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PAMAM dendrimer derivatives as a potential drug for antithrombotic therapy

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ABSTRACT

Platelets are anucleated blood cells that play an important role both in the pathogenesis of atherosclerosis and subsequent thrombosis. Dendrimers have attracted great interest in biomedical applications. However, their interactions with cell compounds and compartments are nonselective, thus causing cytotoxicity and hemotoxicity. We derivatized PAMAM G4 and G5 dendrimers to evaluate their interactions with serum metabolites, their effects on the viability of red blood cells, and their antithrombotic properties. PAMAM G4 and G5 derivatives showed better hemocompatibility than the PAMAM G4 and G5 dendrimers without any derivatization (NH_2). PAMAM G4-Arginine-Tos and G4-Lysine-Cbz act as potent inhibitors of platelet aggregation induced by ADP. PAMAM G4-Arginine-Tos also showed inhibition of platelet aggregation induced by collagen, TRAP-6 and arachidonic acid. Moreover, G4-Arginine-Tos present inhibition of platelet secretion and thrombus formation under flow conditions. Based on our study, the PAMAM G4-Arginine-Tos derivative is hemocompatible and produces desirable antiplatelet and antithrombotic effects. Thus, this compound has potential applications as an antithrombotic drug or a drug delivery vehicle.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality worldwide. In the USA, nearly 800,000 strokes occur annually with almost one in four being a recurrent event [1]. In Europe, stroke is the third leading cause of death, and stroke complications now account for approximately 5% of the UK's National Health Service budget [2]. In general, the 2008 overall rate of death attributable to cardiovascular disease (CVD) (International Classification of Diseases, 10th Revision, codes I00 –I99) was 244.8 per 100,000 [3].

Cardioembolic stroke, in which the thrombus forms in the heart, occurs primarily in patients with atrial fibrillation and is prevented with anticoagulation [4,5]. Pooling of blood in the fibrillating atria allows formation of a clot that initially adheres to the atrial wall but may later dislodge and embolize. Noncardioembolic ischaemic stroke, in which the thrombus results from arterial plaque rupture, is best managed with antiplatelet therapy [2]. Platelets are anucleate blood cells that retain cytoplasmic mRNA and maintain functionally intact protein translational capabilities [6], playing an important role both in the pathogenesis of atherosclerosis and subsequent thrombosis [7]. Following the rupture of the fibrous cap of the atherosclerotic plaque, prothrombotic materials are exposed to the circulating blood leading to the development of a platelet-rich thrombus over the disrupted lesion [8].

Dendrimers have attracted great interest in biomedical applications because of their unique dendritic structures and multiple surface properties. Because of the presence of a large number of terminal groups, drug molecules can be attached to the dendrimer surface through covalent bonds [9,10], whereas internal cavities are capable of encapsulating small molecules [11]. Nevertheless, their interactions with cell compounds and compartments are nonselective, thus causing cytotoxicity and hemotoxicity [12,13].

However, platelet activation by injected drug delivery nanoconstructs could readily result in unintended consequences in host circulation. This potential was recently demonstrated for PAMAM dendrimers injected into systemic blood circulation in mice [14]. Platelets comprise a major metabolically active blood component,

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circulating constantly at 2.5×10^8 platelets/mL of blood, are highly responsive to diverse physical and biochemical stimuli with potent coagulation and growth factors, and are responsible primarily for hemostasis [14,15].

Dobrovolskaia et al. [15] have demonstrated that decreasing the positive charges on the PAMAM surface, decreases the platelet aggregation and increases the hemocompatibility. Therefore, the aim of this work was to obtain different PAMAM G4 and G5 derivatives, to evaluate their interactions with serum metabolites, their effects on the viability of red blood cells (RBCs), and their antithrombotic properties.

2. Results and discussion

The results of the inhibition of the platelet function are completely new compared to other studies that employed PAMAMs functionalized with agonist or antagonist of G protein-coupled receptors (eg. adenosine receptor) [16,17].

2.1. Dendrimers synthesis

2.1.1. Polyamine-conjugation of the dendrimers PAMAM G5 with folic acid and coumarin

Folic Acid (FA) and Coumarin-3-carboxylic acid (Cou) were coupled to the amine terminals of the PAMAM G5 dendrimers by 1-[3-(dimethylamino)propyl]-3-ethylcarbodi-imide hydrochloride (EDC·HCl) and N-hydroxybenzotriazole (HOBt) coupling reaction [18]. A higher molar ratio of 1:140 reagents were used to get all the 128 terminal amine groups of the PAMAM G5 dendrimer conjugated. However, only 23 FA and 64 Cou molecules were found attached to the PAMAM G5 dendrimers, respectively. The difference in the number of molecules coupled to PAMAM G5 was due perhaps to the steric hindrance caused by the size of FA and Cou, respectively (Fig. 1a and b).

2.1.2. Polyamine-conjugation of the dendrimers PAMAM G4 with lysine and arginine

Lysine-Cbz (Lys-Cbz) and Arginine-Tos (Arg-Tos) were coupled to the amine terminals of the PAMAM G4 dendrimers by EDC·HCl and HOBt coupling reaction [18]. A higher molar ratio of 1:70 was used to get all the 64-amine groups of the PAMAM G4 dendrimer conjugated. The modification of the dendrimers surface was characterized by MALDI-TOF-MS spectrometry analysis (Fig. 2a and b). The spectra analyzes of PAMAM G4 dendrimers showed that 16 Arg-Tos and 39 Lys-Cbz were attached to the derivatives, respectively.

