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A bioconjugated polyglycerol dendrimer with glucose sensing properties

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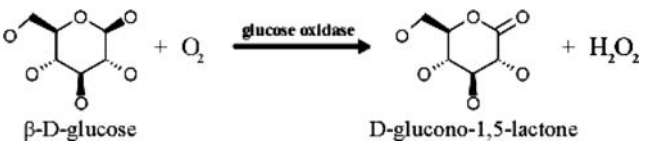
Abstract In this work, the biological and electrochemical properties of glucose biosensor based on polyglycerol dendrimer (PGLD) is presented. Streptokinase (SK), glucose oxidase (GOx) and phosphorylcholine (PC) were immobilized onto PGLD to obtain a blood compatible bioconjugate with glucose sensing properties. The bioconjugated PGLD was entrapped in polyaniline nanotubes (PANINT's) through template electrochemical polymerization of aniline. PANINT's were used as electron mediator due to their high ability to promote electron-transfer reactions involving GOx. Platelet adhesion, fibrinolytic activity and protein adsorption were studied by in vitro experiments to examine the interaction of blood with PGLD biosensor. The PGLD biosensor exhibits a strong and stable amperometric response to glucose. The enzyme affinity for the substrate (K_M^{app}) indicates that the enzyme activity was not significantly altered after the bioconjugation of GOx with PGLD dendrimer. The bioelectrochemical properties suggest that the bioconjugated PGLD developed in this work appears to be a good candidate for providing interfaces for implantable biosensors, especially oxidoreductase-based sensors.

1 Introduction

It is well known that diabetes mellitus is an important health problem worldwide and it is estimated that the number of persons with diabetes could grow to 350 million, in the next 25 years [1]. Enzyme glucose biosensors for in vitro assays have been developed extensively to better monitor the glycemia of diabetic patients and thus reduce the occurrence of long-term complications associated with diabetes improving the life quality of the patients [2].

Glucose oxidase (GOx)-based electrodes represent a major application of immobilized enzymes in medicine for monitoring the glycemia of diabetic patients [3]. Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC1.1.3.4) has long been the key to a variety of blood glucose assays that has had an important role in development of biosensors for diabetes therapy. GOx hydrolyzes glucose according to the following reaction:



The production of hydrogen peroxide (H_2O_2) is in direct proportion to the glucose available and their formation may be detected amperometrically measuring the electrical current produced by the reaction:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$

Recently, a great effort has been made to the development of GOx biosensors that are potentially implantable to monitor glucose levels remotely and continuously for applications in insulin pumps, but the biocompatibility remains an issue [4, 5]. The foreign-body inflammatory response may directly interfere with biosensor function, reducing glucose concentration in the interstitial fluid, or alter the transit time of glucose from blood vessels through interstitial fluid.

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We have recently been interested in the bioconjugation of polyglycerol dendrimers (PGLD) with the fibrinolytic enzyme streptokinase (SK) [6]. Streptokinase (SK), a 47-kDa single-chain protein is a thrombolytic agent commonly used in myocardial ischemia [7]. SK is commonly referred to as plasminogen activator, since their mode of action is through the conversion of the enzymatically inert plasminogen of the fibrinolytic system to an active protease, plasmin that dissolves the fibrin, the main component and skeleton of a blood clot or thrombus [8]. The bioconjugated PGLD-SK have a potential attractiveness for their use in development of implantable glucose biosensors due to their antithrombogenic property [6].

The available hydroxyl functional groups at PGLD-SK surface may be biofunctionalized in a second synthetic step and used as reactive sites for immobilization of phosphorylcholine (PC) and glucose oxidase (GOx). The improvement of the blood compatibility of synthetic polymers by inclusion of PC moieties to the hydroxyl groups of PGLD-SK may be a convenient strategy that aims to defeat the body's multiple defenses. PC is found on the extracellular surface of the lipid bilayer that forms the matrix of the plasma membrane, namely the terminal group of phosphatidylcholine and sphingomyelin [9]. The in vivo biocompatibility of polymers containing such synthetic phospholipids has previously been confirmed [10–12].

