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Antithrombogenic properties of bioconjugate streptokinase-polyglycerol dendrimers

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Dendrimers are monodisperse, spherical and hyperbranched synthetic macromolecules with a large number of surface groups that have the potential to act as carriers for drug immobilization by covalent binding or charge transfer complexation. In this work, a bioconjugate of streptokinase and a polyglycerol dendrimer (PGLD) generation 5 was used to obtain fibrinolytic surfaces. The PGLD dendrimer was synthesized by the ring opening polymerization of deprotonated glycidol using polyglycerol as core functionality in a step-growth processes denominated divergent synthesis. The PGLD dendritic structure was confirmed by gel permeation chromatography (GPC), nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR) and matrix assisted laser desorption/ionization (MALDI-TOF) techniques. The synthesized dendrimer presented low dispersion in molecular weights ($M_w/M_n = 1.05$) and a degree of branching of 0.82 which characterize the polymer dendritic structure. The blood compatibility of the bioconjugate PGLD-Sk was evaluated by *in vitro* assays such as platelet adhesion and thrombus formation. Uncoated polystyrene –microtitre plates (ELISA) was used as reference. The epifluorescence microscopy results indicate that PGLD-Sk coating showed an improved antithrombogenic character relative to the uncoated ELISA plates.

1. Introduction

Today, polymers represent the most important and largest family of materials being used in medical technology. Applications of polymers in medicine and surgery include temporary, permanent devices and complex devices simulating the physiological function.

The materials requirement for the above biomedical applications varies markedly according to the application being considered. One of the major problems encountered with artificial organs is blood compatibility since many of these organs either handle blood directly or come into contact with the blood in a membrane exchange reaction.

The blood contact with a device surface may result in clotting that impairs the performance of the device and harms the patient who depends on it. Today it is well known that the coagulation and inflammation induced by polymer surfaces may be locally controlled by binding of anticoagulants on surfaces like as heparin or antiplatelet drugs that can suppress platelet adhesion and activation [1–3]. Some coatings rely on biologically active materials that are loaded into a polymer matrix or bonded to the surface of a device [4, 5]. These bioactive materials prevent clot formation by altering the physiological responses of blood.

Recently, vigorous research activity in the area of polymers with controlled architectures has been observed. Actually, a high level of structural control can be attained in the synthesis of highly branched macromolecules denominated dendrimers [6, 7]. Due to their similarity with globular proteins and regular micelles, a rapid increase of interest to use dendrimers in biomedical field has been observed [8–11].

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Dendrimers are complex monodisperse macromolecules with a regular and highly branched three-dimensional architecture. The large number of active chemical groups located at the periphery and the cavity structures make the dendrimers an interesting macromolecule for the design of biologically active biomaterials [12].

Polyglycerol (PGL) is a water-soluble polymer that contains both pendant hydroxyl groups and ether linkages. The low cytotoxicity and the FDA approval of PGL as emulsifiers in the pharmaceutical and food industries make it a promising polymer for use in the biomedical field [13]. Dendritic polyglycerols with low glass transition temperature (T_g) may be interesting polymers due to the high density of local hydroxyl groups. These functional groups may be modified in a second synthetic step and used as sites for immobilization of bioactive compounds. In this manner it is possible to tailor bioactive materials suitable for coating of implantable biomaterials devices.

An interesting approach to the development of clot-lysing surfaces may be the use of thrombolytic agents to pharmacological dissolution of the blood clot by use of plasminogen activators [14]. Streptokinase (Sk) is a catalytically inert bacterial protein commonly used in the thrombolytic therapy [15]. Streptokinase can form an activator complex with plasmin or plasminogen (Pg), which induces the formation of a functional active site [16]. Plasmin is an enzyme that dissolves fibrin, the protein matrix of blood clots. Although the activator complex formed by streptokinase is the most efficient activator of plasminogen, its success as a therapeutic agent seems to be influenced by the risks of hemorrhagic complications due to the degradation of other circulating clotting factors and their extremely short half-life [17].

The aim of this work was to study the blood compatibility of the bioconjugate streptokinase—polyglycerol dendrimer (PGLD-Sk) for the development of clot-lysing coatings for cardiovascular applications.

