Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 1744–1750
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Surface plasmon resonance immunosensor for human cardiac troponin T based on self-assembled monolayer

Rosa Fireman Dutra [a,b], Renata Kelly Mendes [b], Valdinete Lins da Silva [c], Lauro Tatsuo Kubota [b,*]

[a] *Departamento de Patologia, Instituto de Ciências Biológicas, Universidade de Pernambuco, Rua Arnóbio Marques, 310, Santo Amaro, 50100-130 Recife, PE, Brazil*
[b] *Departamento de Química Analítica, Instituto de Química, Universidade Estadual de Campinas, CP 6154, 13083-970 Campinas, SP, Brazil*
[c] *Departamento de Engenharia Química, Universidade Federal de Pernambuco, Av. Prof. Moraes Rego, s/n, Cidade Universitária, 50740-521 Recife, PE, Brazil*

Received 11 July 2006; received in revised form 11 December 2006; accepted 13 December 2006
Available online 21 December 2006

Abstract

The cardiac troponin T (cTnT) is specific biomarker important for trials of acute myocardial infarctions (AMI). In this paper, a SPR sensor in real time to detect the biomarker was developed on a commercially available surface plasmon resonance AUTOLAB SPIRIT®. The cTnT receptor molecule was covalently immobilized on a gold substrate via a self-assembled monolayer (SAM) of thiols by using cysteamine-coupling chemistry. This biosensor presented a linear response range for cTnT between 0.05 and 4.5 ng/mL ($r = 0.997$, $p \ll 0.01$) with a good reproducibility (CV = 4.4%). The effect of the cysteamine (CYS) concentrations on the SAM coated gold sensor was studied as a function of the amount of the immobilized cTnT monoclonal antibodies. Analysis using serum samples undiluted was carried out at room temperature showing a well agreement with the ECLIA methods and the sensor surface could be regenerated by using a solution of 1% (w/v) sodium dodecyl sulphate (SDS) without losing the sensor immunoreactivity. These studies open new perspectives of using SAM to develop regenerable immunosensor with a good reproducibility allowing its use in the clinical applications.

Keywords: SAM; SPR; Troponin T; Cardiac injury

1. Introduction

Cardiac troponins are part of the new definition of acute myocardial infarction (AMI) by the European Society of Cardiology and the American College of Cardiology (ESC/ACC). They are released into the bloodstream from injured muscle cells during cardiac ischemia with no overlap with skeletal muscle troponins under normal conditions [1]. Multiple studies have demonstrated that both cTnI and cTnT are important prognostic indicators in patients presenting chest pain, even when creatine kinase (CK), MB fraction is not elevated [2]. Particularly, the troponins T are gaining wider acceptance as a tool to stratify patients with chest pain, moreover the detection of cardiac troponin T might also be useful prognosticator in high risk patients whose standard evaluation of myocardial ischemia is not always accurate [3,4]. Several methods have been shown to be able to detect the troponin T such as enzyme-linked immunosorbent assay (ELISA) [5], radioimmunoassay (RIA) [6] and immunochromatographic [7] tests. The first two are more usually used; however, they involve several steps and time consuming, whereas the second is a qualitative test. During the myocardiac infarctions (MI) the troponin T is immediately released to bloodstream. Then a sensor able to monitor this cardiac marker in less than 10 min would improve the patient care by allowing a definitive diagnosis of MI in real-time, and having a lower detection limit is more advantageous in providing more sensitive results (Table 1).

Surface plasmon resonance (SPR) immunosensors have been widely recognized as potential analytical tool due to this technique is extremely sensitive to small changes in the refractive

* Corresponding author. Tel.: +55 19 37883127; fax: +55 19 37883127.
E-mail address: kubota@iqm.unicamp.br (L.T. Kubota).

0731-7085/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.12.013

Table 1
Values of cTnT measured by ECLIA methods and average on three replicates in the SPR sensor from different serum samples