2.2. Biochemical and red blood cells interactions

The circulatory system seems to be the most convenient way of drug administration because an active compound within a relatively short time is able to reach distant tissues, which are unavailable directly. However, blood constituents can be the first and unwanted targets of drug action. Binding to plasma proteins, erythrocytes, leukocytes, platelets, and blood vessel walls, may lead to serious problems, or at least dramatically lower the amount of drug available for therapy [19].

2.2.1. Biochemical measurements

Of all the studied dendrimers, only PAMAM G4 and G5-Cou presented interaction with glucose (Gl), total cholesterol (TCh), and urea (Ur), and PAMAM G5 with TCh, Gl and creatinine (Cr) (Table 1). However, the variations found in the different metabolites are not enough to cause physiological changes in the human body.

2.2.2. Assay of red blood cells lysis

Fig. 3 shows the hemolysis of RBCs expressed as percentage of released hemoglobin compared to the positive control Triton X-100 (0.2%, V/V). These findings indicate that PAMAM dendrimers G4 and G4-Lys-Cbz (final concentration of 0.25 mg/mL in saline) have the highest percentage of hemolysis (9.8% and 8.7%, respectively). Morphological differences between RBCs, negative control and RBCs exposed to the PAMAM dendrimers (G4, G5, and their derivatives) are shown in Fig. 4. The optical microscopy data shows that the commercially available PAMAM G4 and G5 dendrimers cause intense agglutination of RBCs, whereas PAMAM G4 derivatives do not have much interaction with the RBCs. In comparison, the PAMAM G5 derivatives showed moderate agglutination of the RBCs.

According to the hemolysis and morphological changes of RBCs, we found that the PAMAM G4 dendrimer produced the highest percentage of hemolysis compared to the positive control (100% hemolysis). However, PAMAM G4 dendrimer derivatives showed a reduction in the percentage of hemolysis. The PAMAM G5-Cou and FA derivatives also displayed reduction in hemolysis; they showed 4.7% and 5.3% of hemolysis, respectively, while non-derivatized G5 dendrimers displayed 6.6% hemolysis. Although the commercially available PAMAM G4 and G5 dendrimers exhibit low hemolysis, they cause a great agglutination of the RBCs as observed by optical microscopy. The agglutination effect was considerably decreased in the PAMAM G5 derivatives and not observed at all in the PAMAM

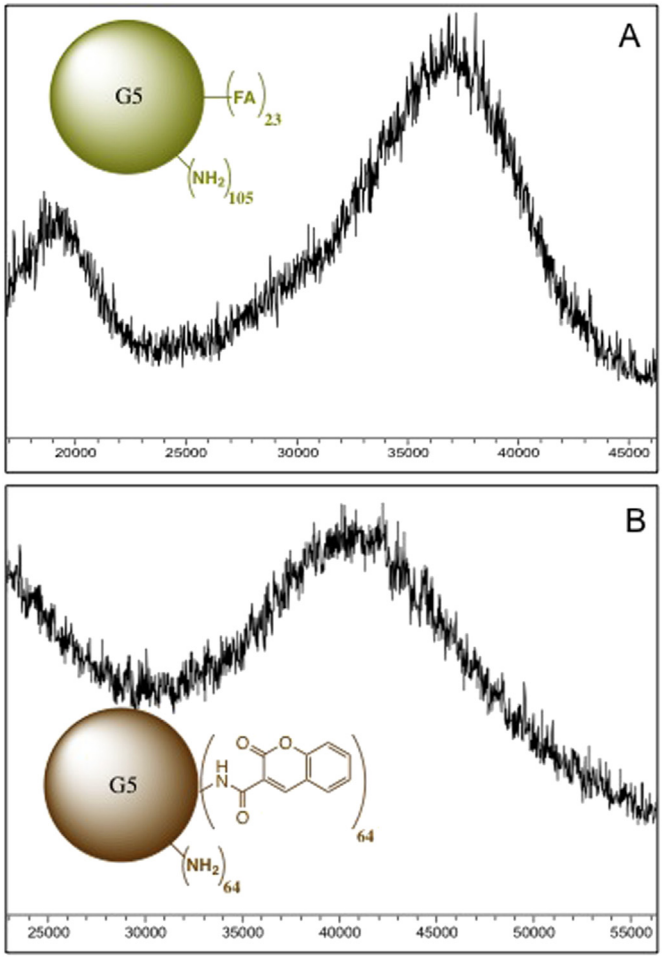

Fig. 1. MALDI-TOF spectra. A) PAMAM G5 dendrimer coupled to 23 molecules of folate and B) PAMAM G5 dendrimer coupled to 64 molecules of coumarin.

G4 derivatives. Results that are in agreement with the studies of Dobrovolskaia et al. [15], in which the substitution of the terminal groups (decreasing the cationic charge) of the dendrimer benefit their biocompatibility by reducing the interaction with cell membranes [15,20].