2. Materials and methods

2.1. Polyglycerol dendrimer synthesis and characterization

The polyglycerol dendrimer (PGLD) was prepared by following the divergent synthetic methodology due to the control degree provided by this technique. Polymerizations were carried out in a batch reactor equipped with a mechanical stirrer (200 rpm) and a dosing pump under nitrogen atmosphere. Glycerol (Aldrich) was extensively dried before use. The PGL core was synthesized by glycerol etherification at 533 K and nitrogen atmosphere in the presence of NaOH as catalyst and distilling the reaction water in a Dean-Stark system. The synthesized polyether was exhaustively dried in high vacuum at 120 °C for 24 h in the reaction vessel. Then, PGL was partially deprotonated (15%) with potassium methylate solution (3.7 M in methanol, Fluka) by distilling off methanol from the melt. A 50 mL aliquot of glycidol (Fluka) was slowly added at 90 °C over 12 h. The slow glycidol addition and the partial deprotonation allowed a better control over molecular weight and polydispersity of the dendrimer. After completion of the reaction, termination was carried out by addition of a drop of acidified methanol. PGLD was dissolved in methanol, neutralized by filtration over cation-exchange resin and precipitated from methanol solution into diethyl ether. Finally, the product was purified by dialysis (benzoylated cellulose membrane with molecular weight cutoff in the range of 25000 $g \cdot mol^{-1}$, Sigma) and subsequently dried for 15 h at 80 °C in vacuum. The polymer was obtained as a transparent and viscous liquid at room temperature (50 cps at 25 °C).

The average molecular weight values (M_n, M_w) were obtained by using Waters chromatograph μSTYRAGEL columns of pore sizes 50, 102, 103 and 104 Å. PGLD was dissolved in DMF as mobile phase at concentrations of 5 $mg \cdot mL^{-1}$. The flow rate was 1.0 mL.min-1. Poly(propylene oxides) 1,000, 2,000, 4,000 (Aldrich), 8,000, 12,000 (ARCO Co) standards were used for the molecular weight calibration. The nature of the PGLD structure was also characterized by ^{13}C-NMR (Bruker, 500 MHz) and MALDI-TOF spectroscopy (Bruker, BIFLEX^{TM}).

2.2. Streptokinase immobilization

Covalent attachment of the streptokinase (Sk) on PGLD dendrimer was made by using 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate (CDAP) [17]. The total amino groups in the streptokinase molecule are 34, including ε-aminogroup in lysine residues and 1 terminal amino group [18]. Thus, streptokinase was immobilized changing the molar ratio of PGLD to amino groups in streptokinase molecule (PGLD/-$NH_2$1.0). The degree of modification of $-NH_2$ groups in the molecule was determined by measuring the amount of amino groups with trinitrobenzene sulfonate [18].

The bioconjugate PGLD-Sk was used as fibrinolytic coating in solid phase immunoassays polystyrene microtitration (ELISA) plates with free $-NH_2$ groups. Thus, PGLD-Sk was dissolved in 0.2 M NaCl (1.0 mL) and cooled at 4 °C. The pH of PGLD-Sk solution was 9.5 (0.1 M sodium phosphate buffer). Then, 10 mg CNBr (Fluka) dissolved in acetonitrile (100 μL) was added to the solution and pH maintained at 9.5 for 6 min. The solution of freshly activated PGLD-Sk was diluted with 0.5 M $NaHCO_3$ (pH 8.5) to a final concentration of 20 $\mu g \cdot mL^{-1}$. Elisa plates were coated with 100 μL of 20 $\mu g \cdot mL^{-1}$ in 0.5 M $NaHCO_3$ (pH 8.5) and incubated overnight at 4 °C. At the end of the PGLD-Sk immobilization reaction the well washings were performed four times with 0.1 M sodium chloride-0.05% Tween 20–0.01 M sodium phosphate pH 9.0–9.5 to

eliminate any PGLD-Sk simply adsorbed, without covalent immobilization. Control Elisa plates were run without addition of PGLD-Sk dendrimer. In this sense, the wells from which PGLD-Sk was omitted were used as blanks. The streptokinase concentration on the PGLD-Sk coating was determined using the extinction coefficient of the enzyme, $E^{1}\%$ cm at 278 nm of 19.4 [19].

Amidolytic activities of free and immobilized streptokinase were determined using the chromogenic assay using as substrate, N-α-carbobenzoyl-L-lysine-ρ-nitrophenyl ester (CLN, Sigma Co) [20]. One CLN units of Sk was defined as a 0.01 increase per minute in the absorbance at 340 nm in the above conditions.