Up to now, the relationship between blood compatibility and glucose sensing properties of a multibiofunctional PGLD containing the immobilized biomolecules GOx, PC and SK have not been thoroughly investigated. In this study, a multibiofunctional PGLD in which streptokinase (SK), glucose oxidase (GOx) and phosphorylcholine (PC) were simultaneously immobilized were prepared and the response of the amperometric glucose biosensor as well as their blood compatibility was evaluated.

2 Experimental

2.1 Dendrimer synthesis and immobilization of phosphorylcholine

The polyglycerol dendrimer (PGLD) of generation 4.0 [$M_n = 3{,}724$, $M_w/M_n = 1.2$ (GPC), average 48 hydroxyls each molecule] was prepared by the slow addition of glycidol to a solution containing a partially deprotonated polyol-core at 120°C in argon atmosphere for 24 h [6]. Afterward, the phospholipids derivative, 11-mercaptoundecylphosphorylcholine (SPC), synthesized by reduction of 1 mmol phosphorylcholine-ended disulfide developed in our laboratory using borohydride, was added to PGLD under mild shake overnight to immobilization of phosphorylcholine (PC) groups on PGLD periphery. The PGLD-PC was purified chromatographically and their purity was analyzed by high performance chromatography (HPLC).

2.2 Covalent immobilization of glucose oxidase and streptokinase

The enzymes glucose oxidase (GOx E.C.1.1.3.4, 148 U mg^{-1}, Sigma–Aldrich) and streptokinase (SK, ≥3,500 units/mg solid, Sigma–Aldrich) were chemically immobilized onto PGLD dendrimer surface through a condensation reaction using activation by a cyanylating reaction to the hydroxyl groups on dendrimer surface via amide bond using 1-Cyano-4-(dimethylamino)-pyridinium tetrafluoroborate (CDAP) [13]. Leaving the activated PGLD in the GOx solution (3 mg ml^{-1}) and streptokinase (1 mg ml^{-1}) at 4°C overnight completed the enzymes immobilizations. The bioconjugated PGLD was purified by dialysis. The amount of immobilized GOx or SK were calculated by measuring the concentration of the proteins in the supernatant and then subtracting from the total free enzyme amount using HPLC.

2.3 Preparation of enzyme electrode

The glucose biosensing electrodes were prepared by immobilizing the bioconjugated PGLD within a ramified network of protonated polyaniline nanotubes (PANI) electrochemically deposited at thin aluminum films (Alcoa, 99.99%, 30.0 μm). The electrochemical polymerization solution consisted of redistilled aniline (0.1 M), the bioconjugated dendrimer (GOx: 4.2 U ml^{-1}) and *p*-toluene sulphonate (1 M) in sodium phosphate buffer saline (NaPBS) (0.1 M, NaH_2PO_4/Na_2HPO_4, 0.05 mol l^{-1} NaCl, pH 7.4) as electrolyte. The nanotubes growth was terminated in the potential cycle at −100 mV vs a saturated calomelan electrode (SCE), so that the PANINT's were in the reduced state and to achieve an estimated charge loading of 30 mC. The electrodes were thoroughly washed with PBS pH 7.4 buffer and stored in NaPBS for 24 h to remove any excess on residual monomer and some bioconjugated PGLD weakly bound to the PANINT's. After the electrochemical entrapment of the bioconjugated dendrimer within a ramified network of PANINT's, the glucose sensing property of the biosensor was measured amperometrically.

The morphology of the resultant glucose biosensors was characterized by scanning electron microscopy (SEM). SEM measurements were conducted on a Phillips XL 30 instrument using an accelerating voltage of 20 kV.

2.4 Photoelectron spectroscopy

The presence of the bioconjugated PGLD in PANINT's were analyzed using an ESCA-36 (McPherson Co) X-ray Photoelectron Spectrometer. The X-ray gun was operated at 15 kV voltages and 20 mA. Survey and high-resolution spectra were collected using 80 and 40 eV pass energy, respectively. The pressure in the analyzer chamber was of the order of 10^{-7} and 10^{-8} Torr. The curve fitting was carried out using a nonlinear least squares curve-fitting program with a gaussian/lorentzian product function. The C_{1s} binding energy was taken as 284.6 eV for calibration purposes.

