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Immobilization of streptavidin in sol–gel films: Application on the diagnosis of hepatitis C virus

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Abstract

Recent advances have accelerated the development of biosensors for the analysis of specific gene sequences. In this kind of biosensor, a DNA probe is immobilized on a transducer and the hybridization with the target DNA is monitored by suitable methodology. In the present work, the streptavidin (STA) was encapsulated in thin films siloxane–poly(propylene oxide) hybrids prepared by sol–gel method and deposited on the graphite electrode surface by dip-coating process. Biotinylated 18-mer probes were immobilized through STA and a novel amperometric DNA biosensor for the detection and genotyping of the hepatitis C virus (genotypes 1, 2A/C, 2B and 3) is described. The HCV RNA from serum was submitted to reverse transcriptase-linked polymerase chain reaction (RT-PCR) and biotin-labeled cDNA was obtained. Thus, the cDNA was hybridized to the target-specific oligonucleotide probe immobilized on the graphite electrode surface and following the avidin–peroxidase conjugate was added. The enzymatic response was investigated by constant potential amperometry at −0.45 V versus Ag/AgCl using H_2O_2 and KI solutions. HCV RNA negative and positive controls and positive samples of sera patients were analyzed and the results were compared to commercial kit. The proposed methodology appeared to be suitable and convenient tool for streptavidin immobilization and diagnose of HCV disease.

Keywords: Sol–gel method; Streptavidin immobilization; Hepatitis C virus; Genosensor; DNA biosensor

1. Introduction

Anchoring biomolecules at the surface of the transducer induces a high sensitivity and recent advances have accelerated the development of biosensors for the analysis of specific gene sequences. In this kind of biosensor, a DNA probe is immobilized on a transducer and the hybridization with the target DNA is monitored by optical, electrochemical, piezoelectric, impedimetric and surface plasmon resonance techniques. Various methods for the attachment of oligonucleotide probe DNAs to the surface of the electrode, such as adsorption, direct covalent binding, entrapment in a polymer matrix or indirect binding by the use of intermediate systems of polypyrrole films, have been describe in the literature [1,2].

A strong interaction occurs between the avidin (or streptavidin) and biotin [3]. Immobilization of DNA probe based on this interaction has been investigated. A graphite epoxy composite electrode with streptavidin [4], streptavidin immobilization at screen-printed carbon electrodes [5] were proposed for detecting a specific DNA sequence. The immobilization of biotinylated DNA probe on an avidin layer previously anchored to a electropolymerized biotinylated polypyrrole film coating the quartz crystal surface [6] and streptavidin immobilized onto quartz crystal through the modification with cysteamine/glutaraldehyde [7] have been also described.

The immobilization of the biological molecules (enzymes, antibodies, antigens, non-catalytic proteins, oligonucleotides and animals, vegetals and bacterials cells) in matrices prepared by the sol–gel process has made this technique a potential tool for the development of new biosensors [8,9]. The silicate matrix derivated by the sol–gel method has been frequently used. Modifications of the sol–gel matrix have been commonly carried out by impregnation, covalent bonding and chemical doping [10].

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Recently, the biosensors, based on the new immobilization materials, sol–gel organic–inorganic hybrid materials, have emerged [11–13].

The hepatitis C virus (HCV) is responsible for most of the cases of post-transfusion non-A and non-B hepatitis [14] and the infection affects approximately 170 million individuals worldwide [15]. The major causes of HCV infection worldwide are organ transplantation, blood transfusions, renal dialysis and intravenous drug abuse [16].

The monitoring of HCV RNA in serum or plasma is indicated for diagnosing or confirming active infections and for assessing patient response to therapy [17,18]. A variety of commercial assays have been developed for the qualitative detection of HCV RNA based on different nucleic acid amplification methodologies such as reverse transcription and polymerase chain reaction (RT-PCR), transcription-mediated amplification (TMA) and nucleic acid sequence based amplification (NASBA) [19–22]. The commercial tests available for the qualitative detection of HCV RNA are based on detection of the amplified RNA product through the use of a target-specific capture probe bound to magnetic particles, in conjunction with a ruthenium-labeled detection probe and an instrument capable of measuring electrochemiluminescence [23] and HCV RNA qualitative spectrophotometric detection by Amplicor® HCV (Roche, USA) which offers detection of HCV RNA levels as low as 50 IU/mL by spectrophotometric technique [24]. Commonly used tests for detecting HCV RNA are based on qualitative (PCR: Amplicor HCV v2.0 Roche Molecular Systems or Transcription-mediated amplification: Versant HCV RNA Bayer Diagnostics) and quantitative (PCR: LCx HCV RNA Abbott Diagnostics, SuperQuant National Genetics Institute, Amplicor HCV Monitor v2.0 Roche Molecular Systems and Cobas Amplicor HCV Monitor Roche Molecular Systems, or Branched DNA: Versant HCV RNA 3.0 Bayer Diagnostics) [25].