2.3. Blood compatibility assay

2.3.1. CD62P expression

Platelet adhesion *in vitro* experiments were conducted to examine the interaction of blood with PGLD-Sk coated and uncoated ELISA plates. *In vitro* platelet activation measurements were performed using a modified method of Murakami *et al.* [21]. 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 $\times$ g for 10 min at 22 °C to obtain platelet rich plasma (PRP). A volume of 200 μL was pipetted into PGLD-Sk coated 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 flow cytometer. A group of platelets was gated, and the expressions and the fluorescence intensities of FITC and PE were measured at 525 nm and 575 nm, respectively. The ELISA assay detection limit for CD62P in this work was 0.93 $ng \cdot L^{-1}$.

2.3.2. Platelet adhesion and microscopy imaging

The coated PGLD-Sk surfaces and uncoated ELISA surfaces were incubated in the previously prepared platelet rich plasma (PRP) (200 μL) for 30 min at 37 °C. After washing in PBS, platelets were fixed by glutaraldehyde (1.5% solution in PBS) at room temperature (25 °C). After addition of 10 μL of acridine orange the epifluorescence of the labeled platelets was detected by using a digital camera (DN 100, Nikon Co) mounted on the front port of an inverted microscope. A vapor mercury lamp selectively filtered within the range 450–490 nm was used as excitation light. A long-pass filter allowed the fluorescence signal to be detected for wavelengths longer than 515 nm. The platelet morphology, platelet density and pseudopodium leaching were observed and evaluated.

TABLE I Physicochemical characteristics of the PGLD synthesized in this work

Degree of branching[a]	M_n[b]	M_w[b]	(M_w)[c]	PI[b] M_w/M_n	OH value (mg KOH $\cdot$ g^{-1})
0.82	16,724	17,564	17,280	1.05	495

[a]Calculated from NMR analysis.
[b]Calculated from GPC analysis.
[c]Calculated from MALDI-TOF.

3. Results and discussion

The dendritic structure was confirmed by matrix assisted laser desorption/ionization (MALDI-TOF) techniques and nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR). The degree of branching, molecular weights, polydispersity index and hydroxyl number results are presented in Table I. The low dispersion in molecular weight ($M_w/M_n = 1.05$) and a degree of branching of 0.82 characterize the PGLD dendritic structure.

It is well known that GPC calibrated with linear standards, understimates Mn for branched systems. In an effort to be more certain about the polyglycerol dendrimer molecular weights, MALDI-TOF mass spectroscopy was attempted. Satisfyingly, the spectrum showed a Gaussian distribution of peaks centered around 17,280 g $\cdot$ mol^{-1} in a reasonable concordance with GPC analysis.

In order to monitor the mechanism of termination, the hydroxyl values of products were measured using chemical analysis. The value of 495 mg KOH $\cdot$ g^{-1} suggests that in the polymerization system no side reaction occurs during the termination process.

Fig. 1 illustrates the reaction scheme for the PGLD synthesis and the immobilization of the bioconjugate PGLD-Sk onto PS surfaces. The reactive NCO groups were introduced in the PGLD surfaces after contacting the dendrimer with CDAP using triethylamine (TEA) as a mild catalyst. The activated PGLD coating was used as a support for streptokinase immobilization.

In the biological conditions, 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 [23]. Upon activation with a wide range of agonists, platelets rapidly become activated and expose receptors/molecules to support adhesion, spreading and aggregation at sites of vascular damage. Both platelets and endothelial cells have been reported to mobilize the surface adhesion