2.5 Bioelectrochemical characterization of the biosensor

The sensitivity of the glucose biosensor was assayed by measuring the current response due to the formation of hydrogen peroxide during the GOx-catalyzed reaction of glucose oxidation at surface PGLD biosensor. The electric current produced in biosensor was proportional to the glucose concentration.

The background current, I_b, of the enzyme electrode, which was allowed to decay to a steady state, was first determined at a given potential, 37°C and pH 7.4. Then, the enzyme electrode was moved immediately into a separate PBS containing a known concentration of glucose to measure the response current. The response current I of the enzyme electrode was considered to be the difference between the measured current I_b and I_S, i.e., $I = I_S - I_b$.

The current measurement across the biosensor was performed with a programmable electrometer (Keithley model 237). The working electrode was the PGLD bioconjugated/PANINT's and a saturated calomelan electrode (SCE) was used as reference electrode.

2.6 Amidolytic activities

Amidolytic activities of free and immobilized streptokinase were determined using the chromogenic assay using as substrate, N-α-carbobenzoyl-L-lysine-*p*-nitrophenyl ester (CLN, Sigma Co) [14]. One CLN units of Sk was defined as a 0.01 increase per minute in the absorbance at 340 nm in the above conditions.

2.7 CD62P expression

Platelet adhesions in vitro experiments were carried out to examine the interaction of blood with PGLD biosensor surface in ELISA plates [15]. Platelet rich plasma was prepared from fresh blood drawn obtained from healthy adult donors who had not taken aspirin containing drugs for at least 10 days prior to donation. Blood was collected from antecubital veins into siliconized tubes containing citrate phosphate dextrose anticoagulant (0.7 mM citric acid, 9.3 mM sodium citrate and 13.6 mM dextrose) and adenine (CPD-A1, Sigma Co) at a ratio of 1.4 and subjected to centrifugation at 800 × *g* for 10 min at 22°C to obtain platelet rich plasma (PRP). A volume of 200 µl was pipetted into PGLD bioconjugated/PANINT's coated ELISA plates and incubated at 37°C for 1 h. Immediately after incubation, 100 µl of PRP was put into tubes containing 20 µl of fluorescein isothiocyanate (FITC) conjugated anti-CD63 monoclonal antibody and the same amount of phycoerythrin (PE) conjugated anti-CD62P monoclonal antibody (Immunotech S.A.) and incubated at 25°C. After the platelets were washed twice in phosphate-buffered saline solution (PBS, pH 7.4) the amount of antigen expressing platelets and fluorescence intensity were measured with a flowcytometer. A group of platelets was gated, and the expressions and the fluorescence intensities of FITC and PE were measured at 525 and 575 nm, respectively. The ELISA assay detection limit for CD62P in this work was 0.96 ng l^{-1}.

3 Results and discussion

The PANINT's may be considered to be an attractive material to the development of biosensors due to their property of rapid electron transfer. Figure 1a shows the SEM micrograph of the PANINT's containing the bioconjugated PGLD deposited at Al electrodes. It is found in SEM studies that PANINT′s have diameters of approximately 90 nm. The length of the fibers range from 1,000 nm and was observed that in the presence of the bioconjugated dendrimers the nanofibers tend to agglomerate into interconnected nanofibers networks. The SEM microstructure analysis demonstrates that the nanotubes formation may be obtained as consequence of the nucleation of aniline monomers into the formed aluminum pores when the oxidation process takes place. The mechanism of the aniline polymerization appears to proceeds with increment of new aniline molecules that can interact with each other, through the amine and iminium nitrogen interaction forming at the same time a fibrous PANI nanostructures (PANINT's). Thus, an electrode with glucose sensing properties may be obtained by the entrapment of the bioconjugated PGLD during the nanotubes growth on metallic surface. Due to their high surface area the electrical properties of PANINT's would drastically enhance the biosensor performance due to the increase of accessible binding sites within the polymer.

