifier organotrialkoxysilanes, for instance TEOS (hydroxyl), MPTMS (thiol), TESPI (isocyanate), APTES (amine), and GPTMS (glycidyl). FTIR spectrum was performed using pure PVA sample as reference and it revealed the major peaks associated with poly(vinyl alcohol) as showed in Fig. 2a. It could be observed C–H broad alkyl stretching band (2,850–3,000 cm^{-1}) and typical strong hydroxyl bands for free alcohol (nonbonded –OH stretching band at 3,600–3,650 cm^{-1}), and hydrogen bonded band (3,200–3,570 cm^{-1}) [7, 8].

All samples of PVA-organosilane hybrids with protein incorporated (Fig. 2b–f) have showed the presence of major bands associated with recombinant glycoprotein G of BoHV-1 and to the silane group. Despite of being superposed by some PVA bands (C–O) and at very low concentration some of major bands associated with protein such as amide-I (1,620–1,680 cm^{-1}) and amide-II (1,480–1,580 cm^{-1}) have been observed [7, 8]. 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%). Moreover, the silane typical broad bands related to Si–O–Si bonds (1,080 and 450 cm^{-1}) and Si–OH bonds (3,500 and 950 cm^{-1}) were verified in all spectra showed in Fig. 2 (except Fig. 2a, of pure PVA). These bands are usually found in most materials obtained via sol–gel route. Furthermore, concerning to the specificity of each silane modifier used on the PVA network most important bands were detected via FTIR spectroscopy. FTIR spectrum (Fig. 2e) of MPTMS showed among several peaks (Si–O–,–CH_2), an important peak at 2,600–2,550 cm^{-1} that is characteristic of the 3-mercaptopropyltrimethoxysilane, associated with the thiol group or sulfhydryl (–SH). Similar results were obtained for PVA hybrids functionalized with GPTMS (Fig. 2b), APTES (Fig. 2c), TEOS (Fig. 2d), and TESPI (Fig. 2f). PVA

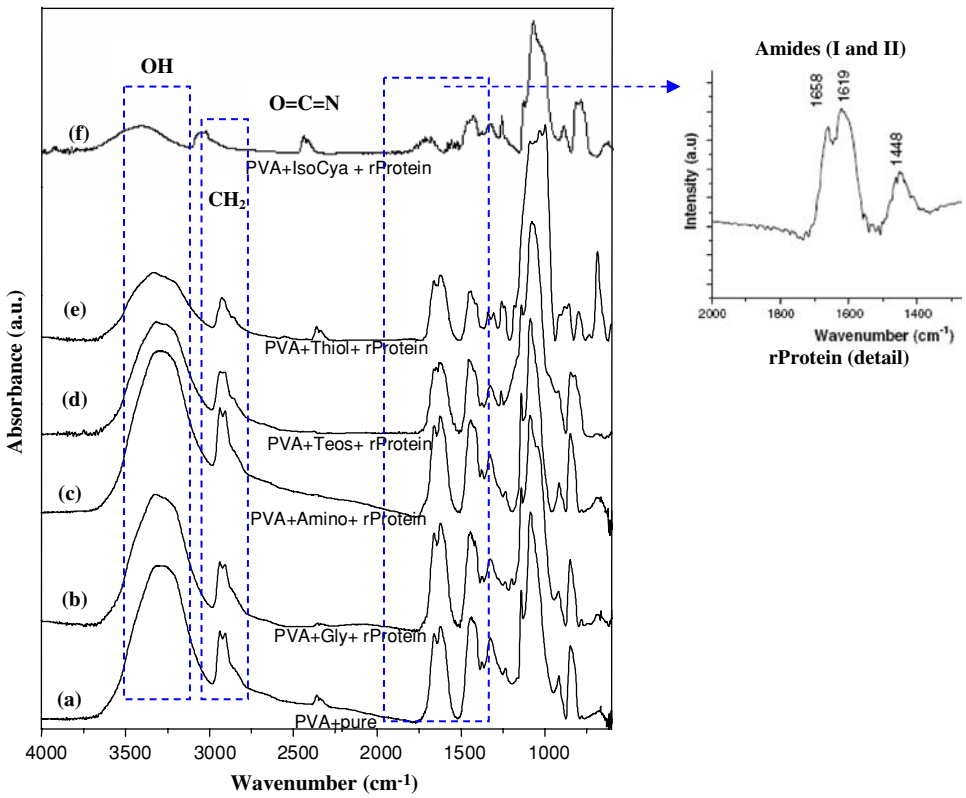

Fig. 2 FTIR spectra of: pure PVA (**a**); GPTMS (**b**); APTES (**c**); TEOS (**d**); MPTMS (**e**); TESPI (**f**)