The bioelectric recognition assay (BERA) has been used for the qualitative and quantitative detection of viruses in humans (hepatitis C virus) by measurement of the change of the electric potential that is caused by the aforementioned [26,27]. The oligonucleotide probes suitable for capture of target sequences were investigated using BIAcore, which is based on surface plasmon resonance [28,29]. Biosensors based on electronic conducting polymers have been successfully applied to the genotyping of HCV in blood samples by fluorescence detection [30], and HCV piezoelectric genosensor for repeated use [7]. As we have known, there are some works on electrochemical genosensor for hepatitis B virus [31–39], but there are few works for RNA HCV detection.

Although the preventive HCV vaccine is economically attractive, its development still remains at an early stage [40] on the other hand the disease is asymptomatic and chronic liver disease may evolve into cirrhosis and hepatocellular carcinoma.

In the present work, we demonstrated that the entrapment of the streptavidin into siloxane–poly(propylene oxide) sol–gel films was suitable for biotinylated oligonucleotide probes immobilization and a convenient analytical tool for HCV diagnostics. The development of a novel amperometric genosensor for qualitative HCV RNA detection and genotyping (HCV 1, 2A/C, 2B and 3), simultaneously, were investigated. The results were compared to commercial kit.

2. Experimental

2.1. Chemicals and solutions

3-(Isocyanatopropyl)triethoxysilane (IsoTrEOS), O,O′ bis(2-aminopropyl)poly(propylene oxide) with molecular weight of 300 and 4000 g mol^{-1}, tetrahydrofuran (THF) were obtained from Aldrich Chemical Co., USA.

Streptavidin (STA) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA). 5′-Biotinylated 18-mer oligonucleotides probes (HCV 1: biotin-CGC TCA ATG CCT GGA GAT, HCV 2A/C: biotin-CAC TCT ATG CCC GGC CAT, HCV 2B: biotin-CAC TCT ATG TCC GGT CAT and HCV 3: biotin-CGC TCA ATA CCC AGA AAT) were supplied by Life Technology (Gaithersburg, MD, USA). All oligonucleotide stock solutions (200 μg mL^{-1}) were prepared in deionized water and kept frozen. Dilute solutions of the oligonucleotides were prepared daily in phosphate buffer solution (0.1 mol L^{-1}, pH 7.0). Buffer solutions were prepared from analytical grade, DNase- and RNase-free reagents and deionized water. Reagents for procedure of the reverse transcription, polymerase chain reaction (PCR) amplification and HCV RNA detection reaction were all purchased from Amplicor® Hepatitis C Virus (HCV) Test, Version 2.0, Roche (microwell format with spectrophotometric detection).

Other reagents were commercially available or laboratory grade ones. The solutions were prepared with high-purity water ($\rho = 18.2$ MΩ cm^{-1}). The H_2O_2 and KI solutions were prepared, immediately before use, in 0.1 mol L^{-1} phosphate buffer solution pH 7.0 (1.0 and 3.0×10^{-3} mol L^{-1}, respectively).

Sera samples were donated by Hepatitis Health Service at Faculty of Pharmaceutical Sciences, Unesp.

