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Influence of fourth generation poly(propyleneimine) dendrimers on blood cells

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Abstract: Dendrimers provide many exciting opportunities for potential biomedical applications. However, owing to their positively charged surfaces, poly(propyleneimine) (PPI) dendrimers show toxic and haemolytic activities. One of the methods for masking the peripheral cationic groups is to modify them using carbohydrate residues. In this study, three types of the fourth generation PPI dendrimers-uncoated (PPI-g4), approximately 35% maltotriose (Mal-III)-coated (PPI-g4-OS), and approximately 90% Mal-III-coated (PPI-g4-DS) were investigated by assessing their effects on red blood cell (RBC) haemolysis in samples of pure RBCs, RBCs in the presence of human serum albumin (HSA) or human plasma, and RBCs in whole blood. Lymphocyte proliferation and platelet (PLT) aggregation were also studied in the presence of various concentrations of dendrimers. Although all dendrimers examined affected all the blood cells studied, the unmodified PPI-g4 had the most damaging effect. It caused high RBC haemolysis rates and PLT aggregation and greatly inhibited lymphocyte proliferation. These effects were caused by the cationic surface of this polymer. The modification of PPI-g4 with Mal-III reduced the effect of the dendrimer on all blood cells. The presence of HSA or plasma in the buffer containing the RBCs or RBC in whole blood significantly decreased the extent of dendrimer-driven haemolysis. J Biomed Mater Res Part A: 100A: 2870–2880, 2012.

Key Words: poly(propyleneimine), PPI, dendrimer, glycodendrimer, lymphocytes, platelets, red blood cells, aggregation, haemolysis, proliferation, toxicity, HSA, blood

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INTRODUCTION

Since their introduction,[1,2] dendrimers have attracted great interest in biomedical applications because of their unique dendritic structures and multiple surface properties. Owing to the presence of a large number of terminal groups, drug molecules can be attached to the dendrimer surface through covalent bonds,[3,4] whereas internal cavities are capable of encapsulating small molecules.[5,6] This makes the dendrimers suitable for drug delivery systems. Because they can interact with nucleic acids, cationic dendrimers can be also used as vectors for gene transfection.[7] Dendrimers can interact effectively and specifically with cell components such as membranes, organelles, and proteins.[8,9] Nevertheless, their interactions with cell compounds and compartments are nonselective, so they also have the potential to cause cytotoxicity and haemotoxicity because of their terminal cationic groups.[4,10] One of the methods of reducing dendrimer toxicity is to modify their surfaces by substitution of amino groups with neutral or anionic moieties such as polyethylene glycol (PEG),[11–13] amino acids,[14–16] or carbohydrate residues.[5,14,17] More information on dendrimer toxicity and biological properties is still needed before they can be used safely and effectively in biomedical applications. In previous studies, we have shown that glycodendrimers with open or dense oligosaccharide shells, created using maltose or maltotriose, exhibit the desired biocompatibility under in vitro and in vivo conditions.[17–21] However, from these studies, further concern was raised about the application of the drug-glycodendrimer complexes/conjugates by different administration processes and their detailed interactions with, and effects on, red blood cells (RBC). This concern arose because the circulatory system seems the best way of administering the drugs for the dendrimer-drug complexes/conjugates to reach distant, directly inaccessible

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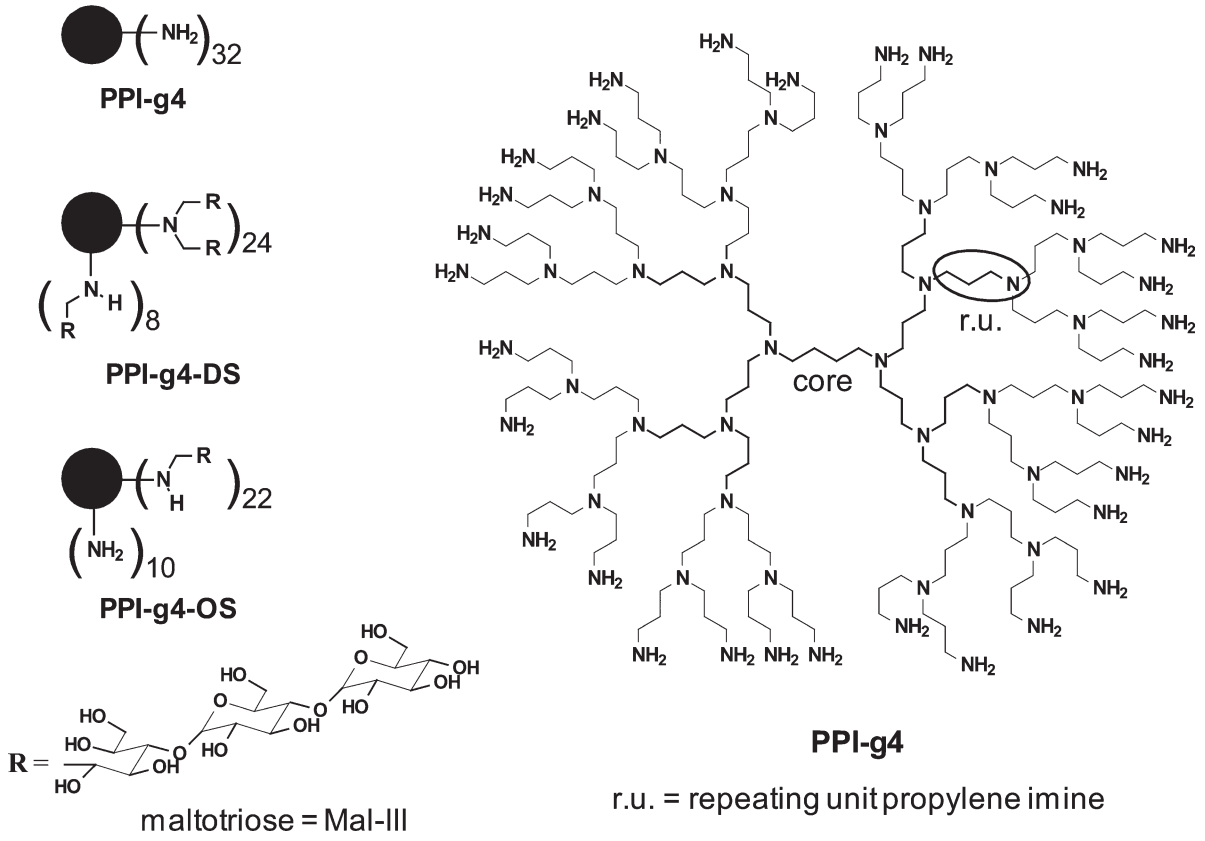

FIGURE 1. Simplified structure of PPI-g4, PPI-g4-OS, and PPI-g4-DS.