Concentration of cTnT in serum (ng/mL)	
ECLIA methods	SPR sensor
1.66 ± 0.09	1.48 ± 0.07
1.91 ± 0.08	2.10 ± 0.09
2.16 ± 0.06	2.42 ± 0.06
3.16 ± 0.08	2.91 ± 0.08

index near the sensor surface caused by variation of the mass on the transducer surface. Different from well-established biochemical immunoassay like ELISA and RIA; SPR does not require label or tracer reducing the number of steps, permitting real-time analysis of interactions, regeneration of the sensor surfaces and low cost analysis. Surface plasmon resonance is an optical based technique that can be employed for interface studies. This phenomenon which occurs because, although the light is reflected off by thin metal films, a component of the light energy incident at a sharply defined angle can interact with the delocalized electrons in the metal film (plasmon) thus, reducing the reflected light intensity [8]. SPR immunosensing involves, immobilizing antibodies by a coupling matrix to the surface of a thin film of precious metal such as gold. Interaction of antigens with immobilized antibodies to the surface will elicit a change in the refractive index of the surrounding medium to cause a shift in the angle of resonance proportional to the mass of antigens bound to the surface [9]. One of the most important aspects on the development of immunosensors is related to the employed immobilization methods. The way how the antigens or antibodies are fixed and maintained during the reaction with the analyte is related to specificity and sensitivity of the biosensor. Self-assembled monolayers (SAM) is a method of formulation of ultra-thin organic films requiring fewer steps than other approaches. Due to its high stability, good orientation, and easy preparation, the SAMs have become well-established method to immobilize sensor elements for biosensor development. They are ordered molecular assemblies formed by the adsorption of an intended compound on a solid surface [10]. The SAM of thiolate compounds has been widely used utilizing 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimidehydrochloride (EDC)/*N*-hydroxysuccinimide (NHS) for the coupling of biomolecules [11–15]. In this article, an attempted to use cysteamine to form a versatile SAM tightly attached to the gold electrode surface by use of mercapto group. The amino groups of cysteamine SAM make it possible to immobilize antibodies through cross-linking by glutaraldehyde.

Due to SPR does not require label or tracer reducing the number of steps, permitting real-time analysis of interactions, regeneration of the sensor surfaces and low cost analysis, etc. innumerous SPR systems were developed including the commercial BIAcore system [16–19]. Although, the BIAcore system has dominated the market since their introduction, a number of SPR instruments more sensitive have been recently introduced. In this commercial system (AutoLab Spirit®), the reflection is measured as a function of the incident angle of the light beam. The incident angle is changed by using a vibrating mirror that permits a broad range of incident angle that is measured within a short time. In the vibrating mirror set-up, the angular change measured for a non-coated gold sensor surface has a resolution of approximately 0.5 millidegree, corresponding to a refractive index resolution of approximately 1×10^{-5}. For a coated gold sensor surface, the angular change is measured with a resolution of approximately 1 millidegree corresponding to 8.3 pg/mm^2 of protein bound to the coated surface.

In this report, the feasibility of a self-assembled monolayer as a ligand of antibodies on the human cardiac Troponin T detection is discussed in more detail.

2. Materials and methods

2.1. Reagents

All the reagents were prepared with chemicals of analytical grade. All chemicals except special remark were purchased from Sigma–Aldrich Chemical (USA). The monoclonal anti-TpT biotin conjugated antibodies (biotinylated mab cTnT) and the human cardiac troponin T were acquired from Roche Diagnostics (Germany). The water used in all solution preparations was obtained from a Millipore unit (USA).

Samples of Human Cardiac Troponin T were collected from venous blood and immediately centrifuged for 120 s at 1150 rad/s. An aliquot of serum was analyzed and the remaining serum was frozen at −20 °C and stored for 3–5 weeks. Donors' sera were provided by Oswaldo Cruz Hospital Central Laboratory (Recife, Brazil).

The quantitative determinations of Troponin T in human serum were processed in an automatic system Roche Elecsys 2010 immunoassay analyser based on ECLIA-Electrochemiluminescence immunoassay [20].

A phosphate buffered saline (PBS), pH 7.4, 10 mM, used in all experiment, except special remark, was prepared by dissolving 0.2 g KCl, 8.0 g NaCl, 0.24 g KH_2PO_4 and 1.44 g Na_2HPO_4, in 1000 mL ultra-pure water.

2.2. Experimental set-up

The molecular interactions between mab-cTnT and cardiac troponins were investigated using a SPR spectroscopy (AutoLab Spirit®, Eco Chemie, The Netherlands). The planar gold SPR discs were purchased from Xantec Bioanalytics (Germany) and are ideal for this system. This equipment is a surface plasmon resonance (SPR) instrument based on Kretschmann configuration that is the most used configuration. The samples and the washing solution were injected in the flow cell using an autosampler equipped with a peristaltic pump. The pumping rate was set to 100 μL/min and the volume of flow cell was 150 μL. The flow of solution was programmed to stop during the incubation steps. The response of the SPR sensor was automatically monitored on the PC by a SPR software 4.1.2 version from Eco Chemie. The schematic representation of the SPR requirement and the detection principle-SPR sensorgram is shown in Fig. 1.