2.2. Preparation of sol–gel thin films

Siloxane–poly(propylene oxide) (PPO) nanocomposites were prepared by the sol–gel method as described elsewhere [41]. IsoTrEOS and PPO in the molar ratio 2:1 were mixed and stirred together in THF under reflux at 80 °C for 24 h. THF was evaporated and the stable hybrid precursor $_3$(EtO)Si–(PPO)–Si(OEt)$_3$ was obtained. 1.0 g of the hybrid precursor was mixed with 2.0 mL of ethanol containing NH_4F ($[NH_4F]/[Si] = 0.001$) used as neutral catalyst. The mixture was stirred until a homogeneous solution was obtained. Finally, 0.2 mL of water was added upon stirring to promote the hydrolysis and polycondensation of the hybrid precursor forming the sol (represented by PPO 300 and PPO 4000). The different concentrations of streptavidin (10, 20, 45 and 90 μg mL^{-1}) were added to sol. The PPO films (without and with encapsulated streptavidin, PPO and PPO/STA, respectively) were deposited on the graphite electrode surface by dip-coating process and were allowed to dry at 4 °C in a refrigerator for 24 h. All the electrodes were washed thoroughly before used in 0.1 mol L^{-1} phosphate buffer solution pH 7.0 and dry stored at 4 °C.

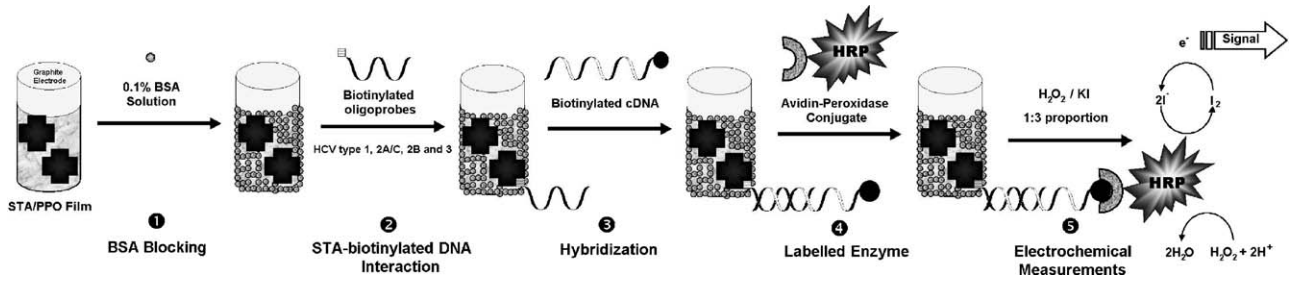

Fig. 1. Scheme of HCV detection by amperometric DNA biosensor.

2.3. Molecular recognition

Preliminary experiments were performed with: (1) bare graphite electrode; (2) PPO films graphite electrodes; and (3) graphite electrode containing streptavidin immobilized into PPO film (PPO/STA film). Sequentially, the construction of amperometric genosensor was developed, as illustrated in the Fig. 1. The methodology consisted on five steps:

(1) Blocking of the modified electrode surface: after immobilization of the streptavidin into PPO films, the modified electrodes surface were incubated in 50 μL of phosphate buffer solution pH 7.0 containing 0.1% bovine serum albumin for 20 min (Fig. 1, step 1). This procedure was accomplished to avoid the unspecific interactions of the oligonucleotide or avidin–peroxidase conjugate adsorptions on the PPO film modified electrode surface.
(2) HCV-specific oligonucleotide probes immobilization: the DNA electrodes were prepared by interaction of the immobilized streptavidin into siloxane–poly(propylene oxide) hybrid film and biotinylated oligonucleotide probes (HCV 1, 2A/C, 2B and 3) as shown in Fig. 1 (step 2). The PPO/STA film modified electrodes were immersed in 50 μL of the biotinylated oligonucleotide probe solution (5 μg mL^{-1}) prepared in 0.1 mol L^{-1} phosphate buffer solution pH 7.0. The incubation was performed for 20 min at 25 °C and the HCV-specific oligonucleotide probes were immobilized individually on the electrode surface. After that, the electrodes were washed in 0.1 mol L^{-1} phosphate buffer solution pH 7.0 under stirring for 5 min.
(3) The HCV RNA was extracted from patient sera. A 244 bp HCV DNA fragment of known sequence was obtained by reverse transcribed polymerase chain reaction (RT-PCR) in the presence of a sense biotinylated primer from commercial assay Amplicor® Hepatitis C Virus Version 2.0 (Roche). Following the PCR amplification, the HCV amplicons were chemically denature to form single-stranded DNA. After that, the hybridization step was performed at 25 °C. The electrodes were incubated in 50 μL of hybridization buffer solution containing (four times diluted) amplified products for 1 h (Fig. 1, step 3). The electrodes were washed with the same solution three times for 5 min under stirring to remove the unbound oligonucleotide.
(4) Avidin–peroxidase labeling: 50 μL of avidin–peroxidase (AV–HRP) conjugate was used to the indirect monitorement of the HCV. The AV–HRP conjugate binds to the biotin-labeled amplicon hybridized to the targeted-specific oligonucleotide probe bound on the electrode surface as shown in Fig. 1 (step 4). The electrodes were washed to remove unbound AV–HRP conjugate.
(5) Amperometric measurements: the electrodes were immersed in hydrogen peroxide and potassium iodide (1.0 and 3.0×10^{-3} mol L^{-1}, respectively) prepared in 0.1 mol L^{-1} phosphate buffer solution pH 7.0 and incubated for 10 min. The enzymatic response was investigated by constant potential amperometry at −0.45 V versus Ag/AgCl reference electrode (Fig. 1, step 5). Therefore, the current intensity obtained is proportional to amount of HCV amplicons in the samples. The background response was evaluated in the absence of HCV amplicons. HCV RNA negative and positive controls and HCV amplicon samples of sera patients were analyzed.