tissues within a relatively short time. Thus, it is important to study the effects of dendrimers on blood cells. Polyamidoamine (PAMAM), poly(propyleneimine) (PPI), or poly-L-lysine dendrimers are known to cause haemolysis and changes in the RBC's shape depending on time and concentration because of their cationic surface.[22–25] It is also confirmed that use of anionic (PAMAM half-generations) or neutral (e.g., PAMAM-OH) dendrimers reduces their haemolytic activity.[4,26] Although haemotoxicity of dendrimers is widely studied, reports about dendrimers influence on platelets (PLT) aggregation[27,28] or lymphocytes proliferation[29–31] are limited. For this detailed study, we have used fourth generation PPI dendrimers—either unmodified (PPI-g4) dendrimers with approximately 35% of the surface amino groups substituted with maltotriose [Mal-III] residues [open shell (PPI-g4-OS)] or dendrimers with approximately 90% of the surface amino groups substituted with Mal-III [dense shell (PPI-g4-DS)] (Fig. 1)—to investigate their influence on RBCs, lymphocytes, and PLT. PPI glycodendrimers with dense oligosaccharide shell (PPI-g4-DS, Fig. 1) have a virtually neutral surface charge on the outer oligosaccharide shell.[14,18,19] Because of this neutral surface charge, they are preferentially involved in H-bond-driven interactions.[17] PPI glycodendrimers with open oligosaccharide shell (PPI-g4-OS – Fig. 1) can undergo simultaneous electrostatic and H-bond interactions. In other studies, these multiple interactions of the cationic dendritic glycopolymers have been successfully used to transform anionic vesicles into tube-like networks.[32]

MATERIALS AND METHODS

Dendrimers

The uncoated fourth generation PPI dendrimer was obtained from Symo-Chem (Eindhoven, The Netherlands). The Mal-III-modified fourth generation PPI dendrimers, PPI-g4-DS and PPI-g4-OS, were synthesized and characterized as

TABLE I. Molar Mass (MM[a]) for Commercially Available PPI-g4 and Synthesized PPI-g4-OS and PPI-g4-DS.[23,24] The Number and the Percentage of Surface Maltotriose Groups

Dendrimer	$MM_{theoretical}$ (g/mol)	$MM_{observed}$ (g/mol)	Number (%) of Surface Mal-III $Groups_{theoretical}$	Number (%) of Surface Mal-III $Groups_{observed}$
PPI-g4	3514	3514	–	–
PPI-g4-OS	19144	14260	32 (50%)	22 (35%)
PPI-g4-DS	34774	31000	64 (100%)	56 (87%)

[a] MM of PPI-g4-OS and PPI-g4-DS determined by ^{1}H NMR approach described in Refs. 17 and 23.

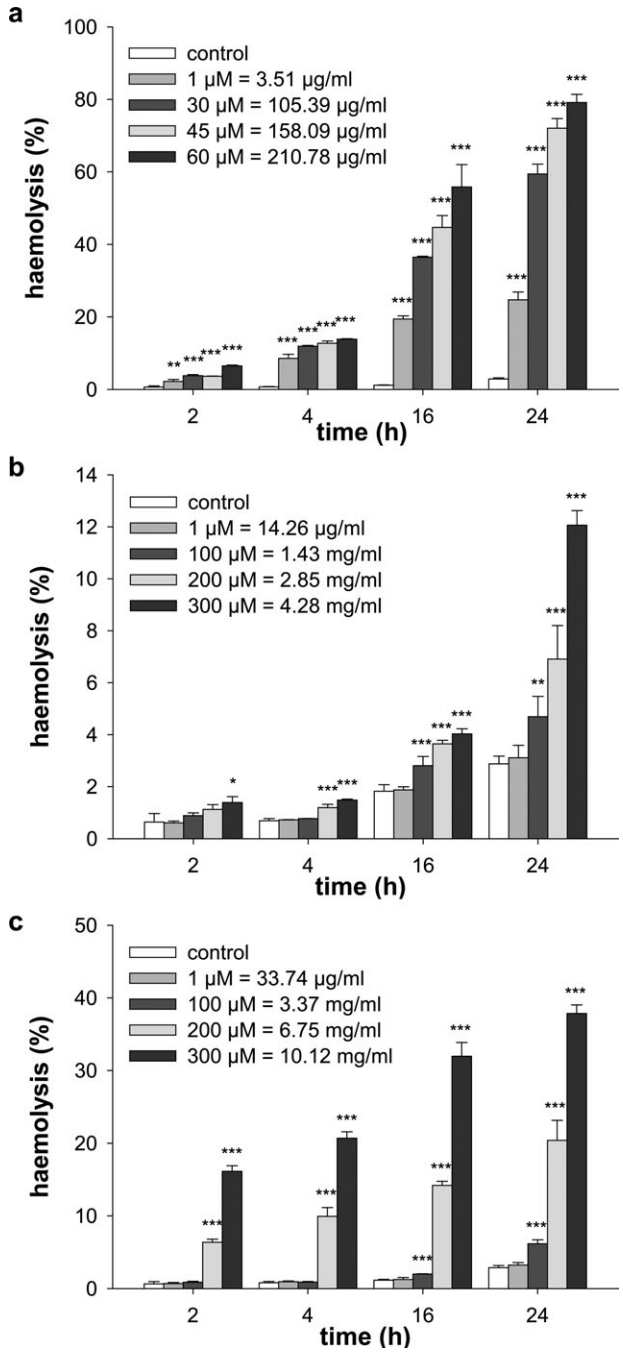

FIGURE 2. Extent of haemolysis induced by PPI-g4 (a), PPI-g4-OS (b), PPI-g4-DS (c) at various concentrations and incubation times. Results are presented as mean and SD. Significant differences at $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

described in Ref. 33 for PPI-g4-DS and in[34] for PPI-g4-OS. The characterization of dendrimers is shown in Table I.

Haemolytic activity

Blood from healthy donors was obtained from the Central Blood Bank (Lodz). It was anticoagulated with 3% sodium citrate. Erythrocytes were separated from plasma and lymphocytes by centrifugation (1000 g, 5 min) at 4°C, 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) and resuspended in PBS. Erythrocytes were used immediately after isolation. To study the effects of dendrimers on haemolysis, RBC resuspended in PBS to a final haematocrit (HTC) of 2% (the proportion of the blood volume occupied by RBC) were treated with different concentrations of each type of dendrimer: 1, 30, 45, 60 μM for PPI-g4 and 1, 100, 200, 300 μM for PPI-g4-OS and PPI-g4-DS. RBCs resuspended in PBS were used as a control. Stock dendrimer solutions were dissolved in PBS. The erythrocyte suspensions were incubated at 37°C for 2, 4, 16, and 24 h. Samples were centrifuged at 1000 g for 5 min and the absorbance of the supernatant was measured spectrophotometrically at 540 nm. The percentage haemolysis was determined on the basis of haemoglobin (HGB) released into the supernatants. For reference (100% haemolysis), RBCs were treated with distilled water.