The presence of the bioconjugated PGLD on PANINT's was confirmed by XPS spectroscopy (Fig. 1b). The

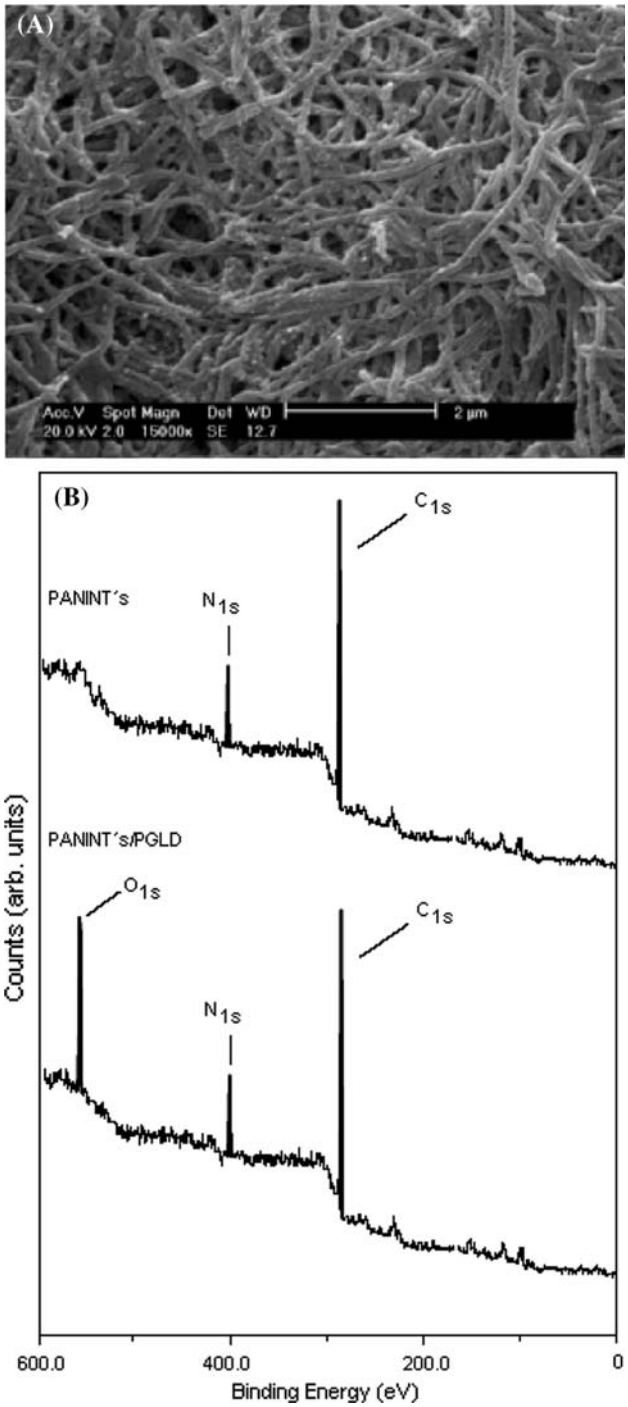

Fig. 1 SEM micrograph (**a**) and XPS survey scans (**b**) of PGLD biosensor surface. Magnification: 15,000×

comparison between PANINT's and PANINT's/PGLD it allows to distinguish the O_{1s} signal at 530–534 eV. The high-resolution O_{1s} XPS spectra (not shown here) are consistent with the C_{1s} profiles, and are comprised mainly of C–O (530.5–532.5 eV). The peak associated with the ethereal carbon in PGLD (C–O) structure may be noted at 285.4–286.2 eV.