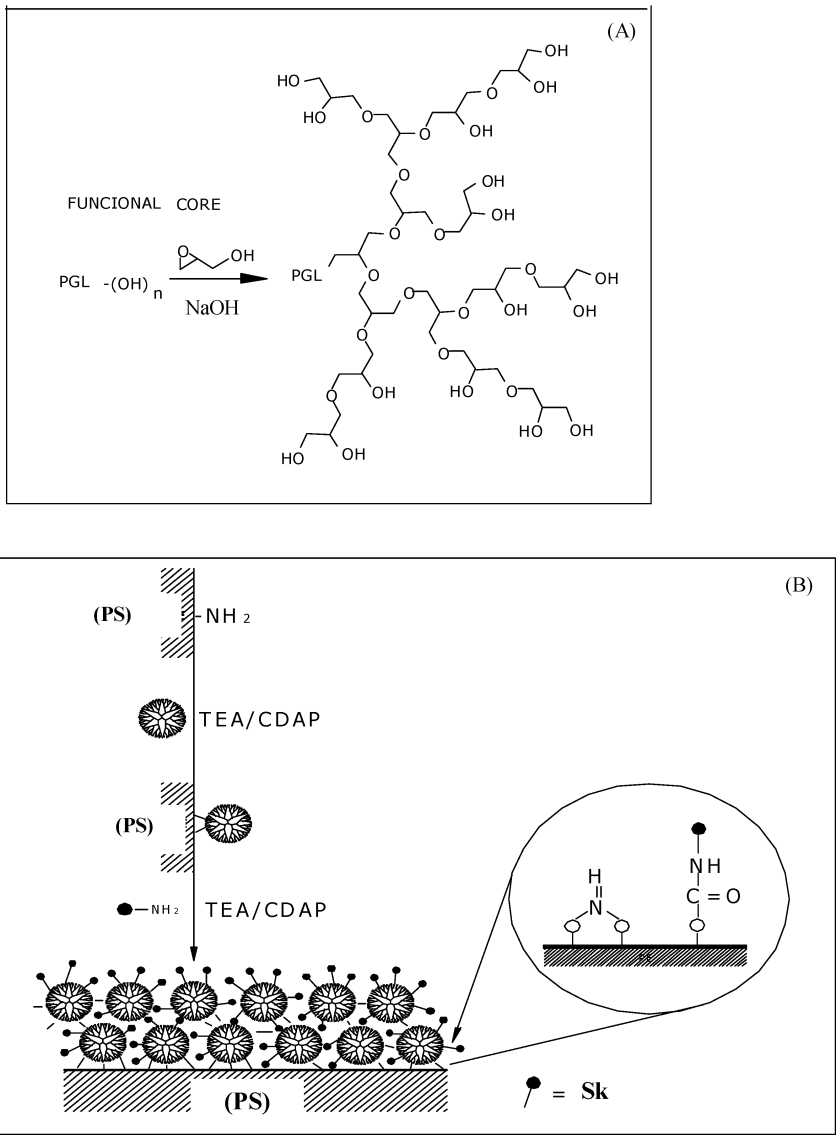


Figure 1 (A) Schematic diagram of the PGLD synthesis and (B) immobilization of the bioconjugate PGLD-Sk onto wells of ELISA (PS) plates.

molecule P-selectin (CD62P) from intracellular granule stores, within minutes 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 140000 dalton single-chain polypeptide [24, 25]. 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 [26].

Fig. 2 shows the results in percentages of CD62P positive cells. Coverslips were used as negative control. Contact with the coated PGLD-Sk Elisa plates caused significantly less CD62P expression than contact with PGLD coated ELISA plates. From a material standpoint, a significant difference was observed in the proportion of CD62P expressing platelets on the PGLD-Sk polystyrene ELISA plates compared to the uncoated Elisa plates. It is obvious that the surfaces having PGLD-Sk show a higher platelet adhesion and activation compared to inert materials.

Platelets are anuclear discoid cells that circulate in the blood in a quiescent state with an average size of 3 μm. In response to an activating stimulus, platelets become activated and accelerate thrombosis by different ways: significant morphological changes, platelet-platelet aggregation and secretion contents from alpha and dense cytoplasm storage granules.

Epifluorescence microscopy of the PGLD-Sk coated and uncoated ELISA plates after incubation with human blood for 15 min at 37 °C are shown in Fig. 3. As can be seen in the picture, the uncoated ELISA surfaces appear to encourage platelet adhesion and activation with thrombus formation. The coated PGLD-Sk ELISA surfaces were essentially free from platelet adhesion as well thrombus formation (Fig. 3(B)).