2.4. Blocking of the genosensor

To evaluate the unspecific adsorption of the biomolecules on the PPO films was used as model a biotin-HRP conjugate. The PPO or PPO/STA films modified electrodes were incubated in 50 μL of 0.1 mol L^{-1} phosphate buffer solution pH 7.0 containing 0.1% bovine serum albumin for 20 min. After blocking, either the PPO and PPO/STA films modified electrodes were incubated with different concentrations of biotin-HRP conjugate (1.0, 2.0, 3.0 or 5.0 units mg^{-1} of protein). The electrodes were washing three times in 0.1 mol L^{-1} phosphate buffer solution pH 7.0 during 5 min under stirring. Therefore, this assay verified all the adsorption processes capable to produce an analytical signal. After optimization of the experimental conditions for blocking of the PPO film electrode surfaces, the effect of bovine serum albumin on the genosensor was investigated.

2.5. Optimization of the genosensor

A good performance of the genosensor depends on the optimum conditions of the amount of the STA and biotinylated oligonucleotide probes immobilized on the electrode surface. Therefore, the concentration of streptavidin (10, 20, 45 and 90 μg mL^{-1}) added to sol before deposition of the PPO films on the electrode surface and the concentration of HCV 1 probe (10, 20, 40 and 80 times diluted in 0.1 mol L^{-1} phosphate buffer

solution pH 7.0) were investigated, simultaneously. Assays were performed as follows: one experiment was carried out with increasing both the STA concentration and the probe dilution and another one with the STA concentration increasing and the probe dilution decreasing. The intersection point between the analytical curves results in the optimum value for the interaction sites of the immobilized STA into the PPO films and the amount of the HCV-specific oligonucleotide probe being used for all subsequent experiments.

2.6. Determination of the cut-off value for the HCV RNA detection

The negative control and HCV RNA negative samples (i.e., HCV RNA not detected by Amplicor® HCV Test Kit) were analyzed by the proposed methodology. The reactivity threshold was established by the current intensity average obtained with these samples plus twice the standard deviation value.

2.7. HCV RNA detection and genotyping (HCV 1, 2A/C, 2B and 3)

Samples were processed by the complete Amplicor® HCV Test procedure and thus obtained PCR-amplified samples of nucleic acid. We selected eight HCV amplicon samples from sera patients for HCV RNA detection and genotyping (HCV 1, 2A/C, 2B and 3) by amperometric genosensor. All samples were positive for HCV antibodies by third-generation HCV enzyme-linked immunosorbent assay. HCV RNA detection was screened according to the manufacturer's instructions by qualitative Amplicor® HCV Test Kit and the HCV genotypes were determined by the line probe assay (InnoLiPA HCV; Innogenetics, Ghent, Belgium).

HCV RNA negative and positive samples of sera patients were analyzed by amperometric genosensor and the results were compared with the standard RT-PCR technique (Amplicor® HCV Test Kit). The quality control of the proposed methodology was done using at least duplicated of either Amplicor® HCV negative and positive controls.