For microscopic observations, RBCs at a final HTC of 2% were suspended in solutions of dendrimers at concentrations of 30 μM for PPI-g4 and 300 μM for PPI-g4-OS or PPI-g4-DS and incubated at 37°C for 24 h. Ten-fold diluted samples were then viewed under an optical microscope at a magnification of 400×. To investigate the influence of human serum albumin (HSA) or human plasma on the haemolysis of RBCs in a buffer, or to study haemolysis in whole blood, dendrimers at concentrations causing 20% haemolysis after 2 and 24 h (as determined by a separate experiment) were used. To study the effect of HSA, 2 mg/ml of HSA was added to the dendrimer solutions. The concentration of HSA was chosen to achieve the same ratio of HTC to HSA as found under physiological conditions.[35] The dendrimer–HSA mixture was left for 5 min to equilibrate before the RBC suspension was added.[36] RBCs resuspended in PBS were used as a control. To study the effect of PPI dendrimers on RBCs resuspended only in blood plasma (with no other morphological elements of the blood), blood samples were centrifuged (1000 g, 5 min) at 4°C. The upper layer of plasma was collected, the lower layer of plasma and lymphocytes was removed, and the RBCs were washed three times in PBS and resuspended in plasma to achieve an initial HTC equal to that found in blood. The suspension was added either to a solution of dendrimers or to PBS (control) to achieve an HTC of 2%. Whole blood was also used to study the effect of dendrimers on RBCs in the presence of plasma and other morphological elements of the blood. RBCs were resuspended to an HTC of 2% by adding whole blood either to a solution of dendrimers or to PBS (control). The samples were then incubated for 2 and 24 h, and haemolysis was determined as described above.

Lymphocytes proliferation

Blood from healthy donors was obtained from the Central Blood Bank (Lodz). It was anticoagulated with 3% sodium citrate. Peripheral blood mononuclear cells (PBMC) were isolated using Histopaque 1077. The viability of the cells was measured by the trypan blue exclusion assay and was found to be approximately 99%. Lymphocyte proliferation was assayed according to Ref. 37. After isolation, the cells

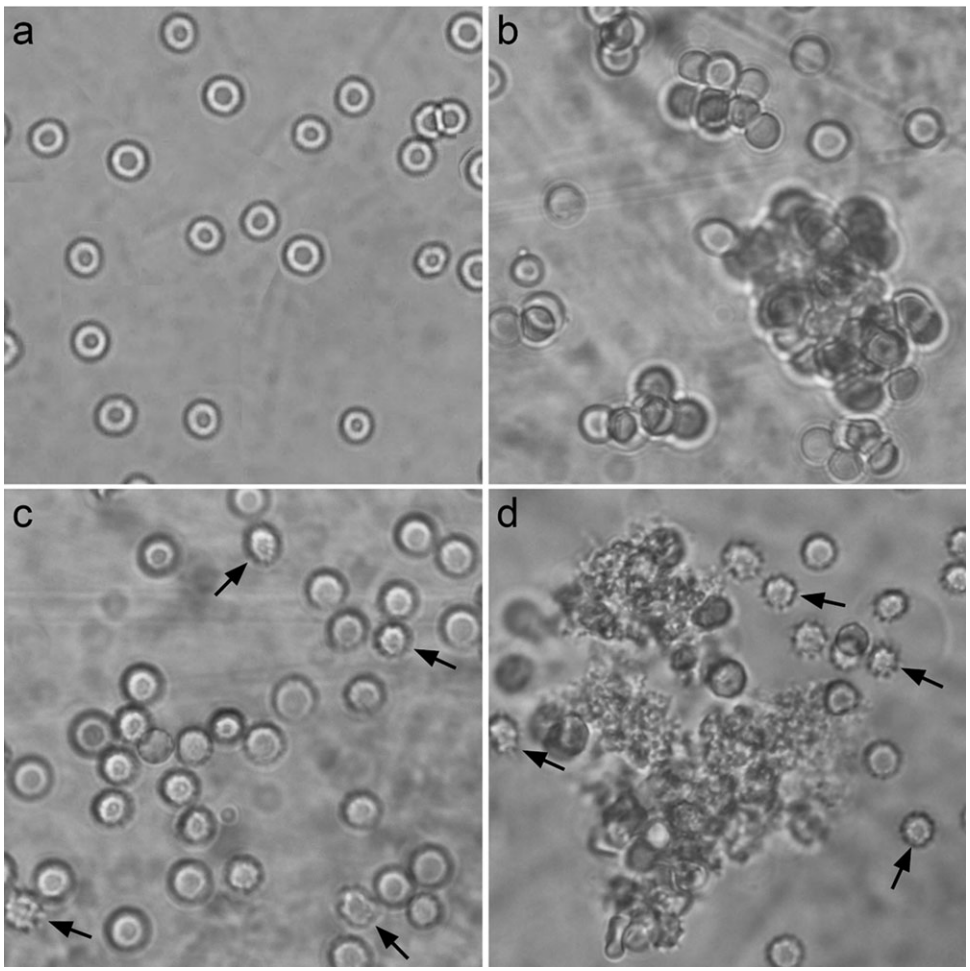

FIGURE 3. Morphological changes in RBC after 24 h of dendrimers treatment: (a) intact cells, (b) after PPI-g4 treatment, (c) after PPI-g4-OS treatment, (d) after PPI-g4-DS treatment. Arrows indicate echinocytes (magnification 400×).

were resuspended in RPMI 1640 supplemented with 10% FBS (heat inactivated), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate at a density of 1×10^6 cells/ml, and 100 μl of the cell suspension per well was dispensed into a 96-well round-bottom plate. The isolated cells were incubated in a humidified 37°C, 5% CO_2 incubator in the presence (test samples) or absence (control samples) of PPI dendrimers, and in the presence or absence of phytohemagglutinin (PHA-M) to assess the inhibition or induction of proliferation, respectively. After 72 h incubation, the samples were analyzed spectrophotometrically using MTT (3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide). The final concentration of PHA-M was 10 μg/ml and the final concentrations of the dendrimers were 1, 0.2, 0.04, and 0.008 mg/ml. A PBMC suspension with PHA-M solution at a concentration of 10 μg/ml in cell culture medium was used for a positive (proliferating) control, and PBMC suspension with PBS was used as the negative (nonproliferating) control. The assay was performed on triplicate cultures.