The results obtained related to the hemolysis and agglutination may be due to the partial incorporation of the dendrimers into the lipid bilayer, or by removing the outer monolayer [21]. However, the interaction of each of the synthesized PAMAM dendrimer derivatives, indicates that these nanoparticles do not cause significant hemolytic reactions at the concentration used (0.25 mg/mL, final concentration). According to previous studies, less than 10% of hemolysis compared to positive control (Triton X-100 at 0.2%) is not considered as cytotoxic [22].

2.3. Effect on platelet function

The effects of PAMAM-G4, PAMAM G4 amino acid (Arg-Tos and Lys-Cbz) derivatives, and net amino acids on platelet aggregation induced by the agonist ADP, collagen, TRAP-6 and arachidonic acid, respectively, are presented in Table 2 (final concentration of all samples were at 100 μg/mL in saline). The inhibition of platelet aggregation induced by ADP compared to negative control reached the following order: G4-Arg-Tos 57 ± 3% ($p < 0.05$), G4-Lys-Cbz 21 ± 1% ($p < 0.05$), while the PAMAM G4 only showed 2 ± 1% of inhibition of platelet aggregation. Whereas for collagen, TRAP-6 and arachidonic acid only G4-Arg-Tos inhibited platelet aggregation compared to control with a percentage of inhibition of 44 ± 4% ($p < 0.05$), 59 ± 8% ($p < 0.05$) and 52 ± 6% ($p < 0.05$), respectively. Also, G4-Arg-Tos inhibited platelet secretion in an order of 25 ± 12% ($p < 0.05$) (Fig. 5).

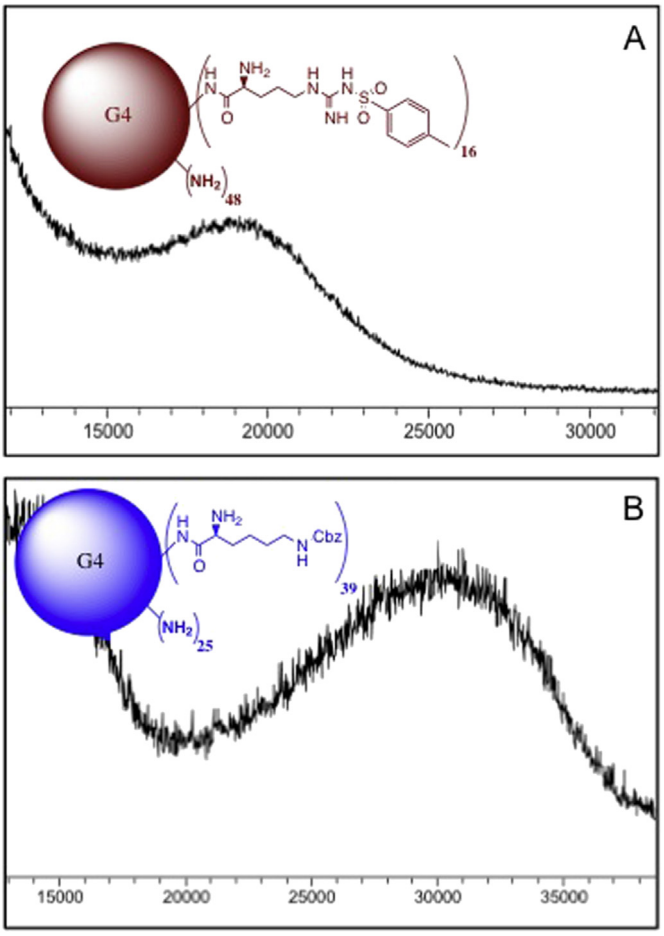

Fig. 2. MALDI-TOF spectra. A) PAMAM G4 dendrimer coupled to 16 molecules of Arg-Tos and B) PAMAM G4 dendrimer coupled to 39 molecules of Lys-Cbz.

The effects of G4-Arg-Tos on human collagen-induced platelet aggregation and thrombus formation under arterial flow conditions are shown in Fig. 6. After the perfusion of citrate-anticoagulated blood (negative control) over plaque-coated surfaces with collagen and a wall shear rate of 1000 s^{-1} for 10 min, rapid platelet adhesion and aggregate formation was observed (Supplemental Material, file Movie C, Fig. 6). G4-Arg-Tos reduced collagen-induced platelet adhesion and aggregation under controlled flow. After G4-Arg-Tos incubation on whole blood, the platelet coverage was inhibited by 74 ± 2% ($p < 0.05$) (Supplemental Material, file Movie P, Fig. 6). In comparison, PAMAM G5 and derivatives showed no effect on platelet function (results not shown).

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.ejmech.2013.08.047.

Both PAMAM G4-Arg-Tos and G4-Lys-Cbz act as potent inhibitors of platelet aggregation induced by ADP. However, only G4-Arg-Tos (100 μg/mL in saline, final concentration) inhibited platelet aggregation induced by collagen, TRAP-6 and arachidonic acid. Furthermore, G4-Arg-Tos exhibited inhibition of platelet secretion, indicating that G4-Arg-Tos increases the cyclic nucleotide concentration (cAMP) of human platelet. The importance of cAMP in modulating platelet reactivity is well established as inhibitor of most of the platelet responses. Furthermore, elevated levels of cAMP decrease intracellular Ca^{2+} concentration by the uptake of Ca^{2+} into the dense tubular system and negatively affect the action of PKC [23].