2.8. Electrochemical measurements

The amperometric measurements were performed with a Potentiostat–Galvanostat EG&G model 263. A one-compartment cell with working volume of 5.0 mL and a thin film PPO modified working electrode, Ag/AgCl reference electrode and a platinum wire auxiliary electrode were used for the measurements. The HRP response was monitored by using H_2O_2 and KI. In the presence of hydrogen peroxide and horseradish peroxidase, iodide is efficiently transformed in iodine. The amount of I_2 generated is detected constant potential amperometry at −0.45 V versus Ag/AgCl. The experiments were performed at 25 ± 1 °C in 0.1 mol L^{-1} phosphate buffer solution pH 7.0. The hybridization of immobilized HCV-specific oligonucleotide probe and the biotin-labeled cDNA which bind to the avidin–peroxidase was monitored amperometrically by I_2 generated. Data are given in vertical bar charts, where each bar shows the mean and the standard deviation of the signal values obtained with different replicates of the genosensor.

3. Results and discussion

3.1. Optimization of the entrapment of biomolecules into siloxane–poly(propylene oxide) thin films

Recent advances in the development of DNA biosensors trends for development of technology for gene sequence analysis and nucleotide sequence-specific DNA hybridization have been evaluated. The immobilization method determines the selectivity, sensitivity and reproducibility of the DNA modified electrode response. There are few investigations about the biological molecules immobilization into organic-inorganic hybrids prepared by sol–gel method [9]; in particular there is no article about streptavidin and the proposed siloxane–poly(propylene oxide) hybrids. Thus, preliminary experiments have been done in the present work. Firstly, the optimization of experimental parameters using as model a streptavidin–peroxidase conjugate immobilized into PPO 300 and PPO 4000 matrices prepared by sol–gel method and deposited on the electrode surface by dip-coating process were evaluated. Every deposition involved one dip at a lifting speed of 600 mm min^{-1} providing a total thickness of 5.0 μm, which was determined using a Talystep profilograph (Taylor-Hobson, UK) apparatus with accuracy of 3%.

Fig. 2 shows the cyclic voltammograms in the potential range from 0.20 to −0.45 V of the PPO 4000 modified electrode recorded in phosphate buffer solution (0.1 mol L^{-1} pH 7.0) containing H_2O_2 and KI (1.0 and 3.0×10^{-3} mol L^{-1}, respectively). PPO 4000 film modified graphite electrode in the absence and presence of the encapsulated STA–HRP conjugate are represented by Fig. 2 (curves a and b), respectively. It was observed an increase of the cathodic current intensity (I_{pc} = −11.9 μA and

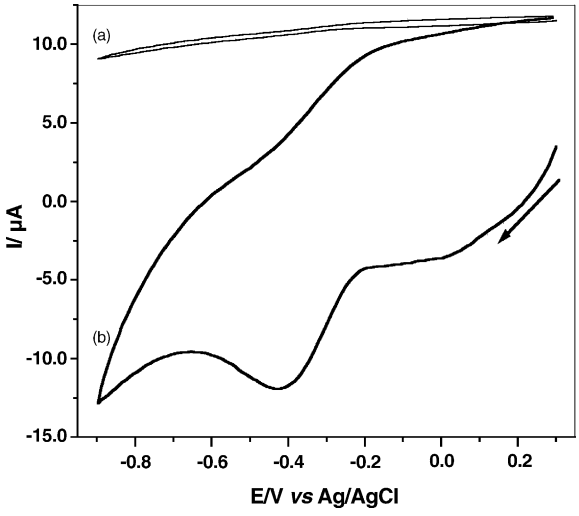

Fig. 2. Cyclic voltammograms recorded in 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) containing H_2O_2 and KI (1.0 and 3.0×10^{-3} mol L^{-1}, respectively) of the PPO 4000 modified graphite electrode in the (a) absence and (b) presence of the encapsulated STA–HRP conjugate, Scan rate: 0.050 V s^{-1}. Film deposition conditions: lifting speed of 600 mm min^{-1} and one dip.