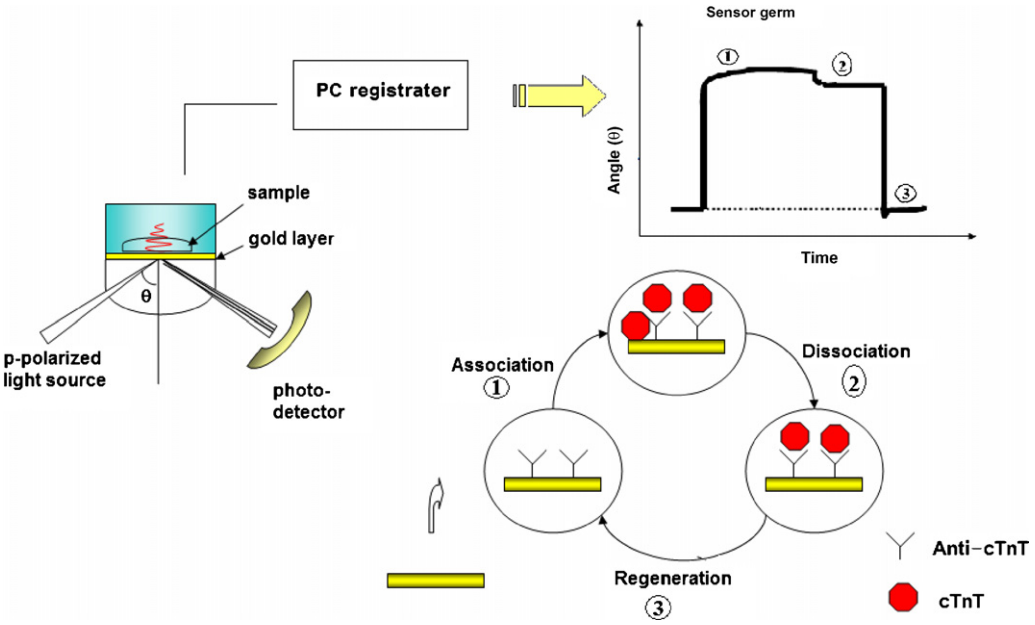

Fig. 1. Schematic representation of the principle of SPR immunosensor for cTnT determinations.

2.3. Immobilization of mab-cTnT on the sensor

After placing the bare gold sensor disc onto the prism of SPR system, its surface was cleaned with ethanol at flow 100 μL/min during 200 s to remove all organic contaminants and the resonant angle at this point was recorded as baseline. The SAM layer of thiols in the bare gold sensors as prepared by injection of a solution freshly prepared 25 mM CYS in ethanol (100 μL) into the flow cell; the change of resonance angle was monitored during an analytical run for 2 h of deposition. After that, the gold substrates were thoroughly rinsed with PBS at flow during 2 min to remove excess of material unbounded. The change of resonance angle was monitored in this point. At following, the sensor surface was incubated with 100 μL of a solution also freshly prepared 2.5% (v/v) glutaraldehyde (GLUT) in PBS (pH 7.4; 50 mM) during 50 min. The sensor disc was rinsed in PBS by 2 min at flow. Finally, the SAM on sensor disc was ready. Immobilization of the mab-cTnT on the SAM was carried out in the SPR system. Then, 25 μL of mab-cTnT (1.5 μg/mL) in PBS was injected in the flow cell for 1 h. The experiment was conduced at temperature of 21 ± 1 °C. Then, a covalent cross-linking by amino reactive group of mab-cTnT with the aldehyde terminals was achieved. Fig. 2 shows a schematic representation of mab-cTnT immobilization on the SAM. After that, the free reactive aldehyde groups were blocked using a solution of 10 mM glycine in PBS during 30 min.