PLTs aggregation

Blood from healthy donors was obtained from the 'Republic Research & Production Center for Transfusiology and Medical Biotechnologies' (Minsk, Republic of Belarus). It was anticoagulated with CPDA-1 (110 mM glucose; 55 mM mannitol; 25.8 mM K_2HPO_4; 14.7 mM KH_2PO_4; 17.9 mM potassium citrate). Blood plasma was centrifuged at 360 g for 5 min to pellet the PLTs, and the pellet was resuspended in 200 μl Tris buffer containing EDTA (0.12 M NaCl, 0.0154 M KCl, 0.006 M glucose, 0.0015 M Na_2EDTA, 0.0133 M Tris,

TABLE II. Concentrations of Dendrimers that Caused Approximately 20% of Haemolysis

Dendrimer	PPI-g4		PPI-g4-OS		PPI-g4-DS	
Time (h)	2	24	2	24	2	24
Concentration (μM)	150	0.80	Not achieved	470	375	200
Concentration (mg/ml)	0.527	0.0028	Not achieved	6.70	12.65	6.75

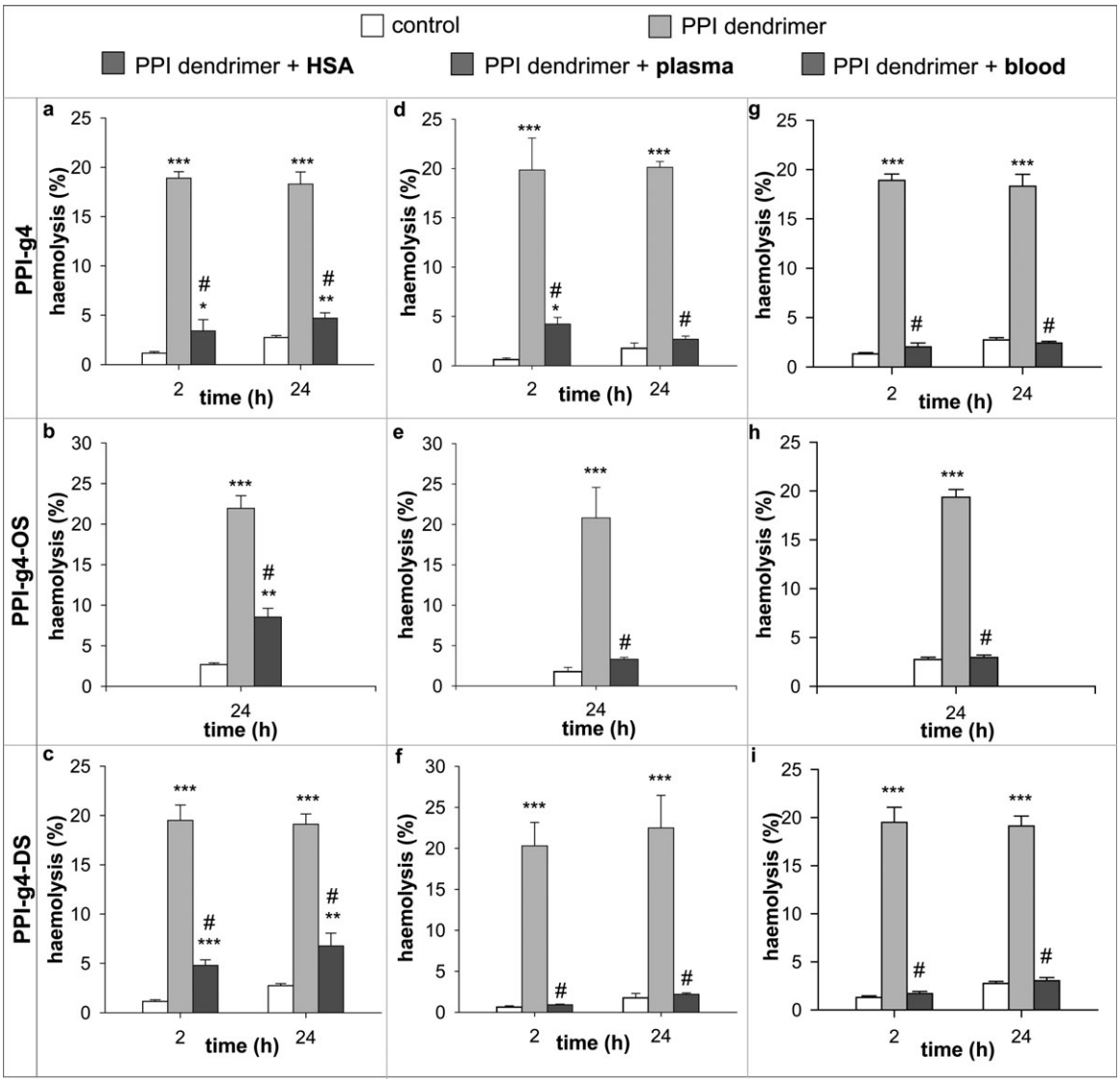

FIGURE 4. Haemolysis in the presence of PPI-g4 (a, d, g), PPI-g4-OS (b, e, h), and PPI-g4-DS (c, f, i) and of HSA (a, b, c), plasma (d, e, f), and blood (g, h, i). Results are presented as mean and SD. Significant differences at $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ when compared with control and $\#p \leq 0.001$ when compared to samples with dendrimer only.

pH 6.5[38]). The suspension was centrifuged at 360 g for 5 min. The supernatant was removed and the pellet was resuspended in the buffer to a concentration of 2.0×10^9 cell/ml. Aggregation of PLTs was studied using an automatic aggregometer AP2110 (SOLAR, Belarus). To assess aggregation, 400 μL phosphate-saline buffer containing Ca^{++} (0.137 M NaCl, 0.0027 M KCl, 0.0087 M Na_2HPO_4, 0.00148 M KH_2PO_4, 0.001 M $CaCl_2$, pH 7.35)[38] and 50 μL of PLT suspension were added to a thermostatic (37°C) plastic tube, to a final PLT concentration of 2.0×10^8 cells/ml. Trypsin (1 μg/ml) or dendrimers at various concentrations (for PPI-g4—0.00001, 0.0001, 0.001, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 mg/ml, for PPI-g4-OS—0.001, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 mg/ml, and for PPI-g4-DS—0.0001, 0.001, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 mg/ml) were then added to the PLT suspension.

TABLE III. The Reduction of RBC Haemolysis in the Presence of HSA and Plasma and in Whole Blood (Mean ± SD)[a]

Dendrimer	PPI-g4		PPI-g4-OS	PPI-g4-DS	
Time (h)	2	24	24	2	24
Reduction of haemolysis (%) in presence of HSA	81.8 ± 6.67	74.3 ± 1.79	60.7 ± 7.92	75.5 ± 1.16	64.4 ± 7.84
Reduction of haemolysis (%) in presence of plasma	78.7 ± 3.3	86.4 ± 2.1	82.8 ± 3.7	95.3 ± 0.7	90.1 ± 2.1
Reduction of haemolysis (%) in whole blood	89.2 ± 2.1	86.8 ± 0.9	84.7 ± 1.8	91.2 ± 1.7	84.1 ± 0.9

[a] Measurements have been performed with dendrimers at concentrations causing 20% haemolysis in PBS.