Generally, an important aspect for platelet aggregation and for platelet adhesion mechanisms is the rheological (blood flow) condition operating at sites of vascular injury. Platelets are exposed to a broad range of hemodynamic conditions *in vivo*, ranging from relatively low flow situations in veins to stenosed arteries with high shear rates. Platelets have the unique capacity to form stable adhesion contacts over all shear conditions operating *in vivo*, and show to be indispensable for hemostatic plaque formation and thrombosis at elevated shear rates. This phenomenon has been instrumentalized by the use of a specific instrument named BioFlux, which reproduce the adhesive steps mediating platelet adhesion to extracellular matrices, including a key role for collagen through engagement of GPVI and integrin $\alpha_2\beta_1$ in promoting platelet arrest. These experimental systems have also confirmed an essential role for integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) in mediating platelet aggregation over the full range of shear conditions operating *in vivo* [24]. Moreover, the platelet adhesion to collagen led to rapid thrombus formation, which was inhibited by G4-Arg-Tos through inhibition of specific intracellular signaling pathways, or by blocking GPVI or GPIIb/IIIa on the matrix.

Thus, it seems likely that inhibition of the collagen–phospholipase C – inositol 1,4,5-trifosfato-thromboxane A2–Ca^{+2} plays an important role in mediating the inhibitory effect of G4-Arg-Tos in collagen-induced platelet adhesion and aggregation [25].

3. Conclusions

This study shows that a previously non-active compound (Arg-Tos) produces desirable biological effects, ie, hemocompatibility and antithrombotic action when conjugated to a PAMAM dendrimer. A PAMAM G4 derivative (G4-Arg-Tos) inhibits platelet function completely: platelet adhesion, secretion, and aggregation. In addition, the PAMAM G4-Arg-Tos has significant antithrombotic activity coupled with almost no cytotoxicity in RBCs. This nano

Table 1
Concentration of the different metabolites studied in the serum after the interaction with the derivatives of PAMAM G4 and G5 dendrimers.

	TCh (mg/dL)	Gl (mg/dL)	Trg (mg/dL)	Ca (mg/dL)	TB (mg/dL)	UrA (mg/dL)	Ur (mg/dL)	Cr (mg/dL)	TP (g/dL)
C(−)	166 ± 11	77 ± 6	164 ± 12	10.7 ± 0.9	0.8 ± 0.4	4.5 ± 0.6	24.2 ± 2.6	1.4 ± 0.3	7.4 ± 0.6
G4	145 ± 10*	63 ± 6*	163 ± 15	11.0 ± 0.6	0.6 ± 0.5	4.3 ± 0.5	17.0 ± 3.7*	1.0 ± 0.2	6.8 ± 0.7
G5	143 ± 10*	63 ± 7*	155 ± 14	9.7 ± 0.8	0.6 ± 0.4	4.6 ± 0.4	19.4 ± 3.1	0.9 ± 0.2*	6.5 ± 0.8
G4-Arg-Tos	159 ± 13	73 ± 11	160 ± 16	8.8 ± 0.7*	0.7 ± 0.3	4.3 ± 0.6	23.3 ± 3.5	1.2 ± 0.2	7.4 ± 0.7
G4-Lys-Cbz	161 ± 15	68 ± 10	166 ± 11	8.9 ± 0.6*	0.6 ± 0.4	4.6 ± 0.5	21.8 ± 2.8	1.1 ± 0.3	7.2 ± 0.4
G5-FA	165 ± 12	76 ± 9	164 ± 12	9.3 ± 0.6	0.8 ± 0.4	4.4 ± 0.5	26.1 ± 3.6	1.5 ± 0.2	7.4 ± 0.6
G5-Cou	144 ± 11*	62 ± 8*	165 ± 13	9.2 ± 0.7	0.7 ± 0.5	4.7 ± 0.4	17.8 ± 3.4*	1.0 ± 0.3	6.8 ± 0.6

TCh: Total Cholesterol; Gl: Glucose; Trg: Triglyceride; Ca: Calcium; TB: Total Bilirubin; UrA: Uric Acid; Ur: Urea; Cr: Creatinine; TP: Total Proteins. $n = 3$, $^*p < 0.05$, compared to the negative control.

compound emerges as a good candidate for future *in vivo* studies of antithrombotic activity on endothelial damage or thrombosis *in situ*, where it can act as an antithrombotic drug or as a drug carrier. *In vivo* studies are ongoing to fully characterize the applicability of this class of nanomaterial in cardiovascular diseases.

4. Experimental

4.1. Chemistry

All reagents were obtained from Sigma Aldrich Co. (Sigma Aldrich Co. Saint-Louis, MO), PAMAM G4 and G5 were obtained from Dendritech (Dendritech, Midland, MI), Mass spectra of dendrimers were recorded on a MALDI-TOF (matrix assisted laser desorption/ionization-time of flight) (Bruker, USA), platelet aggregation and secretion were done in a lumi-aggregometer (Chrono-Log, Havertown, PA, USA), platelet adhesion and thrombus formation were done using a BioFlux 200-flow system (Fluxion, San Francisco, California, USA). Kits for biochemical analyzes were obtained from Valtek (Valtek, Nuñoa, Chile), the biochemical analyzes were recorded on a Spectrophotometer (Clima Plus, RAL S.A, Barcelona), the counts of platelets and red blood cells were obtained in an hematologic counter (Bayer Advia 60 Hematology System, Tarrytown, NY, USA).