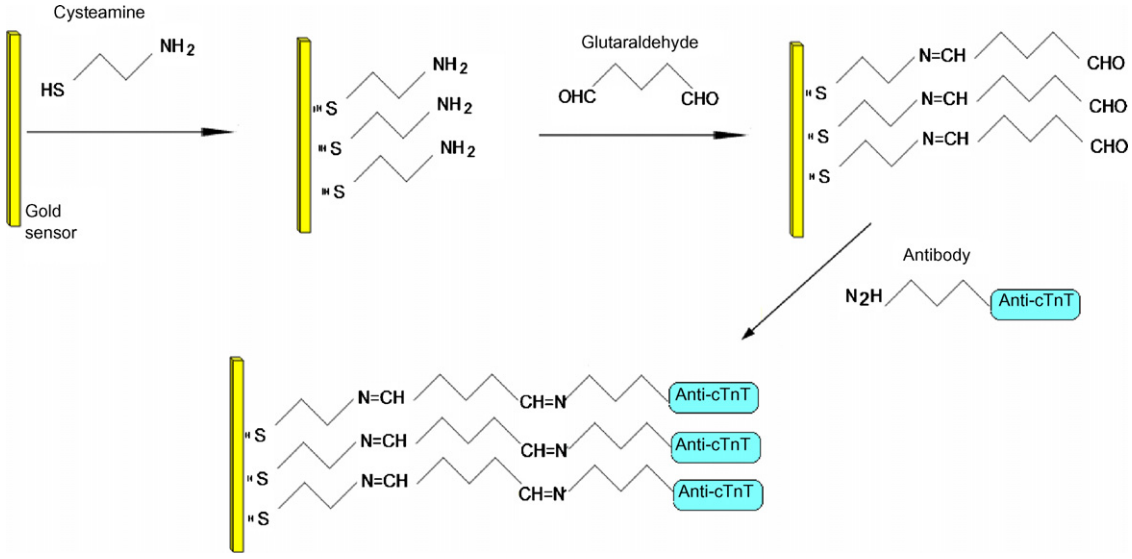

Fig. 2. Ilustrative scheme for the mab-cTnT immobilization.

2.4. Kinetic analyses of the cTnT and mab-cTnT

The kinetic analyses of the reaction of cardiac biomolecules with SPR biosensor included the following steps: (a) establishment of the baseline—after coated mab-cTnT, the flow cell was washed with PBS during 2 min at flow rate of 100 μL/min. The resonant angle was monitored until the baseline was stabilized, (b) association—undiluted sample containing human cardiac troponin (20 μL) was injected in the flow cell and incubated during 1000 s (about 14 min) at stopped flow, while the SPR signal was monitored. Meanwhile, the mixer was turned on every 2 min during 20 s. The interval of incubation was established in our previous studies with serum samples; at this time, the monitored SPR signal reaches the plateau, when the highest concentration of the measured cTnT by this system was bound to the sensor surface. After that, the flow cell was PBS washed during 2 min, the baseline was set-up about 120 s resonant angle monitoring; (c) dissociation—the non-specific adsorptions were removed by two PBS washing for 120 s at flow of 100 μL/min; (d) regeneration—the interaction between immobilized mab-cTnT and cTnT was removed by injection of a solution of 1% (w/v) SDS dissolved in PBS in the flow cell (100 μL) during 2 min; afterwards the flow cell was washed with PBS and the baseline was restored. Then, the coated sensor disc was ready to new runs. This experiment was performed at room temperature (21 ± 1 °C).

3. Results and discussion

3.1. Preparation of the SAM

The adsorption of SAM on gold sensor was accomplished by changing in the resonant angle continuously monitored at the SPR system in six replicates analysis. When the solution of 25 mM CYS was injected in a flow cell during approximately 2 h, the resonant angle shifted about 125 millidegree with a low coefficient of variation (CV = 10.3%) as a result of the SAM tightly attached to the gold electrode surface by using thiol group. The use of ethanol instead of water or buffers to dilute CYS promoted a more homogeneous layer. After GLUT incubation followed by PBS washing, a shift of 238 millidegree was observed. The incubation time of glutaraldehyde can affect the multilayer of the immobilized component on the surface. In this assay, the incubation time was controlled at 45 min and the reproducibility achieved in six assays showed a good CV = 12.0%. Although more amount of mab-cTnT was immobilized when the incubation time was up to 45 min, the CV was higher as a result of the uncontrolled multilayer. Fig. 3 shows an illustrative sensorgram of the SAM preparation on gold sensor. The function of GLUT was immobilizing the antibody through cross-linking by amino reactive groups [21]. Then, based on the shift on the resonant angles, a SAM layer was performed on the gold sensor.

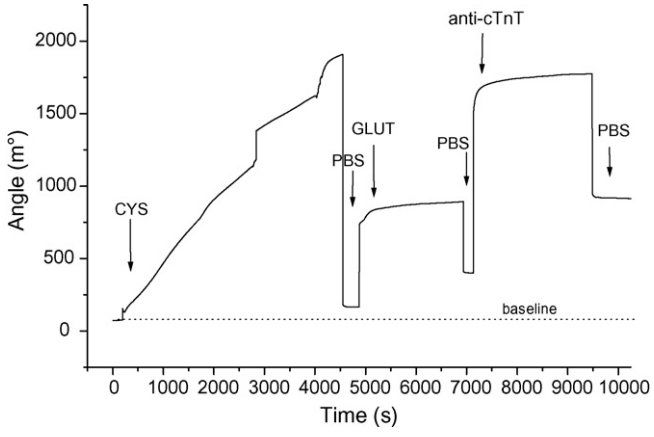

Fig. 3. Typical sensorgram for the SAM preparation and immobilization of mab-cTnT.