modified with GPTMS (Fig. 2b) with glycidyl at 1,055–1,060 cm^{-1} (C–O–C broad band) and at 1,250 cm^{-1} (epoxy ring). In the same way, APTES (Fig. 2c) has indicated the contribution of amine groups from in-plane NH_2 scissoring absorptions at 1,550–1,650 cm^{-1}, out-of-plane wagging at 650–900 cm^{-1} (broad) and stretching at 3,100–3,400 cm^{-1} (N–H and C–N). In Fig. 2f, the TESPI spectrum has showed the presence of isocyanate band at 2,100–2,270 cm^{-1} (weak, N=C=O) and C=O at 1,680 cm^{-1} (strong) [7, 8]. Hence, FTIR spectroscopy has given evidence that the experimental procedure developed in this work was successful in obtaining and altering the organic–inorganic structure of hybrid based on PVA. It should be pointed out that a deeper investigation is required to properly address the actual structure of those hybrids as far as the nano-order is concerned. That was not the goal of this research but the influence of different chemical functionalities on the overall behavior in potential immunoassay tests.

3.3 ELISA performance in PVC commercial and modified PVA-silane hybrids

The effect of chemical modification of the plates on adhesion and presentation of recgG-BoHV-1 protein was measured comparing an indirect ELISA performance using polyclonal antibodies using commercial PVC and PVA-based hybrids. Tests were done with at least four replicates. The specificity of serum response was transformed in percentage using the followed equation: OD homologue sera response (anti-BoHV-1)—OD heterologue sera response (anti-BoHV5)/OD homologue sera response, where OD = Optical density obtained by UV-vis spectroscopy absorbance at $\lambda = 492$ nm. Figure 3 shows the specificity results of ELISA assay comparing different PVA hybrids developed in the present research and PVC commercial microplates for gG-recBoHV-1 antigen. As it can be observed (Fig. 3) all PVA-derived hybrids materials (except thiol) have given some specific response to the immunoassay, although only the TESPI based hybrid has strongly enhanced the specificity accessed by polyclonal bovine sera for BoHV-1 comparing to commercial PVC commercial solid support. Isocyanate is a group of very reactive chemicals with the functional group N=C=O (abbreviated as NCO) that has been extensively used in the manufacture of various materials, such as coatings, and polyurethane based biomaterials. In fact, some authors [9–12] have reported the specificity regarding to the interaction of proteins with isocyanate derived materials such as polyurethane and co-polymers. Despite of not being fully understood, they believe that the behavior might be associated with proteins preferentially adsorb to the apolar microdomains in the material network. The

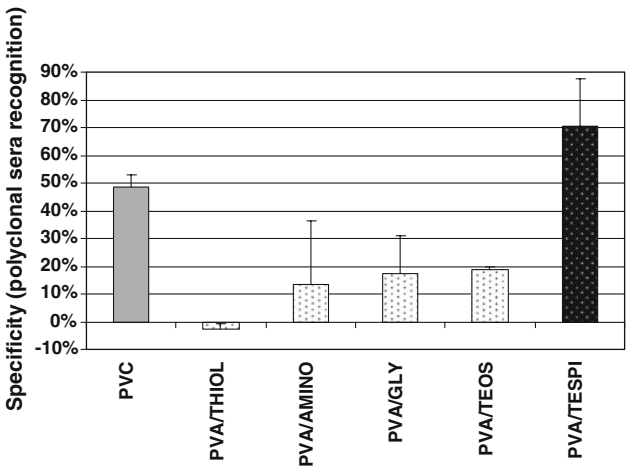

Fig. 3 Histogram of ELISA immunoassay conducted in five different PVA-derived hybrids modified with organosilanes using recombinant BoHV-1 glycoprotein G, obtained by UV-Vis spectroscopy absorbance at $\lambda = 492$ nm. Commercially available PVC support showed in solid bar