TABLE IV. Lymphocytes Proliferation in the Presence of PPI Dendrimers and PHA-M

c (mg/ml)	1	0.2	0.04	0.008
PPI-g4	45.0% ± 5.72	42.8% ± 5.63	42.5% ± 5.34	64.5% ± 5.13
PPI-g4-OS	69.2% ± 5.73	87.0% ± 6.77	94.8% ± 4.45	99.7% ± 5.27
PPI-g4-DS	95.1% ± 7.27	96.6% ± 6.51	97.4% ± 6.90	96.9% ± 8.28

Results are presented as percent of cell viability compared with proliferating control (100%) (mean ± SD).

RESULTS

Haemolysis

Haemolysis was caused by PPI-g4 as well as PPI-g4-OS and PPI-g4-DS and was time- and concentration-dependent (Fig. 2). PPI-g4 was the most haemolytic dendrimer, causing high haemolysis at the lowest concentration (1 μM) even after only 2 h of incubation. The surface modification of the dendrimer by Mal-III significantly reduced haemolysis. After 24 h incubation, the glycodendrimers at the highest concentration (300 μM) were significantly less damaging than the unmodified PPI-g4 at 30 μM. However, the increase in the degree of surface modification was not proportional to the decrease in the percentage haemolysis. PPI-g4-DS at 200 and 300 μM caused much greater haemolysis than PPI-g4-OS at the same molar concentrations, even after only 2 h incubation. The influence of dendrimers on RBC morphology was assessed by optical microscopy (Fig. 3). The control cells had their normal physiological biconcave disc shape, and no aggregates were observed. After PPI-g4-OS and PPI-g4-DS treatment, echinocytes appeared, but no echinocytes were present in the cell suspension after PPI-g4 treatment. The main change observed was the cell aggregation caused by PPI-g4 and PPI-g4-DS dendrimers, leading to cluster formation. To investigate the influence of HSA or human plasma on haemolysis, dendrimer concentrations that caused approximately 20% haemolysis after 2 and 24 h were selected, based on the previous results. The dendrimer concentrations used are shown in Table II. Adding HSA to the system markedly reduced the extent of haemolysis caused by all the dendrimers tested [Fig. 4(a–c)]. Although the amount of HGB released in samples containing HSA was still significantly higher than in the control, it was significantly lower than in the samples with dendrimers alone. When the RBCs were suspended in dendrimer-plasma solution, haemolysis was significantly lower than in samples without plasma. This behaviour was comparable with the control samples [Fig. 4(d–f)]. The percentage haemolysis induced by the dendrimers in whole blood was markedly lower than in the samples with RBCs resuspended in PBS. There was no difference between tested samples and controls [Fig. 4(g–i)]. The rates of haemolysis reduction in the presence of HSA, blood plasma, and in whole blood are shown in Table III. The reduction of PPI-induced haemolysis in the presence of HSA, blood plasma, and whole blood, compared with PBS, ranged from 60.7 to 91.2% depending on the incubation time and dendrimer type.

Lymphocytes proliferation

All the dendrimers examined influenced lymphocyte proliferation, but the extent of the effect depended on the dendrimer end groups. PPI-g4 significantly inhibited lymphocyte proliferation over the whole concentration range ($p \leq 0.001$); PPI-g4-OS [except concentration of 1 mg/ml ($p \leq 0.001$)] and PPI-g4-DS only slightly inhibited cell proliferation and when compared with the effect caused by PPI-g4, we can consider this effect as not important from the biological point of view, even though in a few cases, a statistical significance was observed [e.g., for PPI-g4-OS at concentration of 0.2 mg/ml ($p \leq 0.001$) and 0.04 mg/ml ($p \leq 0.003$) and for PPI-g4-DS at 1 mg/ml ($p \leq 0.05$)]. Detailed results are presented in Table IV and Figure 5.

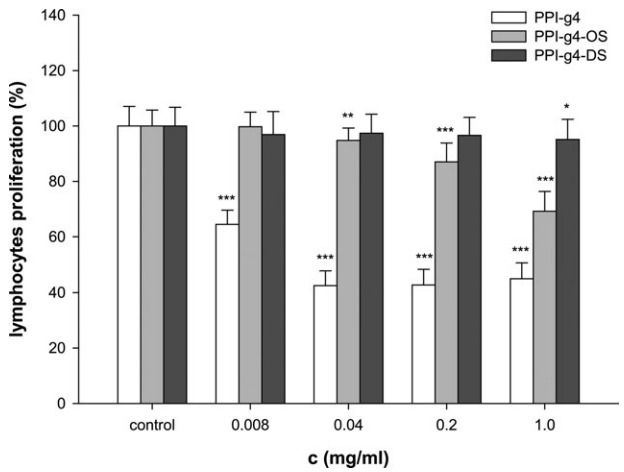

FIGURE 5. Extent of lymphocytes proliferation after 72 h of PPI dendrimers treatment (mean, SD). Control cells were treated with PHA-M only. Significant differences at $*p \leq 0.05$, $**p \leq 0.003$, $***p \leq 0.001$.

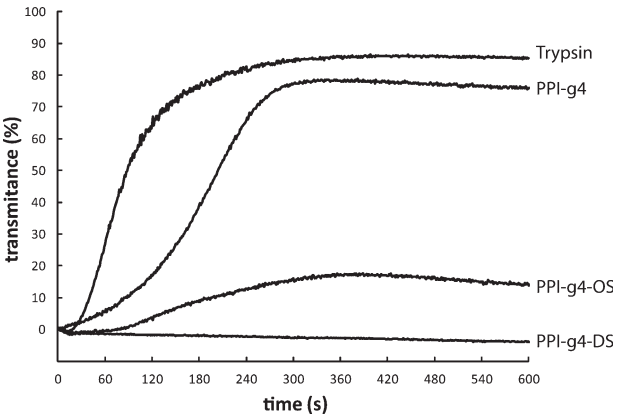

FIGURE 6. Exemplary aggregatogram of PLTs aggregation induced by trypsin and PPI dendrimers: trypsin at concentration of 1 μg/ml; PPI-g4, PPI-g4-OS, and PPI-g4-DS at concentration of 50 mg/ml.