3.2. Analysis of mass loading by immobilization of mab-cTnT

The SPR biosensor has been widely used for the monitoring of various affinity bindings [22]. Using the SPR biosensor, the amounts of mass loading by mab-cTnT could be estimated. During the layer preparation, the SPR signal was measured online. The mass loading was calculated from the baseline changes before and after sample injection. The SPR signal can be converted to the mass loading on the sensor surface by the following relation: 120 millidegree = 1 ng/mm^2 [23]. As also shown in Fig. 3, the chemical adsorptions of mab-cTnT to the SAM layer-gold surface resulted in changes on average of 538 millidegree that was presumed be 4.48 (±0.35) ng/mm^2.

The evaluation of association constant (k_{ass}) of m_{Ab} cTnT were calculated according to the formulas of kinetic analysis [24] and was processed in the ESPIRIT KINECTIC EVALUATION software, module from the AutoLab company (Eco Chemie, The Netherlands). The linear relationship is used for linear regression; the slope of linear regression straight line is used to calculate k_{ass}. The slope of the linear regression straight line the product of k_{ass} and the maximum angle shift (R_{max}). Therefore k_{ass} can be calculated as $k_{ass} = X_{slope}/R_{max}$ and $k_{ass} = 1.32\ mol^{-1}\ L\ s^{-1}$.

3.3. Optimisation of the coupling condition

The effect in the amount of antibodies immobilized as a function of the CYS concentration was investigated. Then, a higher concentration of CYS than 25 mM was tested so that more amounts of antibodies could be immobilized. The concentration of CYS was duplicated to 50 mM. As noted in Fig. 4, it was a discrete increase in the shift of resonant angle, for the CYS monolayer. However, after injection of 25 μL mab-cTnT, the difference in amount of immobilized antibodies was not significant as compared to the 25 mM CYS. Then, the concentration of 25 mM was chosen for the SAM preparations.

The improvement on the orientation of the immobilized antibody with the poly-L-lysine employment was previously observed [25,26]. In this work, when the L-lysine was added on the adsorbed CYS-GLUT layer on gold sensor in a concentration of 0.1% (w/v) in 0.1 M carbonate buffer (pH 9.5) and incubated during 20 min, the results was in contrast to the observed in the literature. As noticed in Fig. 5, after samples of cTnT were

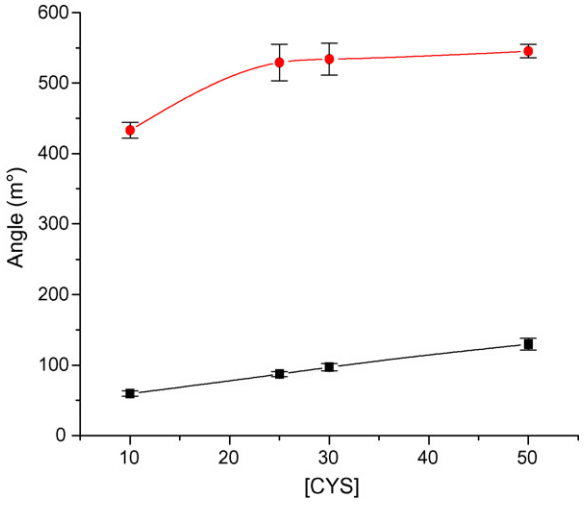

Fig. 4. Concentration of CYS as function of the resonant angle for thiol monolayer (-■-) and amount of immobilized mab-cTnT (-●-). Means in three replicates assays.

injected in a flow cell, and the calibration curves were plotted, the sensitivity determined by the channel with lysine was 38.7% lower than the control.