4.2. Dendrimers synthesis

4.2.1. PAMAM-G5-folate

Conjugation of PAMAM-G5 dendrimer with FA was carried out by a condensation between the γ-carboxyl group of FA and the primary amino group of PAMAM G5. Thus, 104 mg (0.23 mmol) of FA reacted with 150 mg (1.28 mmol) of EDC·HCl and 150 mg of HOBt, in a mixture of 2.7 mL of dry N,N-dimethylformamide (DMF) and 1.0 mL of dry dimethyl sulfoxide (DMSO), under a nitrogen atmosphere for 1 h. This mixture was added dropwise to a solution of 40 mg (1.38×10^{-3} mmol) of PAMAM G5 in 3.0 mL of water. The reaction mixture was vigorously stirred for 72 h. The functionalized dendrimer was purified by membrane dialysis against water (membranes with a cut-off of 500 Da) to remove the excess of non-reactive folate and coupling agents. After the lyophilization, the amount of the PAMAM G5-FA obtained was 52 mg.

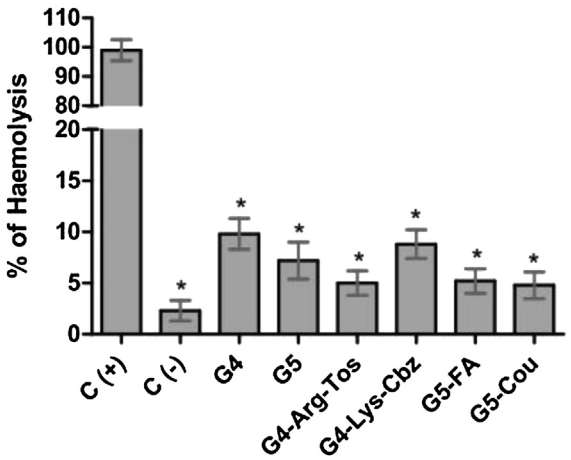

Fig. 3. Percentage of hemolysis obtained from the interaction of the derivatives of PAMAM dendrimers with washed RBCs, compared to the positive control Triton X-100 at 0.2% (100% hemolysis). $n = 3$, $^*p < 0.05$.

4.2.2. PAMAM-G5-coumarin

Conjugation of PAMAM-G5 dendrimer with Cou was carried out by a condensation between the carboxyl group of Cou and the primary amino group of PAMAM G5. Thus, 39.5 mg (0.208 mmol) of Cou reacted with 32 mg (0.208 mmol) of EDC·HCl in a mixture of 2.7 mL of dry DMF and 1.0 mL of dry DMSO under a nitrogen atmosphere for 1 h. The mixture was added dropwise to a solution of 40 mg (1.38×10^{-3} mmol) of PAMAM G5 in 3.0 mL of water. The reaction mixture was vigorously stirred for 72 h. The functionalized dendrimer was purified through dialysis (using water) membranes with a cut-off of 500 Da to remove the excess of Cou. After the lyophilization, the amount of the PAMAM G5-Cou obtained was 55 mg.

4.2.3. PAMAM G4-arginine-Tos-OH

Conjugation of PAMAM-G4 dendrimer with Boc-Arginine (Tos)-OH was carried out by a condensation between the carboxyl group of the Arginine and the primary amino group of PAMAM G4. Thus, 64 mg (0.208 mmol) of Boc-arginine (Tos)-OH reacted with 150 mg (2.9 mmol) of EDC·HCl and 150 mg of HOBt in a mixture of 2.7 mL of dry DMF and 1.0 mL of dry DMSO, under a nitrogen atmosphere for 1 h. The mixture was added dropwise to a solution of 40 mg (1.38×10^{-3} mmol) of PAMAM G4 in 3.0 mL of water. The reaction mixture was vigorously stirred for 72 h. The functionalized dendrimer was purified through dialysis membranes with a cut-off of 500 Da to remove the excess of the amino acid. Then, the product was lyophilized and the amount of the PAMAM G4-Boc-Arginine (Tos)-OH obtained was 55 mg. Next, a solution of HCl/dioxane (4.0 mL, 4.0 M) in a 25 mL round-bottom flask equipped with a magnetic stirrer was cooled by an ice-water bath under nitrogen. PAMAM G4-Boc-Arginine (Tos)-OH (0.2 mmol) was added in one portion with stirring. The ice-bath was removed and the mixture was kept stirred for 1 h, thin layer chromatography (TLC) indicated that the reaction was completed. The reaction mixture was condensed by rotary evaporation under high vacuum at room temperature. The residue was then washed with dry ethyl ether and collected by filtration (for oil products, a simple decantation was used instead) [26], affording 95% yield of PAMAM G4-Arg-Tos.