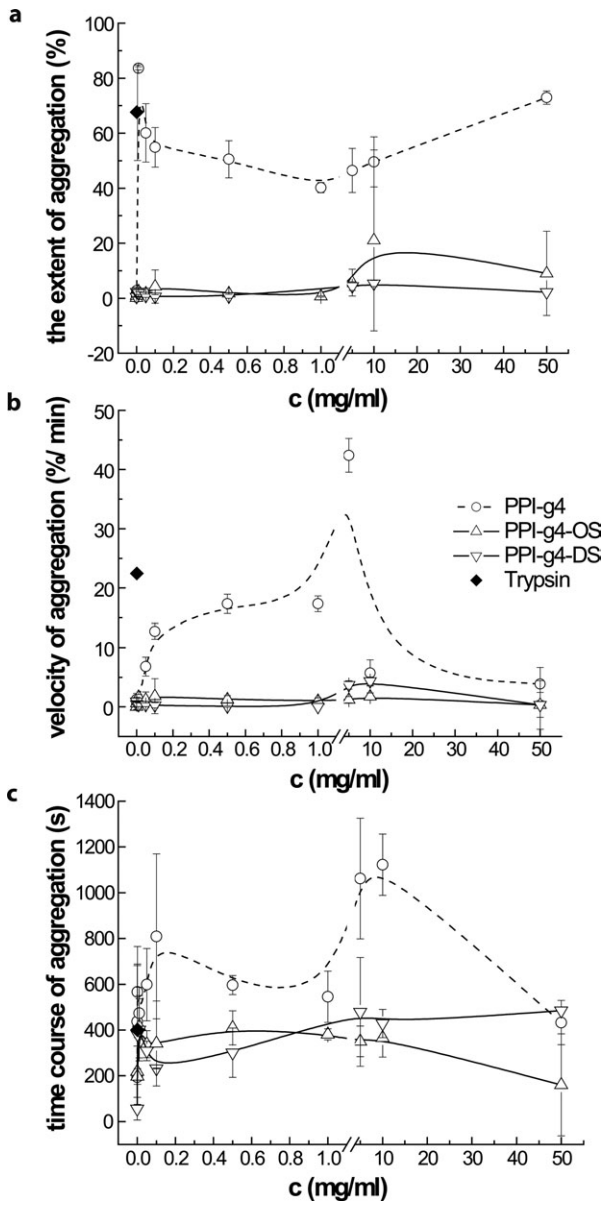

FIGURE 7. The extent (a), velocity (b), and time course (c) of PLT aggregation induced by trypsin (1 μg/ml) and PPI-g4, PPI-g4-OS and PPI-g4-DS.

PLTs aggregation

The extent, velocity, and time course parameters of PLT aggregation induced by trypsin (as a control) and PPI dendrimers were measured. A typical aggregatogram of PLT aggregation induced by trypsin at a concentration of 1 μg/ml and PPI dendrimers at a concentration of 50 mg/ml is shown in Figure 6. The extent of PLT aggregation induced by PPI dendrimers and the trypsin (as a control) is shown in Figure 7(a).[39] PPI-g4 caused aggregation comparable with that of trypsin even at low concentrations. The modification of PPI dendrimers by Mal-III drastically reduced their ability to induce PLT aggregation. At concentrations higher than 10 mg/ml, PPI-g4-OS induced 20% of PLTs to aggregate. No PLT aggregation was seen in the presence of PPI-g4-DS at any of the concentrations studied. The velocity of PLT aggregation induced by the PPI-g4 dendrimer gave a bell-shaped curve; it increased significantly with increasing concentrations up to 10 mg/ml and then abruptly decreased at higher concentrations [Fig. 7(b)]. The time course of PLT aggregation induced by the PPI-g4 dendrimer showed a similar character [Fig. 7(c)]. As expected, the modification of PPI dendrimers with Mal-III resulted in a loss of changes in the aggregation time course. The results obtained were confirmed by microscopic images of PLT morphology. Images of intact PLTs, PLTs treated with trypsin (1 μg/ml) and those treated with the dendrimers PPI-g4, PPI-g4-OS, and PPI-g4-DS, are shown in Figure 8. The activity of unmodified dendrimers at 50 mg/ml resulted in the formation of large cell aggregates. At 1 mg/ml, PPI-g4 induced cell aggregation with the formation of multiple small aggregates. When PPI-g4-OS and PPI-g4-DS were used at the same concentration, images of the cell suspensions were practically identical to those of untreated PLTs.

DISCUSSION

The aim of this study was to investigate the effects of fourth generation PPI dendrimers on blood cells: RBCs, lymphocytes, and PLTs. All dendrimers (unmodified, partially, or fully modified by Mal-III on the surface) affected blood cells. However, owing to their positively charged surfaces, PPI-g4 and PPI-g4-OS had greater effects. The data on dendrimer-driven HGB leakage give a qualitative indication of the potential damage that could be caused by dendrimers administered intravenously. The high density of cationic surface groups on dendrimers allows them to interact strongly with RBC cell membranes[40,41] where negatively charged glycolipids and some membrane glycoproteins are located.[22] The negative charge of the RBC cell surface prevents self-aggregation and adhesion to the endothelia of blood vessels.[42] In our study, the interactions between the cationic PPI dendrimers (PPI-g4 and PPI-g4-OS) and the RBC membrane are thought to be due to electrostatic attractions, but for the dense-shell glycodendrimer PPI-g4-DS it is not electrostatic but H-bond-driven interactions that are thought to be responsible.[17] Additionally, the H-bond features of PPI-g4-OS cannot be totally excluded from this study, as demonstrated in other studies.[32] Increased dendrimer concentrations and longer incubation times induce stronger PPI-RBC interactions. This effect does not depend on the PPI surface as, unexpectedly, PPI-g4-OS proved less haemolytic than PPI-g4-DS at the same molar concentrations (200 and 300 μM). This can be explained by the difference in their molecular weights corresponding to the molar concentrations: PPI-g4-OS: 200 μM ≈ 2.85 mg/ml, 300 μM ≈ 4.28 mg/ml; PPI-g4-DS: 200 μM ≈ 6.75 mg/ml, 300 μM ≈ 10.12 mg/ml. Thus, as well as surface modification, the size and molecular weight of the particle are also important. In other words, the greater number of maltotriose units on the PPI surface in PPI-g4-DS may induce a stronger response in RBCs but may also induce stronger cluster effects against RBCs [Fig. 3(d)] when larger masses of PPI-g4-DS (200 and 300 μM) are used for the haemolysis assay (Fig. 2). Thus, in further

