

Mini review

INFLUENCE OF DENDRIMERS ON RED BLOOD CELLS

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Abstract: Dendrimers, highly branched macromolecules with a specific size and shape, provide many exciting opportunities for biomedical applications. However, most dendrimers demonstrate toxic and haemolytic activity because of their positively charged surface. Masking the peripheral cationic groups by coating them with biocompatible molecules is a method to reduce it. It was proven that modified dendrimers can even diminish haemolytic activity of encapsulated drugs. Experiments confirmed that anionic dendrimers are less haemotoxic than cationic ones. Due to the high affinity of dendrimers for serum proteins, presence of these components in an incubation buffer might also influence red blood cell (RBC)-dendrimer interactions and decrease the haemolysis level. Generally, haemotoxicity of dendrimers is concentration-, generation-, and time-dependent. Various changes in the RBCs' shape in response to interactions with dendrimers have been observed, from echinocytic transformations through cell aggregation to cluster formation, depending on the dendrimer's type and concentration. Understanding the physical and chemical origins of dendrimers' influences on RBCs might advance scientists' ability to construct dendrimers more suitable for medical applications.

Paper authored by participants of the international conference: 18th Meeting, European Association for Red Cell Research, Wrocław – Piechowice, Poland, May 12-15th, 2011. Publication cost was covered by the organizers of this meeting.

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Abbreviations used: AFM – atomic force microscopy; CSi – carbosilane dendrimers; DOX – doxorubicin; FA – folic acid; HSA – human serum albumin; MRI – magnetic resonance imaging; PAD-PPI – dextran conjugated PPI dendrimers; PAMAM – polyamidoamine dendrimers; PEG – poly(ethylene glycol); PEO – poly(ethylene oxide); PPI – poly(propyleneimine) dendrimers; PPI-DAB – PPI dendrimers with diaminobutane core; PPI-DAE – PPI dendrimers with diaminoethane core; RBCs – red blood cells; Rms – AFM roughness values

Key words: Dendrimer, Erythrocytes, Haemolysis, Red blood cell, Red blood cell morphology, Toxicity

INTRODUCTION

Dendrimers are highly branched, perfectly monodisperse, three-dimensional macromolecules with a precisely controlled chemical structure. They were first synthesised by Tomalia *et al.* [1] and Newkome *et al.* [2]. The term dendrimer is derived from Greek ‘*dendron*’, meaning a tree or a branch, due to its resemblance to a tree, and ‘*meros*’, meaning a part [3]. Since their introduction, dendrimers have attracted great interest because of their unique structure and properties. The globular shape of dendrimers is a result of their internal structure, in which all bonds emerge radially from a central core with repeat units contributing a branching point which provides the possibility to attach at least two monomers [4, 5]. The number of branch points counted from the core to the periphery of a dendrimer defines its generation (G1, G2, G3, G4). When the synthesis of dendrimers consists of two steps (as in the case of PAMAM dendrimers), one can distinguish so-called half generations. PAMAM dendrimers’ branched units are constructed from both methyl acrylate and ethylenediamine. First, amino groups react with methyl acrylate monomers to give a half generation with carboxyl groups on the surface, then ethylenediamine is added and a full generation is obtained (Fig. 1). The higher the generation, the larger and more branched the dendrimer becomes and the more end groups it possesses on the surface [6, 7] (Tab. 1). A high number of surface functional groups makes dendrimers very attractive for applications where multivalency

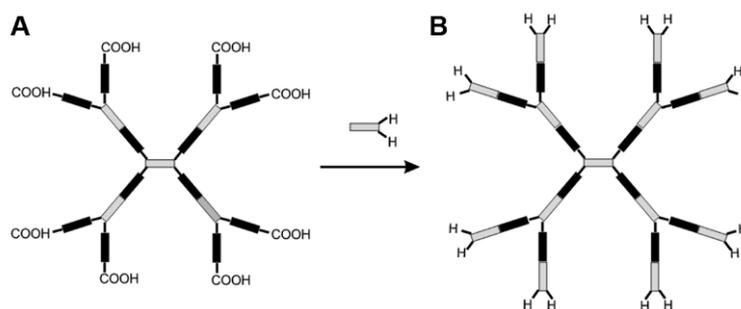


Fig. 1. The structure of a half-generation PAMAM dendrimer (A) and a full-generation PAMAM dendrimer (B).