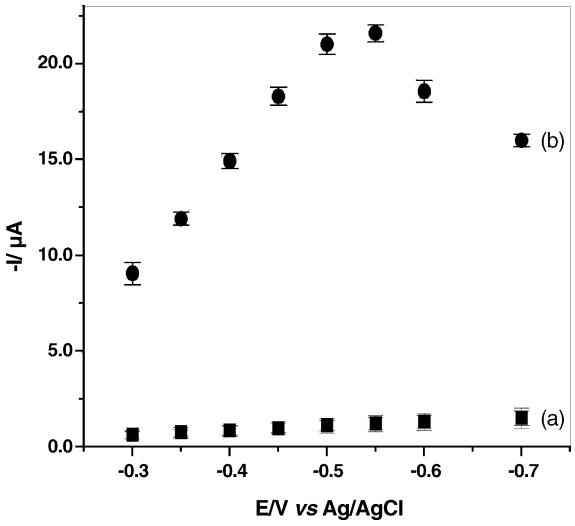

Fig. 3. Influence of applied potential on the enzymatic response. 0.1 mol L^{-1} phosphate buffer solution pH 7.0 containing H_2O_2/KI in the absence (a) and presence (b) of STA–peroxidase conjugate.

$E_{pc} = -0.42$ V) compared to system in the absence of the immobilized STA–HRP conjugate. This behaviour is indicative of the enzymatic activity of HRP.

The investigation of applied potential on the enzymatic response indicated that the current intensities increased significantly with increasing potential from −0.30 to −0.55 V (Fig. 3b). No effect was observed in the PPO 4000 film without the immobilized enzyme as shown in Fig. 3a. The potential of −0.45 V was selected for the further experiments.

Following, the performance of the STA–HRP conjugate in respect to pH solution of the phosphate buffer solution used as support electrolyte was investigated. The enzymatic response exhibited an optimum enzymatic response at 6.5–7.5. The current intensity is constant and close to zero when no peroxidase is immobilized into the PPO 4000 film. Therefore, subsequent experiments were carried out at pH 7.0.

Experiments were also performed using the PPO 300 hybrid. The enzymatic response of the STA–HRP/PPO 300 modified electrodes was lower than PPO 4000. Probably, it could be explained by conformation of the immobilized STA–HRP conjugate into PPO matrices due to the size of the molecular chains (molecular weight of 300 and 4000 g mol^{-1}). Therefore, the PPO 4000 presented a suitable porous which increase efficiently the STA–HRP immobilization [42,43].

3.2. Blocking of the genosensor

Previously, it was observed an adsorptive effect of biotin–HRP conjugate on the PPO film surface. Therefore, the effect of BSA (0.1 mol L^{-1} phosphate buffer solution pH 7.0 containing 0.1% BSA) on the electrode surface blocking was investigated. After blocking, either PPO or PPO/STA films were incubated with different concentrations of biotin–HRP conjugate (1.0, 2.0, 3.0 or 5.0 units mg^{-1} of protein). As can be seen in Table 1, the concentration of biotin–HRP conjugated has no effect on the current intensity values obtained with the films

Table 1
Effect of bovine serum albumin on the electrode surface

Biotin–HRP conjugate (units mL^{-1})	$-I$ (µA)	
	PPO film	PPO/STA film
1.0	0.041 ± 0.004	0.093 ± 0.007
1.5	0.045 ± 0.001	0.106 ± 0.002
2.0	0.049 ± 0.003	0.117 ± 0.002
3.0	0.050 ± 0.001	0.136 ± 0.002
5.0	0.055 ± 0.001	0.165 ± 0.001

$n = 2$.

without STA, indicating that BSA minimizes the unspecific adsorption by blocking the free sites on the sol–gel modified electrodes. On the other hand, the PPO/STA electrode exhibited an enzymatic response proportional to increase of biotin–HRP conjugate concentrations. The percentage of BSA (0.1, 0.5, 1.0 and 2.0%) in the phosphate buffer solution used to blocking of the PPO/STA modified electrode surfaces was also studied. It was not observed any significative difference in the effective blocking of the PPO/STA modified electrode surfaces. Therefore, 0.1% of BSA was established to blocking of the genosensor.