3.4. Response to the cTnT by SPR sensor

The calibration curves of the immunosensor were obtained from successive injections of cTnT at the concentration of 0.5 ng/mL (20 μL) followed by PBS washing during 120 s at 100 μL/min after each injection. The cTnT samples prepared in PBS (pH 7.4; 10 mM) were incubated during 800 s at stopped flow, while the SPR signal is being monitored. Meanwhile, the mixer was turned on every 2 min during 20 s. The interval of incubation was established in our previous studies with serum samples; at 800 s the monitored SPR signal reaches the plateau when the highest concentration of the cTnT measured by this

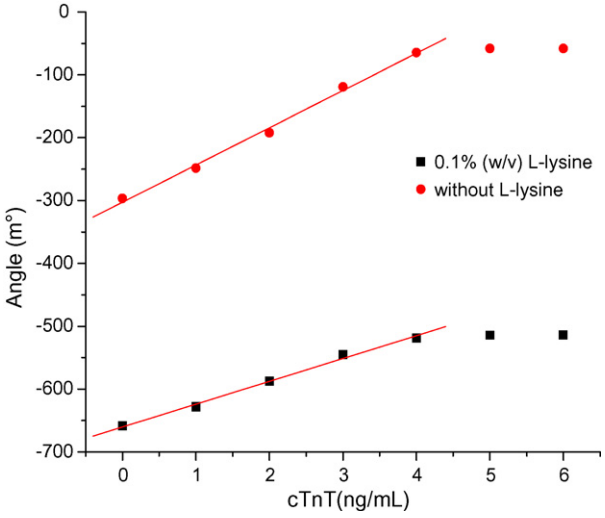

Fig. 5. Effect of the L-lysine use after SAM. (cysteamine plus glutaraldehyde) on the sensitivity of the immunosensor. The calibration curves obtained with 1.5 μg/mL concentration of the immobilized mab-cTnT.

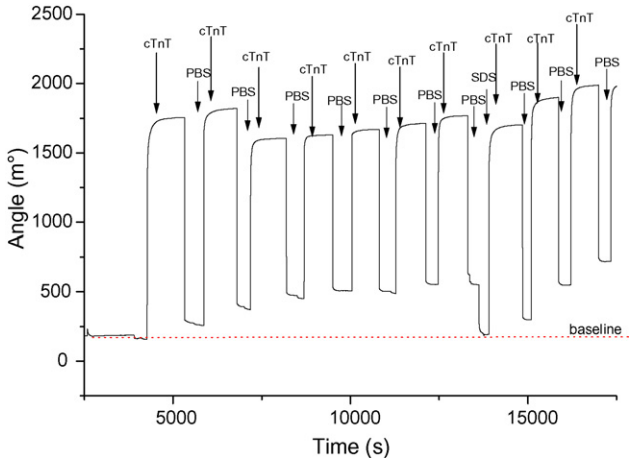

Fig. 6. Sensorgram of response of immunosensor for cTnT in successive injections of cTnT. The change in resonance angle was considered after the injection of PBS. The experiment was conduced at room temperature (22 ± 1 °C) at pH 7.4.

system was bound to the sensor surface. The washing with PBS had the function of dissociating the excess of non-bounded antigens, which imply on false response of the sensor. Moreover the effect due to the different refractive indexes of the samples and PBS stated as baseline are compensated when the flow filled with PBS is monitored, then the changes in the resonant angle were calculated from difference between PBS and previous baseline (Fig. 6). According to the calibration curve, the immunosensor reached a plateau at 6.0 ng/mL of cTnT and the linear range was up to 4.5 ng/mL (Fig. 7a). The dates from the plotted curve presented a high linearity: $r = 0.997$ ($p < 0.0001$, $n = 5$), combined with a low relative error of 4.4% (Fig. 7b).

The effect of temperature was studied as function of the anti-cTnT and cTnT interaction. Fig. 8 shows the thermody-

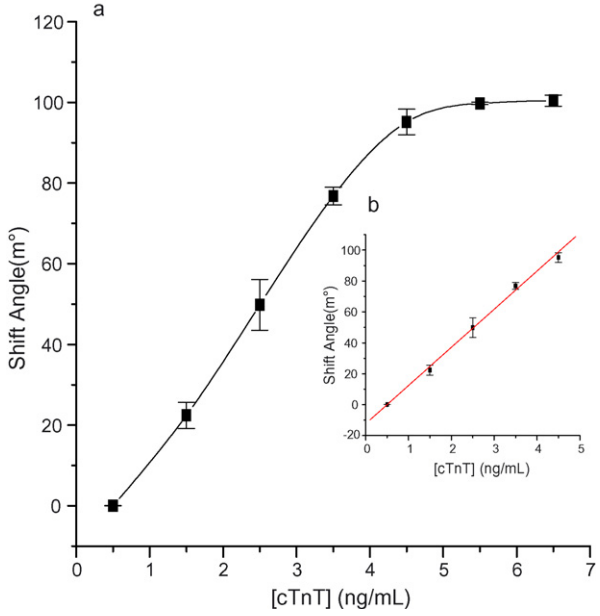

Fig. 7. (a) Calibration curve of SPR sensor for cTnT injections with immobilized mab anti-TpT (1.5 μg/mL); (b) linear fit as function of cTnT concentration in three replicates run.