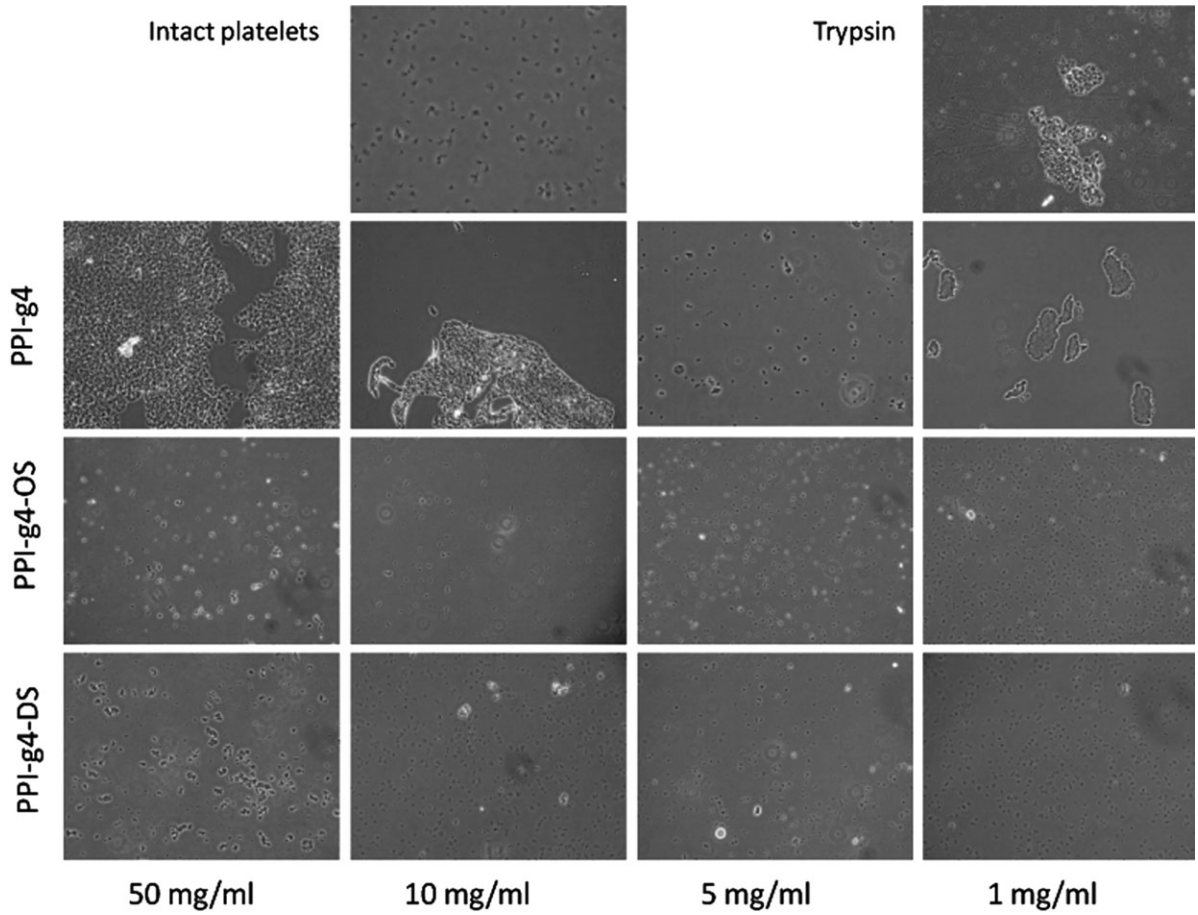

FIGURE 8. Microscopic images of human PLTs treated with trypsin (1 μg/ml) and PPI-g4, PPI-g4-OS, and PPI-g4-DS.

studies, we only used a mass concentration of PPI dendrimers to compare their effects on the other blood cells. It is also a possibility that intramolecular H-bonds and subsequent decrease in polarity result in penetration of the PPI-g4-DS dendrimer into a cell membrane. The neutral PPI-g4-DS may easily form intramolecular H-bonds which modify the structure and size of the dendrimers and decrease their polarity. In such a situation, these dendrimers may enter the cell membrane and cause haemolysis. Dendrimer-induced haemolysis was accompanied by changes in RBC morphology. Under physiological conditions, human RBCs form biconcave discs that are highly deformable, as this is necessary to enable them to pass through the capillaries. The influence of intrinsic or extrinsic factors may lead to cell transformation and the creation of echinocytes—morphologically altered RBCs that have numerous uniform spicules throughout the cell membrane.[43] Echinocytes were formed on addition of modified PPI dendrimers at 300 μM. However, PPI-g4, despite its greater haemolytic activity, caused no echinocytic transformation. According to the bilayer couple hypothesis,[44] partial dendrimer incorporation into a lipid bilayer, or the drawing-out of the outer monolayer by PPI molecules, might be a cause of echinocytic transformation. Furthermore, membrane fluidity studies on RBCs by Domanski et al.[45] revealed that fifth-generation thiophosphate dendrimers significantly stiffen the erythrocyte lipid bilayer, both in its interfacial and core regions. The lack of altered RBCs after PPI-g4 treatment might suggest that these dendrimers, being the smallest molecules in our dendrimer series, are incorporated into the erythrocyte membrane and prevent cell shape transformation. When incubated with 30 μM of PPI-g4 and 300 μM of PPI-g4-DS for 24 h, the erythrocytes aggregated and clusters were formed, probably as a consequence of cell-dendrimer cross-linking caused by noncovalently driven interactions—electrostatic attraction in case of the cationic PPI-g4[41] and H-bonding in the case of the neutral PPI-g4-DS.[17] The presence of HSA in the incubation buffer significantly decreased the haemolysis caused by PPI dendrimers in our study. This protective effect could be a consequence of the high affinity of dendrimers for serum proteins.[46,47] Klajnert et al.[48] showed that the presence of HSA significantly decreased the extent of haemolysis induced by PAMAM G5 dendrimers. Thus, dendrimers interacting with HSA are unable to disrupt the RBC membrane to the same extent as free dendrimers. In our study, lack of surface modification means that there are 32 positively charged terminal amino groups on the dendrimer surface that can easily interact with negatively charged HSA. The Mal-III residues on the dendrimer surface in PPI-g4-OS and PPI-g4-DS reduce the number of