isocyanate hydrolysis can also takes place in aqueous system (leading to the production of urea groups) but it is likely to be very slow without the catalyst and the competing reaction of silane in water. Thus, protein adsorption is influenced by surface microenvironments of comparable size to the protein itself. This suggests that the matrix at the nano-order scale may organize the biological-material interface. Besides that, researchers [9, 10, 12] have also claimed that the main interaction between isocyanate and proteins is that of the isocyanate and the free amino-groups, for instance lysine molecules. From the chemical viewpoint it seems to be rather difficult to decide, without further much deeper investigation, which groups in a protein are likely to react with isocyanates or undergo some modification during the interaction, but evident possibilities are: the hydroxyl group of tyrosine, the disulphide group of cystine, the sulphydryl group of cysteine, the iminazole group of histidine, the guanidine group of arginine, the pyrrolidine group of proline and the acid–amide groups of asparagine and glutamine. Hence, in the present work the PVA hybrid modified with isocyanate organosilane (TESPI) has given evidence that the functional group N=C=O has driven the conformation and orientation of the recombinant protein towards the interaction with hybrid at the interface. Thus, PVA-isocyanate hybrid improved the ELISA performance when compared to commercial solid phase. As far as the results of all other hybrids are concerned, one can take into account the amino acids composition of the gG-BoHV-1 recombinant protein: 38 polar negatively charged and 17 polar positively charged (favoring ionic force) besides neutral amino acids, being 87 of hydrophobic and 26 of hydrophilic properties that can explain the good performance observed for PVA-derived

hybrids used in these experiments, once the polymer structure with pendant hydroxyl groups would have favored hydrophilic interactions and organosilanes would have helped more hydrophobic interactions.

As it was verified by immunoassays, all the PVA hybrids except thiol-modified have showed enhanced interaction with the viral recombinant protein produced, and in ELISA assays, the solid phase is a critical element for test performance, once the antigen needs to strongly interact with the surface [13]. Weak interactions induce poor sensitivity and specificity, enhancing the unspecific molecule interactions and compromising the test reproducibility [14, 15]. PVA hybrids developed during this work certainly can be tested in industrial scale to produce immune diagnostic tests. Besides interaction stability required for biological tests such as immunodiagnostic, it is necessary to take into account the adsorption capacity of biomolecules to plastic surface evaluating the total amount of proteins that binds to material surface and within them, the amount that really retain biological activity after binding. The concentration of active molecules is dependent of molecule nature and of biochemical character of the surface in solid phase [14, 15]. Hybrid matrices have been developed as solid support to proteins immobilization in order to improve the sensibility and specificity of diagnostic tests, favoring alternatives to modify the surface very specifically, according the nature of produced molecule. This versatility is not found in any other method of immobilization, once working with hybrid matrix we can control both: biomolecule and behavior of the immobilized material, maximizing the final results. Many authors have reported regulatory proteins, nucleic acids, antibodies and until whole cells immobilized to materials derived of sol–gel process with protection of your biological characteristics and activities, reinforcing the excellence of this materials to develop biological assays [16–19]. Besides these properties, sol–gel hybrids have shown good retention capacity in early biding phase and after long term of storage, and in some cases leading to stability enhancement of label molecules [20]. All of these characteristics besides lower cost production point the biocomposite out as an attractive material to develop and improve immunological methods.

4 Conclusion

FTIR spectroscopy results have indicated that recombinant protein (recgG-BoHV-1) was efficiently incorporated into PVA-organosilane hybrids. Also, it was observed that the developed PVA-derived hybrid based on isocyanate has indeed enhanced the binding of viral recombinant protein to solid-phase support and improved the specificity performance in an immunological assay by serological differentiation. Therefore, these novel hybrids represent a promising alternative to the field of diagnostic tests. Moreover, they are readily synthesized which may be of great advantage for commercial biomedical applications.

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