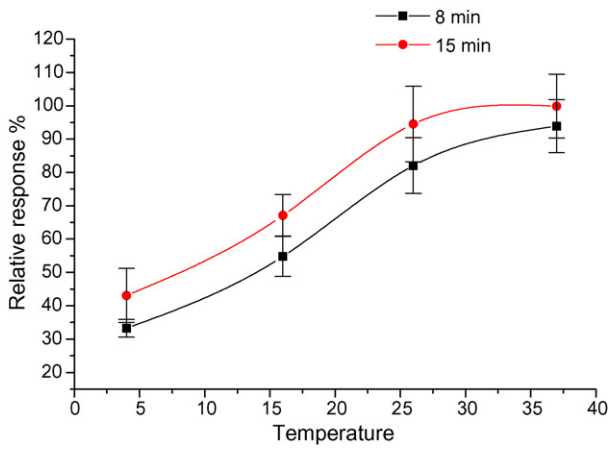

Fig. 8. Effect of temperature and incubation time as function of interaction of anti-cTnT and cTnT. The error bars represent the standard deviation in four replicates.

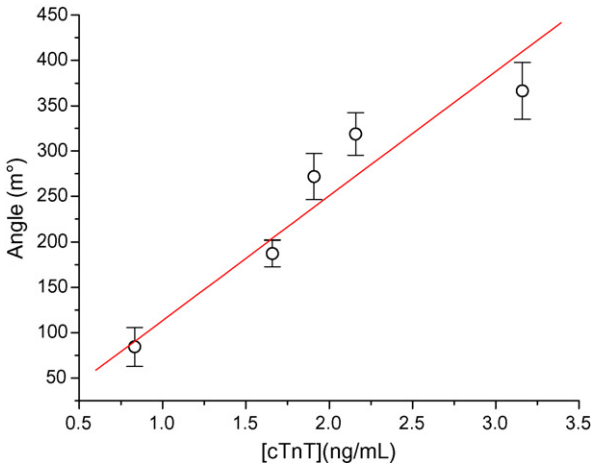

Fig. 9. Response of SPR biosensor for the samples of serum at different concentration of cTnT measured by ECLIA methods. Means of three replicates analysis after regeneration with 1%SDS.

namic curves established at two different time of incubation: 4 and 15 min. As noticed, the curves reached the plateau approximately at temperature up to 30 °C. At incubation time of 15 min an increase of 5.36% as against 11.4% for 8 min was observed. Then, the incubation time of 15 min was chosen to remain studies.

3.5. Measuring in the human serum samples

To accomplish the human cTnT SPR response in complex medium, serum samples (20 μL) was injected in the flow cell and incubated during 800 s, after that the non-specific adsorption was removed washing out with PBS at 100 μL/min and the shift of resonant angle was monitored for 120 s. The experiment was conduced at room temperature (21 ± 1 °C). The main advantage of the stopped flow rate during the incubation steps is the practice, faster and is possible to measure the cTnT without dilution of serum sample. A low volume of human serum used is often desirable in the conventional methods of clinical routine.

The amount cTnT of serum samples was previously measured by Roche Elecsys 2010 immunoassay analysers based on ECLIA—electrochemiluminescence immunoassay. The SPR curve plotted as response to the concentration of cTnT in the serum samples presented a linear correlation, $r = 0.948$ ($p < 0.01$), see Fig. 9. The error bars corresponding to standard deviation from three replicates analysis from a single sample after regeneration with 1% (w/v) SDS. The measurements with this SPR sensor to cTnT determinations showed a good agreement with the ECLIA methods at 95% confident level when paired *t*-test was applied. The CV of regeneration of 8.26% from triplicates analysis of five serum samples of cTnT, at concentrations of 0.83, 1.66, 1.91. 2.16 and 3.16 ng/mL was determined. Comparing to relative error in the calibration curves in Fig. 7, the CV was 4.4%, which implies an increment of 3.85% for equal number of analysis. The results showed that 1% SDS surfactant was needed to restore the baseline and preserved a good sensitivity of immunosensor.

4. Conclusion

In the developing optical sensor, the self-assembly technique offers interesting perspectives. By generating functionalized surfaces through modification of thiols, it was possible to selectively attach the biomolecules of interest. Moreover, the use of SAM to covalently immobilize antibodies permitted repetitive measurements with cost saving. The use of SDS as surfactant makes possible the regeneration of immunosensor implies on a relative error less than 10% in approximately 15 assays. This developed sensor for cTnT determination in human levels is a real time manner to trials AMI without dilutions of serum and with a desirable volume of sample. However several samples need to be validated for set-up in clinical application.