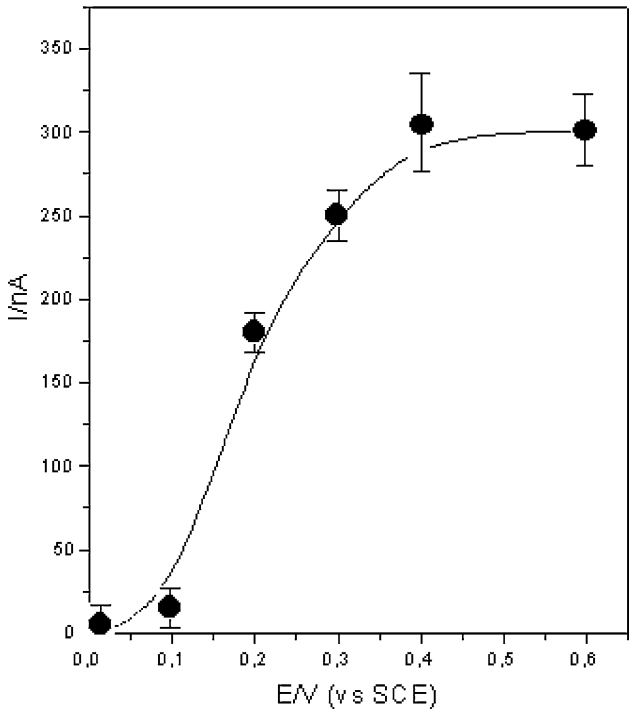

Fig. 2 Relationship between the response current and the applied potential of the PGLD biosensor in PBS pH 7.4 at 37°C and glucose concentration of 20 mM. The results are expressed as mean + standard deviation, $n = 5$

The effect of the operation potential on the amperometric response to glucose at the bioconjugated PGLD electrode is shown in Fig. 2. At the enzyme electrode, hydrogen peroxide produced by GOx reaction was oxidized at potential higher than 50 mV and reaches the maximum value at 400 mV (versus SCE) showing a plateau after this value.

Figure 3a shows the relationship between response current and glucose concentration. It was found that, current increases with increasing glucose concentration in the range 1–50 mM. Considering that the bioconjugated PGLD is uniformly distributed throughout the PANINT's, the reaction appears to be predominantly in the surface of the biosensor in the lower glucose concentration. However, the reaction on the biosensor surface and the diffusion occurring simultaneously at higher concentrations delays the response time. Thus, the increased glucose concentrations also increased the response current and finally reached to steady state value.

The apparent Michaelis–Menten constant (K_M^{app}) gives an indication of the enzyme–substrate kinetics for the glucose biosensor based on the GOx immobilized on PGLD dendrimer. The K_M^{app} can be calculated from the electrochemical Lineweaver–Burk equation [16]:

$$\frac{1}{i_{SS}} = \frac{K_M^{app}}{i_{Max}} \frac{1}{[S]} + \frac{1}{i_{Max}} \quad (1)$$