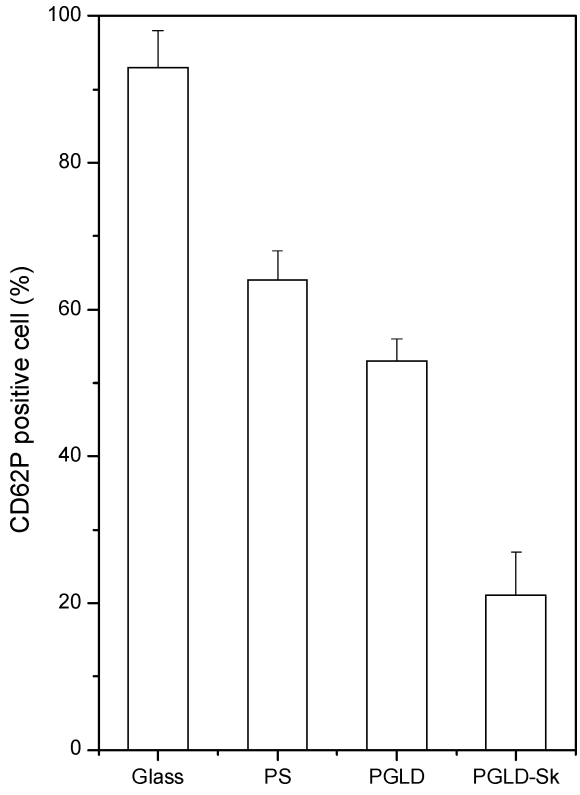


Figure 2 Expression of CD62P positive platelets (%). The Pearson (P) correlation is 0.012.

Streptokinase is a plasminogen activator, which is composed of 414 amino acids. Many studies of Sk have suggested that the Ser60-Lys333 peptide of Sk is required for minimal enzyme activity and that the N-terminal lle1-Lys59 peptide is important in just maintaining and stabilizing the proper conformation of Sk to have its full activation [27, 28].

The activity of the bioconjugate PGLD-Sk is shown in Fig. 4. The fibrinolytic activity of the enzyme decreased significantly with the degree of substitution indicating a higher degree of substitution on amino groups in lysine residues of streptokinase.

An interesting method to obtain more thromboresistant polymers is the modification of polymeric surfaces by coating them with biomolecules with thrombolytic properties. Streptokinase activates the blood fibrinolytic system by activation of the proenzyme, plasminogen, to the active enzyme plasmin. Plasmin is an enzyme that is very active in breaking down fibrin. Plasmin helps to dissolve a thrombus and limits clot formation by digesting the coagulation factors V, VIII, XII, and prekallikrein. The inhibition and activation of plasminogen (the inactive precursor of plasmin) modulate fibrinolysis. The scheme 1 represents the actuation mechanism of streptokinase.

Fig. 5 shows the fibrinolytic activity of PGLD-Sk coating. As can be seen, the bioconjugate PGLD-Sk coating should be able to lyse the blood clots using the immobilized streptokinase indicating that the immobilization procedure does not affect the fibrinolytic property of the enzyme

4. Conclusion

This paper presents data on the interaction of PGLD-Sk-coated and non-coated polystyrene-microtitre plates

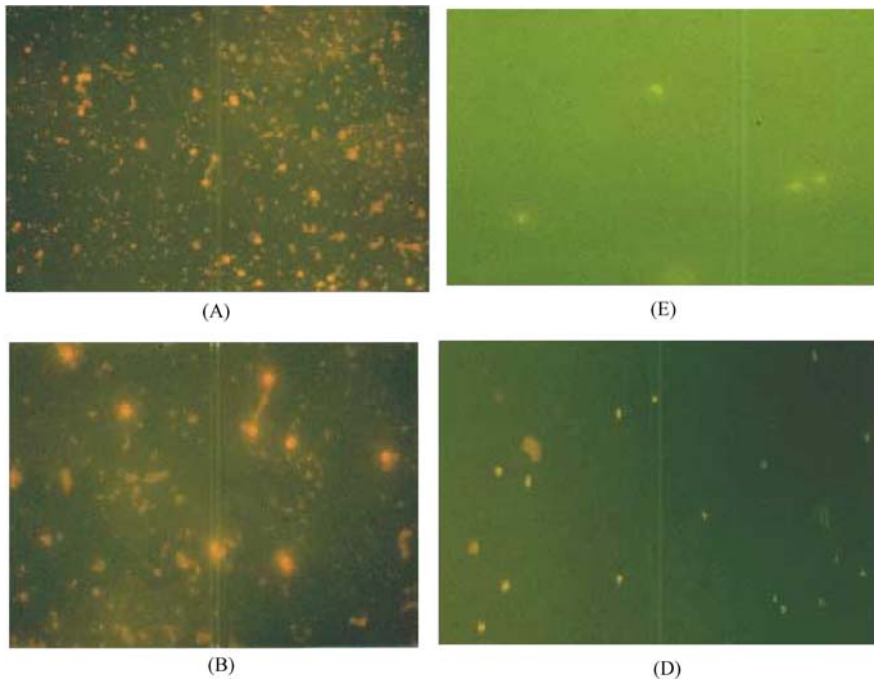


Figure 3 Epifluorescence microscopy of ELISA plates (A)–(B) and PGLD-Sk coated ELISA surface (C)–(D) after blood exposition.