Acknowledgments

The authors acknowledge CNPq, FAPESP and FINEP agencies from Brazil for financial support. The assistance of Doctor Dilenia Cipriano from the Oswaldo Cruz hospital central laboratory (Recife, Brazil) is also acknowledged.

References

[1] J.S. Sarko, C.V. Pollack Jr., J. Emerg. Med. 23 (2002) 57–65.
[2] F.S. Apple, A.H.B. Wu, Clin. Chem. 47 (2001) 377–379.
[3] H. Herkner, U. Waldenhofer, A.N. Laggner, M. Mullner, E. Oschatz, S. Spitzauer, G. Gamper, A. Bur, M.M. Hirschl, Resuscitation 49 (2001) 259–264.
[4] E.M. Ohman, P.W. Armstrong, R.H. Christenson, C.B. Granger, H.A. Katus, C.W. Hamm, M.A. O'Hanesian, G.S. Wagner, N.S. Kleiman, F.E. Harrell, R.M. Califf, E.J. Topol, N. Engl. J. Med. 335 (1996) 1333–1341.
[5] H.A. Katus, A. Remppis, S. Looser, K. Hallermeier, T. Scheffold, W. Kubler, Mol. Cell. Cardiol. 21 (1989) 1349–1353.
[6] B. Cummins, M.L. Auckland, P. Cummins, Am. Heart J. 113 (1987) 1333–1344.
[7] K. Penttilä, H. Koukkunen, A. Kemppainen, M. Halinen, T. Rantanen, K. Pyörälä, I. Penttilä, Intern. J. Clin. Lab. Res. 29 (1999) 93–101.
[8] B. Leidberg, C. Nylander, I. Lundstrom, Biosens. Bioelectron. 10 (1995) 1–9.

[9] M.H.F. Meyer, M. Hartmann, M. Keusgen, Biosens. Bioelectron. 21 (2006) 1987–1990.
[10] J.C. Pyun, S.D. Kim, J.W. Chung, Anal. Biochem. 347 (2005) 227–233.
[11] B. Johnson, S. Lofas, G. Lindquist, Anal. Biochem. 198 (1991) 268–277.
[12] J.E. Pearson, J.W. Kane, I. Petraki-Kallioti, A. Gill, P. Vadgama, J. Immunol. Meth. 221 (1998) 87–94.
[13] D.J. O'Shannessy, M. Brigham-Burke, K. Peck, Anal. Biochem. 205 (1992) 132–136.
[14] B.-K. Oh, Y.K. Kim, W. Lee, Y.M. Bae, W.H. Lee, J.W. Choi, Biosens Bioelectron. 18 (2003) 605–611.
[15] Subramanian, J. Irudayaraj, T. Ryan, Biosens.Bioelectron. 21 (2006) 998–1006.
[16] M.-R. Stephanie, D. Dupont, P. Dulieu, Food Agric. Immunol. 15 (2003) 265–277.
[17] M. Liljeblad, I. Ryden, S. Ohlson, A. Lundblad, P. Pahlsson, Anal. Biochem. 288 (2001) 216–224.
[18] M. Liljeblad, A. Lundblad, P. Pahlsson, Biosens. Bioelectron. 17 (2002) 883–891.
[19] X. Liu, J. Wei, D. Song, Z. Zhang, H. Zhang, G. Luo, Anal. Biochem. 314 (2003) 301–309.
[20] G. Klein, M. Kampmann, H. Baum, T. Rauscher, T. Vucovic, K. Hallermayer, H. Rehner, M. Muller-Bardorff, H.A. Katus, Wien Klin Wochenschr Suppl. 3 (1998) 40–51.
[21] S.-F. Chou, W.-L. Hsu, J.-M. Hwang, C.-Y. Chen., Biosens. Bioelectron. 19 (2004) 999–1005.
[22] R. Karlsson, A. Michaelsson, L. Mattsson, J. Immunol. Meth. 145 (1991) 229–240.
[23] E. Stenberg, B. Persson, H. Roos, C. Urbaniczky, J. Coll. Interf. Sci. 143 (1991) 513–526.
[24] P.R. Edwards, R.J. Leatherbarrow, Anal. Biochem. 246 (1997) 1–6.
[25] K.K. Toyobo (TOYM), Japan, Patent JP2004125624-A (2004).
[26] X.C. Zhou, J.Z. Zhou., Proteomics 6 (2006) 1415–1426.