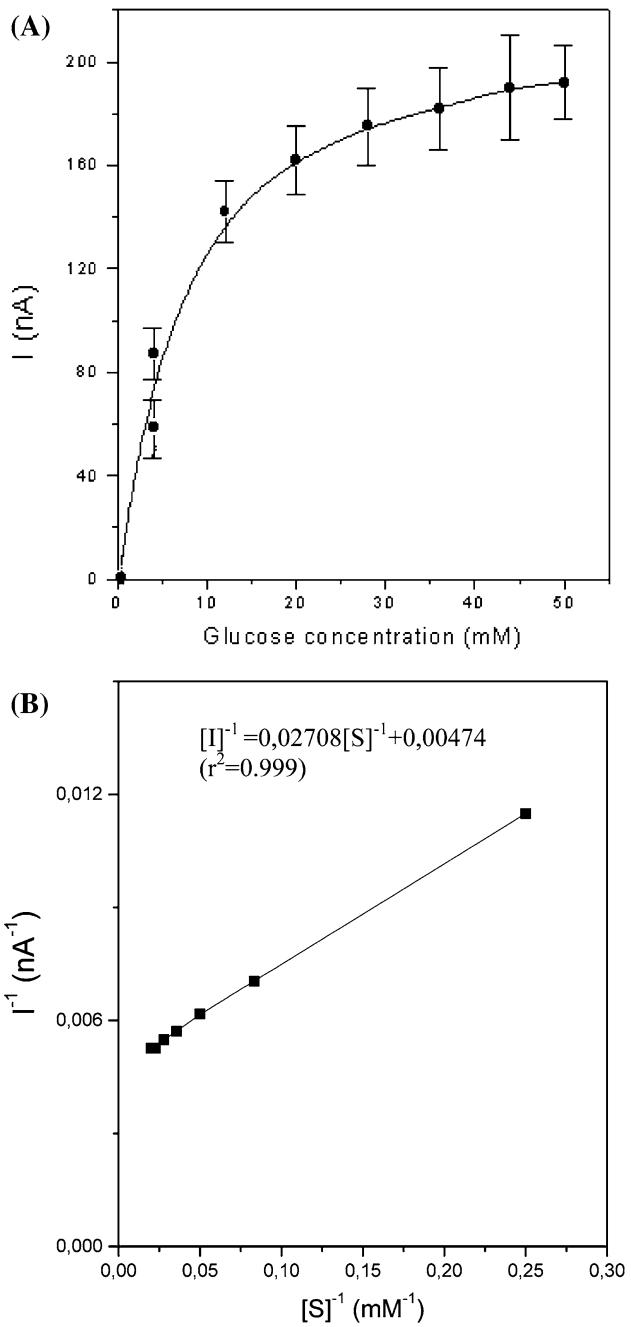

Fig. 3 Relationship between response current and glucose concentration (**a**) and electrochemical Lineweaver-Burk plot (**b**) for the PGLD biosensors in 0.1 M PBS, pH 7.0 and 37°C

where i_{SS} is the steady-state current after the addition of substrate, i_{Max} the maximum current measured under saturated substrate condition and [S] is the bulk concentration of the substrate.

The K_M^{app} value was determined by analysis of the Lineweaver-Burk plots of $1/i_{SS}$ versus 1/[S] (Fig. 3b). The calculated kinetic parameters, V_{Max} and K_M^{app} are 210.97 nA and 5.71 mM, respectively. The K_M^{app} is related to the concentration of the substrate reaching the maximum rate of the enzyme-catalyzed reaction. The smaller K_M^{app} value, the lower is the substrate concentration for reaching the maximum value of the enzyme-catalyzed reaction. Recently, a multiarrayed enzyme films composed of GOx immobilized with glutaraldehyde on periphery of fourth generation PAMAM dendrimer have been reported [17]. The performance of this biossensor was, however; significantly lower relatively to the bioconjugated PGLD with glucose sensing properties developed in this work.

The material–tissue interaction that results from sensor implantation is one of the major obstacles in developing viable, long-term implantable biosensors. It will necessary for indwelling implantable biosensor to be biocompatible, i.e., able to integrate with the biological tissue without evoking inflammation, clot formation in blood vessels and protein adsorption.

In the biological conditions, blood platelets circulate in a quiescent state without interacting with each other or with other cells in the vascular system such as leukocytes or endothelial cells [18]. Upon activation with a wide range of agonists, platelets rapidly become activated and expose receptors to support adhesion, spreading and aggregation at sites of vascular damage. Both platelets and endothelial cells have been reported to mobilize the surface adhesion of the molecule P-selectin (CD62P) from intracellular granule stores following activation with agonists such as thrombin. The CD62P antigen, also known as platelet activation-dependent granule-external membrane (PADGEM) protein or granule membrane protein (GMP-140), is a 140-kDa single-chain polypeptide. Previous studies have shown that CD62P antigen is secreted on the surface of platelet membrane when the platelet is activated by external stimulation such as contact with synthetic surfaces [19].

Figure 4a shows the results of CD62P positive cells. Glass and silicone medical grade (SiGM) was used as negative control. From a material standpoint, a significant difference was observed in the proportion of CD62P expressing platelets by the PGLD glucose biosensor developed in this work compared to the glass or silicone medical grade.