free terminal amino groups. Thus, theoretically, HSA should have much less influence on modified PPI–RBC interactions. Nevertheless, the level of haemolysis caused by modified dendrimers in the presence of this protein also decreased. Klajnert et al.[17] suggested that the H-bond-forming maltose shell in dense shell glycodendrimers can interact with HSA just as strongly through noncovalent interactions as the cationic surface of the unmodified PPI dendrimers; this could explain the phenomenon observed in our study. The surface composition of HSA provides not only the possibility of electrostatic interactions but also the formation of hydrogen bonds between carboxylate groups or oxygen atoms of the protein amide groups and the maltotriose shell of the PPI-g4-OS and PPI-g4-DS. The interaction of HSA with modified dendrimers could explain the reduction in haemolysis in the case of PPI-g4. Unmodified cationic PPI dendrimers tend to form more contacts with HSA than the Mal-III-modified dendrimers. Surprisingly, PPI-g4-OS reduced haemolysis slightly less than did PPI-g4-DS. A possible explanation for this HSA-glycodendrimer interaction depends on the size and molecular shape of the molecule; PPI-g4-OS might not have the necessary spherical shape or rigid molecular architecture because only approximately 35% of its surface is covered with sugar residues, so it cannot interact as strongly with HSA as PPI-g4-DS (Fig. 4). This working hypothesis is well supported by a previous study,[12] in which fifth generation glycodendrimers with dense maltose shells underwent stronger interactions with HSA than the same fourth generation counterpart. When the experiments investigating the effect of PPI on RBC haemolysis were performed in plasma or in whole blood, almost no haemolysis occurred. This suggests that other components of the blood, such as proteins and cells, protect the RBCs from disruption by extrinsic factors. Thus, the buffer composition plays a significant role in haemolysis assays under physiological conditions. The results of this study suggest that the actual haemotoxicity of dendrimers in vitro might be higher than in vivo.[21] This phenomenon is desirable with respect to the toxicity of dendrimers; however, it may be disadvantageous, if we consider using dendrimers as drug carriers. Dendrimers binding to blood components may reduce their effectiveness as transporters. Therefore, it would be advisable to verify, in a similar test, how dendriplexes or dendrimers with attached folic acid[49,50] affect blood cells.

T-lymphocytes (T-cells) are the cornerstone of the adaptive immune system and play an essential role in the host defence against microbial pathogens.[51] T-cell proliferation is the result of cell activation, which initiates the immune response. Disorders of the activation process might be a reason for many irregularities in the immune system.[52] When a new macromolecule is under consideration for a potential biological application, it is important to ensure that it does not constitute a nonspecific antigenic stimulus. We therefore treated PBMCs with different concentrations of PPI dendrimers, looking for induction/inhibition of lymphocyte proliferation. The assay used in this study has allowed us to measure the ability of nanoparticles to induce a proliferative response in human lymphocytes, or to suppress proliferation induced by a mitogen–PHA-M. None of the three types of dendrimers studied presented an antigenic stimulus to the cells, but both PPI-g4 and PPI-g4-OS (at the concentration of 1 mg/ml) inhibited lymphocyte proliferation (Table IV, Fig. 5). There may be a number of reasons for this phenomenon. PPI-g4 and PPI-g4-OS can interact with negatively charged cell membranes owing to their positively charged surfaces and this interaction may block the access of the mitogen to receptors on the cell surface. It is also possible that the lack of proliferation is a consequence of dendrimer genotoxicity.[53–56] Because they are able to interact with nucleic acids,[7] dendrimers bind to DNA or RNA after entering the nucleus. If the PPI-DNA adducts are not excised, they prevent cell division. However, further investigations are required to establish whether the inhibition of proliferation really is a result of the effect of PPI on T-cells or it is due to interactions between PPIs and PHA-M that preclude protein activity. Finding an explanation of the molecular mechanism of PPI dendrimers activity in the inhibition of T-cells proliferation is important because of biomedical implications of this fact, e.g., in the treatment of pathologies where T-cell proliferation is undesirable, such as autoimmune disorders or cutaneous T-cell lymphoma.[57] However, a prolonged dosage of dendrimers, especially PPI-g4, may result in immunodeficiency caused by defects in cell-mediated response, i.e., with reduced activity of specific population of T-cells.[51,52] PLTs are considerably smaller than erythrocytes and leukocytes. Mammalian PLTs have a discoid form and a typical diameter of 2–3 μm.[58] Under physiological conditions, PLTs circulate preferentially in close proximity to vascular walls but they do not interact with endothelial cells, which provide a natural resistance to thrombosis. After disruption of the continuity of the endothelial layer, a coordinated series of events is set in motion to seal the defect. PLTs play the primary role in this process.[59] A PLT aggregation test can be performed to determine how well PLTs stick together.[60] The extent of aggregation (the maximum aggregation), and the steady state value, is used to characterize PLT function. The extent of aggregation is the most widely reported parameter of PLT function, and it correlates with clinical outcomes.[61-63] The velocity of PLT aggregation is determined by measuring the greatest slope of the aggregation curve and is expressed as the change in percentage transmission per min.[64] The bell-shaped character of the velocity of PLT aggregation induced by the PPI-g4 dendrimer was similar to the observed effect of fibrinogen on the velocity of PLT aggregation.[65] The authors proposed a model of saturation of PLT receptors by fibrinogen molecules. The equation describing this simplified scheme is given by: $V = k_{agg} \times S \times (1\text{-}S)$; where V is the initial aggregation velocity at a given fibrinogen concentration, k_{agg} is an overall kinetic constant of the PLT aggregation reaction, and S is the fractional saturation of the receptor. In this equation, the velocity is assumed to be a function of the product of the saturated (S) with the unsaturated ($1\text{-}S$) receptor fraction. This value is maximum when $S = 0.5$ and symmetrically decreases at higher and lower S values.[65] In this study, we observed a decrease in the velocity of PLT

aggregation induced by PPI-g4 dendrimers after saturation of the PLT receptors by molecules of that dendrimer at high concentrations. In contrast, modification of the PPI dendrimers by Mal-III led to a decrease in the velocity of aggregation because there was no aggregation. The time course of aggregation can be explained as a result of concurrent aggregation and disaggregation processes and is directly connected to the velocity of aggregation.[66] The time course of PLT aggregation induced by PPI-g4 dendrimers also has a bell-shaped character [Fig. 7(c)]. This indicates that the time course of aggregation is directly connected with the velocity; the possible reasons for this are discussed above. Results, from morphological images (Fig. 8), show that the modified PPI dendrimers have significantly less effect on PLTs than the unmodified ones, confirming the previous results of our study.

CONCLUSIONS

The biological effects of unmodified PPI-g4 dendrimers and of modified open (PPI-g4-OS) and dense shell (PPI-g4-DS) glycodendrimers on RBC haemolysis, lymphocyte proliferation, and PLT aggregation were studied and analyzed. The results confirmed that the cationic groups on the PPI dendrimers have a significant effect on their toxicity. Surface modification of the dendrimers by Mal-III residues markedly reduced their haemotoxicity and the ability to activate PLTs. Although all examined dendrimers inhibited lymphocyte proliferation, PPI-g4-OS and PPI-g4-DS had much less effect on cell division than PPI-g4. The advantages of surface modification make these dendrimers much more suitable for biomedical applications than their parental PPI-g4. Because the cationic charge of dendrimers is required for electrostatic interactions with DNA or RNA,[55] partial modification of the surface appears to be a perfect solution for the reduction in dendrimer toxicity and retention of activity. The results from our group[48] suggest that the actual impact of dendrimers on blood cells and blood cell components in vivo is lower than that seen in vitro.

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