4.2.4. PAMAM G4-lysine-Cbz-OH

Conjugation of PAMAM-G4 dendrimer with Boc-Lys-Cbz-OH was carried out by a condensation between the carboxyl group

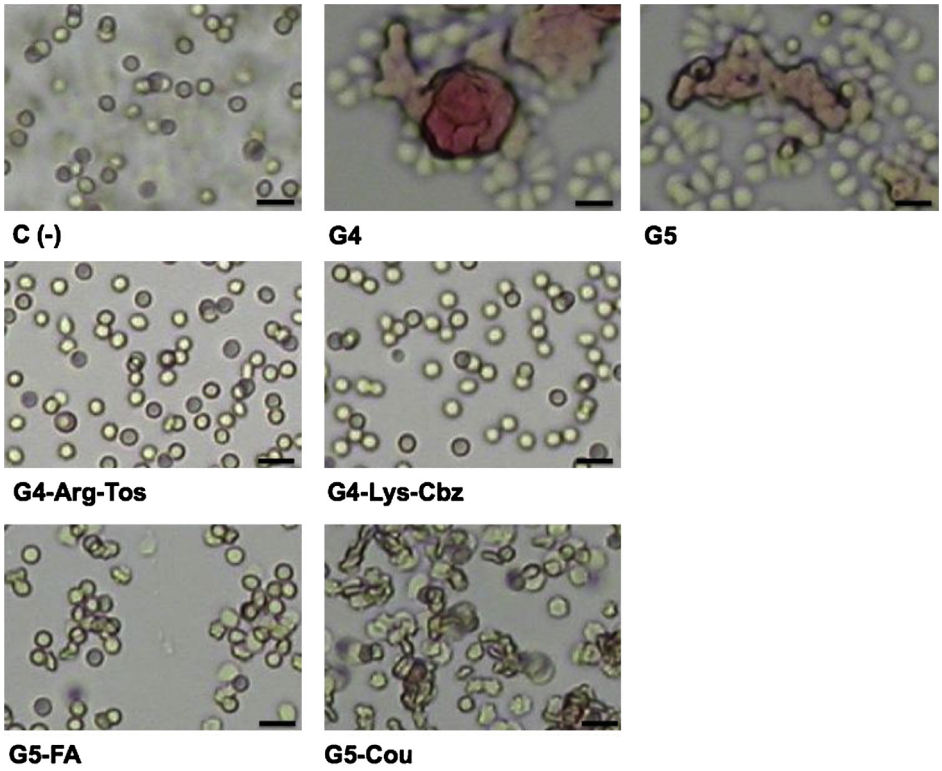

Fig. 4. Optical microscopy of washed RBCs after the interaction with PAMAM derivatives (0.25 mg/mL). Scale bar: 15 μm.

of the lysine and the primary amino group of PAMAM G4. Thus, 56 mg (0.208 mmol) of Boc-Lys-Cbz-OH reacted with 150 mg (2.9 mmol) of EDC·HCl and 150 mg of HOBt, in a mixture of 2.7 mL of dry DMF and 1.0 mL of dry DMSO, under a nitrogen atmosphere for 1 h. The mixture was added dropwise to a solution of 30 mg (2.1×10^{-3} mmol) of PAMAM G4 in 3.0 mL of water. The reaction mixture was vigorously stirred for 72 h. The functionalized dendrimer was purified through dialysis membranes with a cut-off of 500 Da to remove the excess of the amino acid. After the lyophilization, the amount of the PAMAM G4-Boc-Lysine-Cbz-OH obtained was 42 mg. Finally, a solution of HCl/dioxane (4.0 mL, 4.0 M) in a 25 mL round-bottom flask equipped with a magnetic stirrer, was cooled by an ice-water bath under nitrogen, and PAMAM G4-Boc-Lysine-Cbz-OH (0.2 mmol) added in one portion with stirring. The ice-bath was removed and the mixture was kept under stirred for 1 h. TLC indicated that the reaction was completed, the reaction mixture was condensed by rotary evaporation under high vacuum at room temperature. The residue was then washed with dry ethyl ether and collected by filtration (for oil products, a simple decantation was used instead) [26], affording 95% yield of PAMAM G4-Lys-Cbz.

Table 2
Effect of amino acids, PAMAM G4 and derivates on platelet aggregation under different agonists.

	ADP	Collagen	Arachidonic acid	TRAP-6
C(−)	85 ± 1	79 ± 2	84 ± 4	91 ± 1
G4	84 ± 2	94 ± 2	88 ± 2	98 ± 1
Arg-Tos	83 ± 5	91 ± 4	81 ± 3	83 ± 5
Lys-Cbz	80 ± 6	71 ± 3*	82 ± 4	80 ± 6
G4-Arg-Tos	37 ± 3*	46 ± 4*	40 ± 6*	37 ± 8*
G4-Lys-Cbz	67 ± 1*	71 ± 3*	84 ± 6	90 ± 9

$n = 3$, $^*p < 0.05$.

4.3. MALDI analysis

To confirm the molecular weight of surface modified dendrimers, mass spectrometric analysis of the dendrimers was performed on a MALDI-TOF (matrix assisted laser desorption/ionization-time of flight) with a pulsed nitrogen laser (337 nm), operating in positive ion reflector mode, using 19 kV acceleration voltage and the matrix 2,5dihydroxybenzoic acid (DHB).

4.4. Biochemical and red blood cells interactions

4.4.1. Sample collection

The blood samples were obtained from healthy donors. For this, to each donor were extracted 10 mL of whole blood, then was

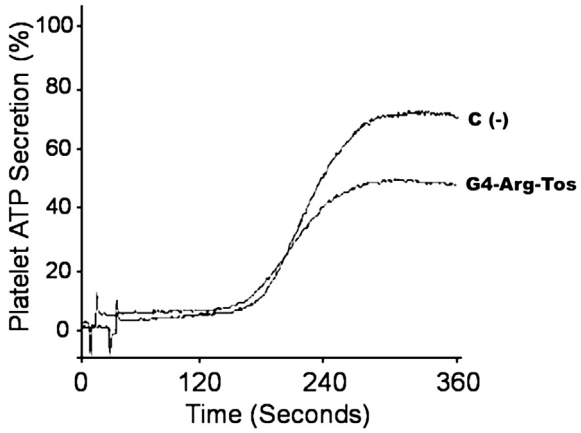

Fig. 5. Effect of the PAMAM G4-Arg-Tos (100 μg/mL) on platelet secretion.