3.3. Optimization of the genosensor

We developed the HCV RNA amperometric biosensor for the detection of HCV genotypes 1, 2A/C, 2B and 3. These specific oligonucleotide probes were chosen due to higher frequency than others genotypes in Brazil [44]. Several strategies were adopted in order to optimize the amperometric genosensor. As the STA could be conveniently immobilized into the PPO 4000, the optimization of the amount of streptavidin, as well as biotinylated oligonucleotide probes (which bind to the STA on the electrode surface) were investigated. The behaviour of the genosensor was evaluated using amplified products (amplicons samples) for the genotype 1 of the HCV. The results are displayed on Table 2. The HCV 1 oligonucleotide probe was prepared in phosphate buffer solution. One set experiment was carried out increasing both the STA concentration (10, 20, 45 and 90 µg mL^{-1}) and the HCV 1 oligonucleotide probe dilution (10, 20, 40 and 80 times diluted). Another one was performed to the STA concentrations increasing and the HCV 1 oligonucleotide probe dilutions decreasing (Table 2). The current intensities values obtained with both experiments were represented versus the STA concentration (Fig. 4). According to Fig. 4b, when both streptavidin concentration and HCV 1 oligonucleotide probe dilution increased, the current decreased slowly. This behaviour indicates that small concentrations of

Table 2
Optimization of STA and probe dilution on the genosensor response

STA (µg mL^{-1})	Probe dilution	$-I$ (µA)	Probe dilution	$-I$ (µA)
10	1:10	1.86 ± 0.02	1:80	1.01 ± 0.03
20	1:20	1.67 ± 0.02	1:40	1.96 ± 0.01
45	1:40	1.37 ± 0.04	1:20	1.47 ± 0.04
90	1:80	1.22 ± 0.01	1:10	1.31 ± 0.05

$n = 2$.

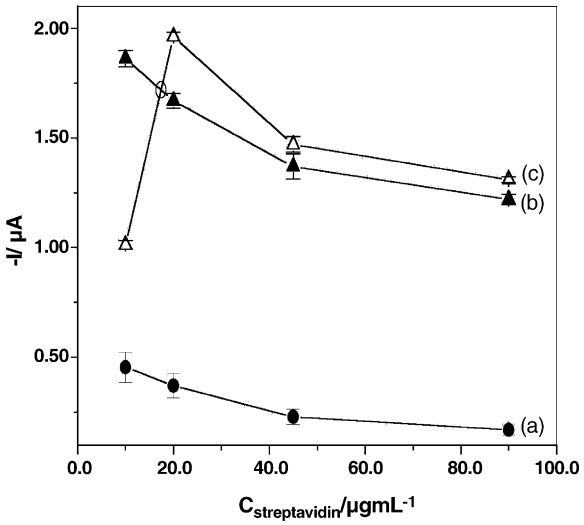

Fig. 4. Optimization of the STA immobilized into the film and the probe dilution. The siloxane–poly(propylene oxide) films containing different concentrations of STA were incubated with c-DNA (a) or the probe HCV 1 (increasing dilution (b) and decreasing dilution (c)) were immobilized through STA, and then incubated with c-DNA.

the immobilized oligonucleotide probe not provide a significant current intensity value. On the other hand, when the STA concentrations increased and the HCV 1 oligonucleotide probe dilution decreased, there was a maximum current intensity at 40 times diluted probe (Fig. 4c). The intersection of the curves (signed as ⊗) was chosen for further experiments, corresponding to the streptavidin concentration of 20 μg mL^{-1} and biotinylated probe 40 times diluted. HCV RNA negative samples presented low values of the current intensities comparable to the background signal (Fig. 4a). It could be observed that the value of current intensity for RNA HCV positive sample was at least four times higher than background signal.

3.4. Determination of the cut-off value for the HCV RNA detection

Following, the establishment of the best conditions for a good performance of the amperometric genosensor, the minimum current intensity value that indicates the presence of HCV RNA in the samples was defined. Thus, the current intensities of five HCV RNA negative samples and negative controls were obtained. The average of current intensity values was -0.37 ± 0.05 μA. Twice the standard deviation was added to average establishing the reactivity threshold as -0.47 μA. Therefore, a sample is considered HCV RNA positive by amperometric genossensor when the current intensity value is equal or higher than -0.47 μA and it is non-infected above that value.