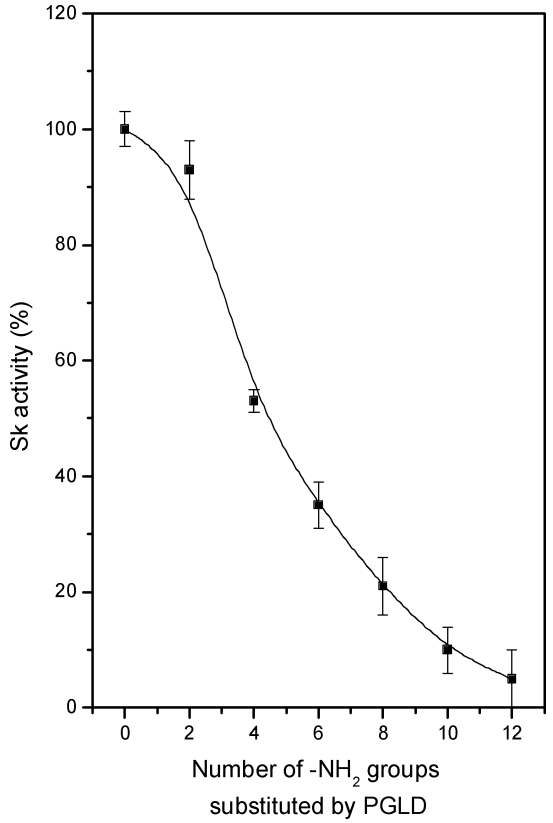


Figure 4 Influence of the amino groups substitution on streptokinase activity. The Pearson correlation is 0.020 ($p = 0.020$).

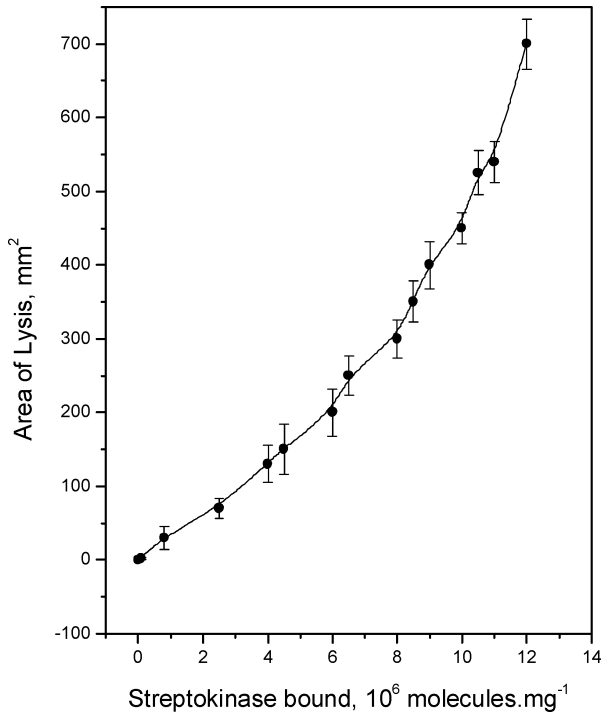
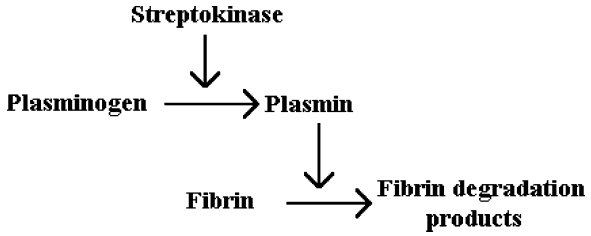


Figure 5 Dependence of fibrinolytic activity of immobilized streptokinase on the amount of streptokinase molecules attached to dendrimer. The Pearson correlation is 0.014.



Scheme 1 Fibrinolysis mechanism of streptokinase.

with blood focusing on the activated platelets taken up by the surfaces as detected by a direct ELISA method. It is shown that the PGLD-Sk coating significantly reduced the proportion of CD62P expressing platelets compared to uncoated surfaces. The biocompatibility of PGLD-Sk may be regulated by many parameters. This study may prove the good biocompatibility of the bioconjugate PGLD-Sk from the platelet activation standpoint of view. Furthermore, the study suggests that research on PGLD-Sk bioconjugate as antithrombogenic coating will yield information of scientific and clinical value.

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