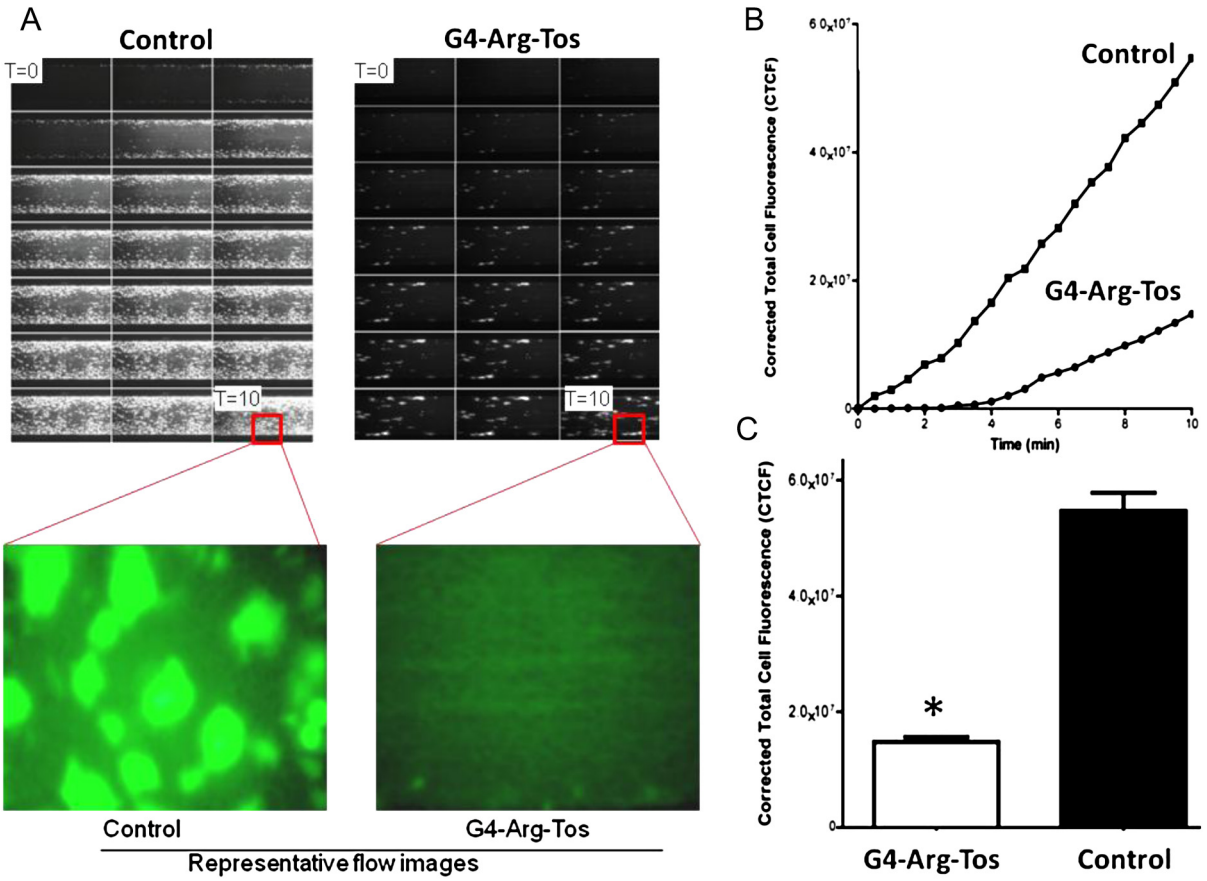

Fig. 6. Effect of G4-Arg-Tos on collagen-induced platelet thrombus formation under arterial flow at a shear rate of 1000 s-1. A) shows the intensity (CTCF) over a time lapse, B) time lapse of 10 min at 1000 s-1, at 30 s intervals and C) bar diagram (values are mean ± SD; $n = 3$). $^{*}p < 0.05$.

centrifuged at 2000 rpm for 15 min, to separate the serum from the rest of the blood. All the serum samples were mixed, aliquotted and frozen at −80 °C until use.

To perform the Assay of Red Blood Cells Lysis, blood from healthy donors was extracted and anticoagulated with 3.2% citrate tubes. Erythrocytes were separated from the plasma and leucocytes by centrifugation (1500 rpm, 5 min) at 4 °C and washed three times with phosphate-buffered saline (PBS: 150 mM NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4).

All the protocols were authorized by the ethic committee of the University of Talca, Chile, in accordance with the Declaration of Helsinki (approved by the 18th World Medical Assembly in Helsinki, Finland, in 1964).