3.5. HCV RNA detection and genotyping (HCV 1, 2A/C, 2B and 3)

The amperometric genosensors were applied to HCV RNA detection in eight samples from HCV-infected patients (Table 3). The samples were previously analyzed by the standard qualitative Amplicor® Hepatitis C Virus Test, and classified as HCV RNA positive (1–5) or negative (6–8) samples. The proposal of this methodology was also develop an amperometric genosensor able to qualitative HCV RNA detection and genotying (HCV 1, 2A/C, 2B and 3), simultaneously. For the PPO/STA modified electrodes prepared with the HCV type 1 (HCV 1), the current intensity values obtained to HCV RNA positive and negative samples were closed to the positive and negative controls, respectively, as shown by Table 3. The HCV RNA positive samples (1–5) were genotyped as HCV type 1 by line probe assay (InnoLiPA HCV) as well as the proposed methodology. All negative and positive controls using oligonucleotides probes HCV 2A/C, 2B and 3 presented similar values and they were also very close to the current intensity values for negative control and negative samples obtained from HCV 1. It means that there were no hybridization between those probes and the positive control because, probably, the standard method (Amplicor® Hepatitis C Virus Test, Roche) uses HCV 1 which is dominant in South America [45]. According to the results obtained by amperometric genosensor prepared with oligonucleotides HCV 2A/C, 2B and 3 all the samples, except sample 7, showed the current intensity values lower than -0.47 μA, i.e., they were HCV RNA negatives for HCV 2A/C, 2B and 3. The sample 7 showed current intensity value twice higher than the cut-off (-0.47 μA) on the

Table 3
Analysis of HCV in sera by spectrophotometric and amperometric methods

Sample	A_{450}[a]	$-I$ (μA)			
		HCV 1	HCV 2A/C	HCV 2B	HCV 3
1	[b]	1.99	0.325	0.329	0.414
2	[b]	2.27	0.456	0.318	0.389
3	[b]	1.89	0.331	0.417	0.451
4	[b]	2.01	0.322	0.364	0.354
5	[b]	2.51	0.417	0.433	0.332
6	0.051 ± 0.002	0.333	0.340	0.421	0.316
7	0.053 ± 0.002	0.326	0.322	0.431	0.863 ± 0.006[a]
8	0.079 ± 0.002	0.424	0.312	0.321	0.341
Positive control[a]	[b]	2.489 ± 0.004	0.331 ± 0.002	0.398 ± 0.002	0.339 ± 0.002
Negative control[a]	0.056 ± 0.002	0.336 ± 0.004	0.412 ± 0.004	0.402 ± 0.002	0.397 ± 0.004

[a] Triplicate.
[b] $A_{450} > 1.0$.

amperometric genosensor for the HCV RNA detection, using the HCV 3 oligonucleotide probe. According to the standard method, the samples 6–8 (HCV RNA not detected by qualitative Amplicor® HCV Test Kit, Roche) cannot be submitted to commercial methods for genotyping because the low absorbance values. Thus, the sample 7 could be considered to patient that demonstrated levels of HCV viremia below of the limit detection of the standard methods for HCV detection.

To determine the variance of repeated runs, the positive (R.S.D. = 0.2%) and negative (R.S.D. = 1.2%) controls were tested five times on different days. Each sample was detected clearly and no significative differences between the results were observed.

In order to application in routine analysis, investigations should be done in respect to quantitative HCV RNA detections and to resolve currently uninterpretable results with commercial methods for HCV genotyping.

4. Conclusions

The precursor 3-(isocyanatopropyl)triethoxysilane and O,O′ bis(2-aminopropyl)poly(propylene oxide) with 4000 g mol^{-1} molecular weight are suitable for streptavidin immobilization. The novel approach described in this work is promising for clinical diagnostics by immobilizing the suitable DNA probe through the streptavidin entrapped into the sol.

The HCV 1, 2A/C, 2B and 3 oligonucleotide probes immobilized on the siloxane–poly(propylene oxide) film prepared by sol–gel route were able to distinguished positive and negative sera samples. The block of the bare surface regions to minimize the unspecific adsorption was very effective by using bovine serum albumin. It was fundamental to guarantee the integrity of the results on genosensor.

The proposed amperometric genosensor is an important tool not only for HCV RNA detection but also as genotyping method.

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