It is very well known that microscopic thrombi and microemboli formation resultants of the blood interactions at synthetic interfaces are the most serious limitations for the clinical applications of bioactive macromolecules in cardiovascular area [20].

The PGLD glucose biosensor exhibits an activity in dissolution of thrombus due to the presence of the enzyme streptokinase (SK) immobilized on dendrimer surface. In this sense, streptokinase activates the blood fibrinolytic system by activation of the plasminogen to the plasmin. Plasmin is an active enzyme in dissolution of thrombus and limits the clot formation by digesting the coagulation

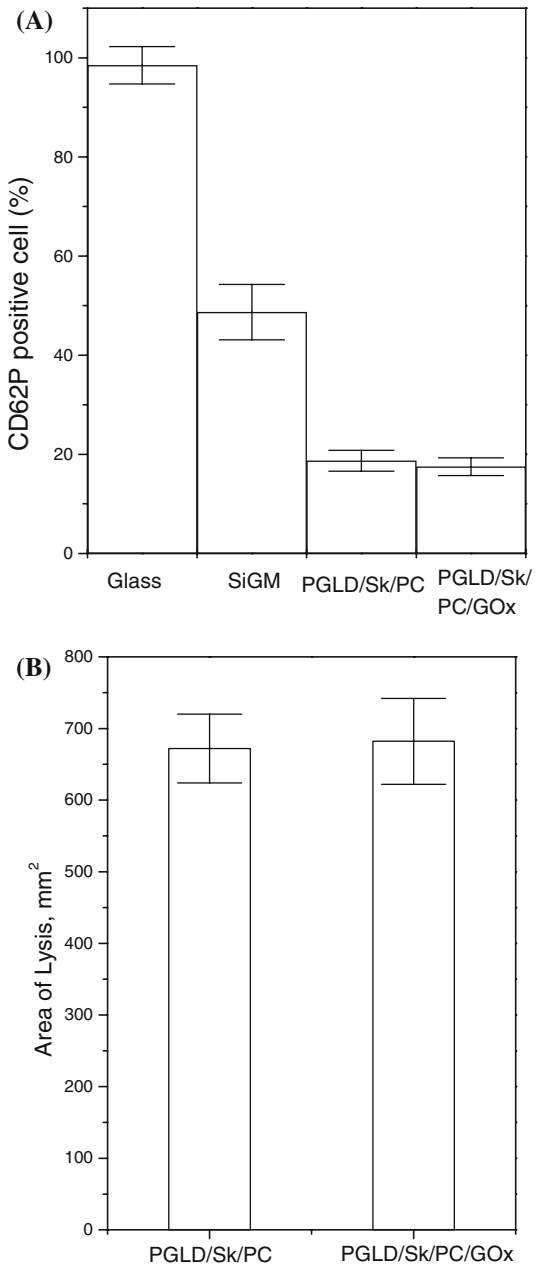

Fig. 4 Expression of CD62P positive platelets (**a**) and fibrinolytic activity (**b**) of PGLD glucose biosensor. Glass and silicone medical grade (SiGM) are the positive and negative controls, respectively. Amount of streptokinase molecules attached to PGLD: 12.10^6 molecules mg^{-1}

factors V, VIII, XII and prekallikrein. Figure 4b shows the fibrinolytic activity of PGLD glucose biosensor. As can be seen, the immobilized enzyme SK has the ability to lyses the blood clots indicating a antithrombogenic character of the biosensor surface.

It is well known that the protein adsorption is a significant obstacle to the in vivo application of biosensors. The adsorbed proteins reduce membrane permeability and block the pathway of analyte molecules [21].