4.4.2. Biochemical measurements

For the biochemical analyzes 3.0 mL of serum were incubated with each dendrimer at a final concentration of 0.25 mg/mL in saline. After 2 h of incubation, samples were centrifuged at 12000 rpm and measured: creatinine (Cr), urea (Ur), total proteins (TP), triglycerides (Trg), uric acid (UrA), total cholesterol (TCh), glucose (Gl), total bilirubin (TB) and calcium (Ca). Commercial available kits were used to perform the analyzes according to the protocols provided by Valtek.

4.4.3. Assay of Red Blood Cells Lysis

The hemolysis assay was performed according to the method of Duncan et al., 2005 [27]. Briefly, washed RBCs at 2% were incubated at 30 °C with a final concentration of 0.25 mg/mL in saline of the selected dendrimer. After 2 h of incubation, the samples were centrifuged at 2000 rpm for 10 min and the absorbance of the supernatant was measured at 550 nm. Hemolysis was expressed as percentage of released hemoglobin. It was used as control (100% of hemoglobin released) a solution of RBCs incubated with Triton X-100 (0.2% V/V). Additionally, morphological changes in the RBCs were determined by optical microscopy.

4.5. Effect on platelet function

4.5.1. Preparation of human platelet suspensions

Blood samples were taken from five volunteers who previously signed informed consent (healthy university students) forms, in 3.2% citrate tubes by phlebotomy with vacuum tube system. The samples were gently homogenized by inversion and allowed to stand for 5 min. Then, were centrifuged at 1000 rpm for 10 min, and 1.0 mL of platelet-rich plasma (PRP) was taken from each tube for platelet count (in triplicate) in an hematologic counter. The original tubes were centrifuged at 3500 rpm for 10 min to obtain the platelet-depleted plasma (PDP). Finally, the PRP was adjusted to 2×10^5 platelets/μL with PDP.

4.5.2. Platelet aggregation assay

Platelet aggregation was monitored by light transmission turbidimetric method according to Born and Cross [28], using a lumi-aggregometer. Briefly, 480 μL of PRP in the reaction vessel were pre-incubated with 20 μL of PAMAM (all samples at final concentration of 100 μg/mL in saline), negative control of inhibition (saline) or positive control (acetylsalicylic acid 110 μM, final concentration). After 5 min of incubation, 20 μL of agonist were added

to initiate platelet aggregation, which was measured for 6 min. ADP, collagen, TRAP-6 and arachidonic acid, were used as agonists. All measurements were performed in triplicate. The results of platelet aggregation were determined by the software AGGRO/LINK, and the relative inhibition of the maximum platelet aggregation was calculated as:

$$\text{Platelet Aggression} = 100 - \left(\%Ag_{\text{Dendrimer}} \times 100\right)/\%Ag_{\text{Neg.Control}}\Big)$$

where, $\%Ag_{\text{Dendrimer}}$: percentage of aggregation of the dendrimer under study, $\%Ag_{\text{Neg. Control}}$: percentage of aggregation of negative control.

4.5.3. Platelet secretion assay

Platelet secretion was determined by measuring the release of ATP using luciferin/luciferase reagent. Luciferin/luciferase (26 μL) was added to 480 μL of platelet suspension (PRP adjusted to 2×10^5 platelets/μL) within 2 min before stimulation. Platelet secretion was recorded in real time in a lumi-aggregometer at 37 °C with stirring (1000 rpm) and luminescence (×0.2). To determine the effects on platelet secretion, platelets were pre-incubated with the different PAMAM derivatives (final concentration of 100 μg/mL in saline) for 2 min prior to the addition of ADP [29].

4.5.4. Analysis of platelet adhesion and thrombus formation in flowing whole blood

For flow experiments, BioFlux 200-flow system with high shear plates (48 wells, up to 20 dyne/cm^2) was used. Using manual mode in the BioFlux software, the microfluidic chambers were coated for 1 h with 50 μL of collagen 200 μg/mL at a wall shear rate of 200 s^{-1}. The plaque coating was allowed to dry at room temperature for 1 h. To remove the interface, the channels were perfused with PBS for 10 min at a wall shear rate of 200 s^{-1}. Then, the channels were blocked with bovine serum albumin (BSA) 0.5% for 10 min at a wall shear rate of 200 s^{-1}.

The plaque-coated microfluidic high shear plates were mounted on the stage of an inverted fluorescence microscope. Control blood and blood with the different dendrimers (final concentration of 100 μg/mL in saline) were pre-incubated at 25 °C for 1 h with calcein-AM 4 μM. Then, the mixture was added to the inlet well, and chambers were perfused for 10 min at a wall shear rate of 1000 s^{-1}. All the experiments were performed at room temperature [30].

Platelet deposition was observed and recorded in real-time (30 frames per min) with a CCD camera. We used bright field and fluorescence microscopy for real-time visualization of platelet adhesion and aggregation in flowing blood. For each flow experiment, perfused surface fields of the size of 237,900 μm^2 (located in the middle of the channels of the viewing window) were recorded, and fluorescence images were later analyzed off-stage by quantifying the area covered by platelets with the ImageJ software. In each field, the areas covered by platelets were quantified.

4.6. Statistical analysis

The results were analyzed statistically, comparing the results of each dendrimer under study with the respective control. The results were expressed as mean ± SE. In the statistical analysis *t* test was used with the software SPSS 15.0 (Statistical Product and Service Solutions). *p* values <0.05 were considered statistically significant.

5. Disclosures

None.

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