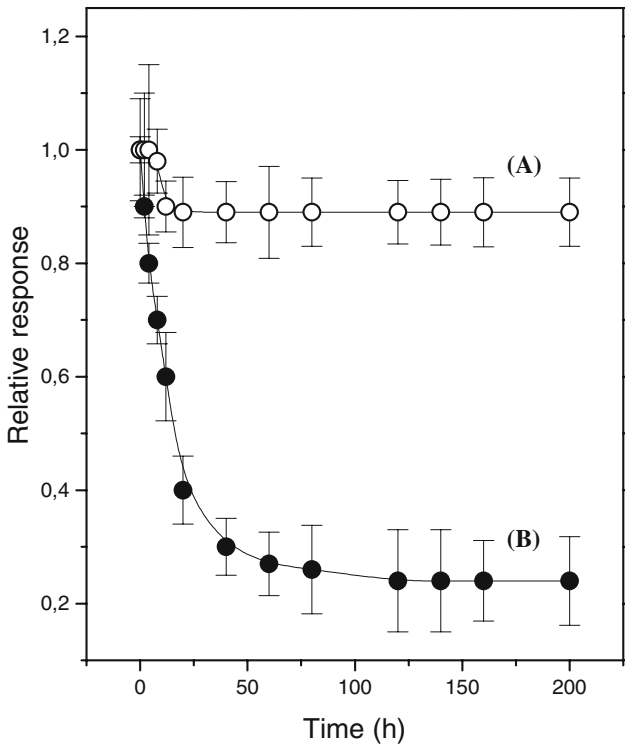

Fig. 5 The PGLD sensor response as function of time in bovine serum with a glucose concentration of 20 mM at 37°C. PGLD/Sk/PC/GOx (**a**) and PGLD/Sk/GOx (**b**)

Figure 5 shows the PGLD biosensor response to glucose in bovine serum with a glucose concentration of 20 mM, magnetically stirred for 200 h at 37°C. Gentamycin (0.5% v/v) was added to the serum to inhibit bacterial contamination. The PGLD biosensor exhibited a relatively steady and stable current output as shown in Fig. 5, suggesting that the phosphorylcholine immobilized onto PGLD has the ability to suppress the protein adsorption avoiding the biofouling on biosensor surface.

Considerable amount of research has been devoted to the development of implantable glucose biosensors. A variety of materials and fabrication techniques have been reported and enzyme immobilization technique is actually used to provide reasonable stable glucose sensor layers. The immobilization of PC is actually a commonly technique used in biomaterials science for the minimization of surface fouling and inhibiting the platelet adhesion and activation thus allowing the use of the glucose sensor for a long time in implantable devices.

Here we have reported our attempts to synthesize glucose biosensor based on the bioconjugation of PGLD dendrimer with PC and SK to favor blood interaction properties and at same time good stability and amperometric response. Evidently, the glucose sensing and antithrombogenic properties of bioconjugated PGLD are due to the proteins absent from mammalian tissues GOx

and SK, respectively, and human immune reactions are observed in clinical uses of these enzymes [22, 23].

Recently, there has been a significant thrust to explore the bioconjugation of SK and GOx proteins with dendrimers and hydrophilic polymers [24, 25]. The water layer adsorbed in these materials seems to prevent the approach of the proteolytic enzymes as well as antibodies or immunological cells, reducing the immunogenic character of the SK and GOx enzymes. These studies motivate into give them continuity of this work through the study of the immuno-histochemistry properties of the bioconjugated PGLD. Such studies will be able to contribute significantly for the design of implantable biosensors.

4 Conclusions

The in vitro results about the blood compatibility and glucose sensing properties suggest that the bioconjugated PGLD could be a very useful material for development of implantable glucose biosensor. Besides the improved biocompatibility of the PGLD biosensor important factors such as the immunogenic character of the immobilized enzymes GOx and SK should be considered in development of implantable biosensors. Much of the experimental work is only of short- or medium-term duration and in vitro conditions, but there are many signs that the bioconjugated PGLD is sufficiently stable to give enhanced performance for glucose sensing applications in in vivo conditions. However, additional and more comprehensive studies about the immunogenic and blood compatibility of the PGLD biosensor are carried out in order to make clear their interaction with biological tissue in in vivo experiments to speculate upon the viability of their use in implantable biosensors and the results will be reported elsewhere.

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