Tab. 1. Characterization of PAMAM dendrimers [7].

Generation	Terminal groups	Number of terminal groups	Molecular weight [Da]	Size [nm]
2	-NH ₂	16	3 256	2.7
3	-NH ₂	32	6 909	3.6
4	-NH ₂	64	14 125	4.5

plays an important role [8]. The development of molecular nanostructures with well-defined particle size and shape is of eminent interest in biomedical applications. One of the earliest medical applications of dendrimers was to exploit them as MRI agents where they were used to prepare diagnostic agents with high relaxivity and long residence times in the blood [9, 10]. A large number of terminal groups enables drug molecules to be attached to the dendrimer surface through covalent bonds [11, 12]. On the other hand, the cavities (empty internal spaces) are able to encapsulate small molecules [11, 13, 14]. Both these strategies make dendrimers suitable for drug delivery systems (Fig. 2). Because of their nanometric size, dendrimers may interact effectively and specifically with cell components such as the membrane, organelles and proteins [15, 16].

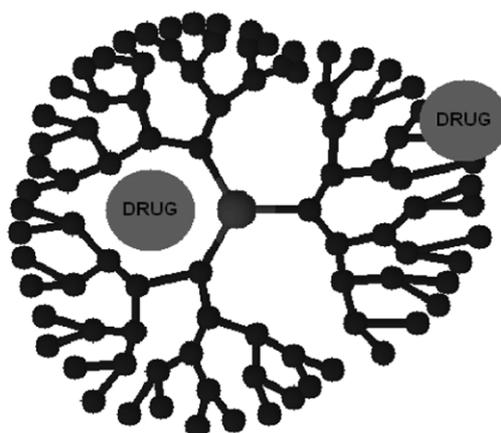


Fig. 2. The two main strategies of drug delivery by dendrimers (within them or on their surface).

Cationic dendrimers can be used as vectors for gene transfection as they have the ability to interact with various forms of nucleic acids and form complexes that protect the nucleic acid from degradation [17, 18]. However, as their interactions with cell components are non-selective, dendrimers also have a potential to cause cyto- and haemotoxicity due to their terminal (e.g. amino) groups and multiple cationic charge [12, 19]. Folic acid is often conjugated to dendrimers to increase the target specificity of these nanoparticles and facilitate dendrimers' location near the tumour, since in various cancer cell lines folic acid receptor are overexpressed [20, 21]. In physiological conditions the erythrocyte outer surface is negatively charged due to the presence of glycolipids and some glycosylated membrane proteins [7]. The negative charge of the cell surface prevents RBCs from aggregating with each other and from adhering to the wall of blood vessels [22]. Cationic dendrimers can come close to the RBC membrane as a result of electrostatic attractions. There are two possible targets on the cell surface for dendrimers: lipids [23] and proteins [24, 25] (Fig. 3). Interactions between dendrimers and the lipid bilayer have been studied using many methods such as

EPR techniques [26-28], leakage, lipid mixing and content mixing assays [29-31], differential scanning calorimetry [32, 33], and atomic force microscopy [34]. Loss of integrity of the RBC membrane which may occur under the influence of dendrimers is accompanied by haemoglobin leakage, which can be measured. The data obtained in such an assay give a qualitative indication of the potential damage that dendrimers can cause when administered intravenously. In this mini-review, we will focus on the *in vitro* interactions between dendrimers and red blood cells (RBCs), the mechanisms by which these macromolecules induce changes in RBC morphology and haemolysis, and possible strategies to alleviate dendrimers' haemotoxicity.

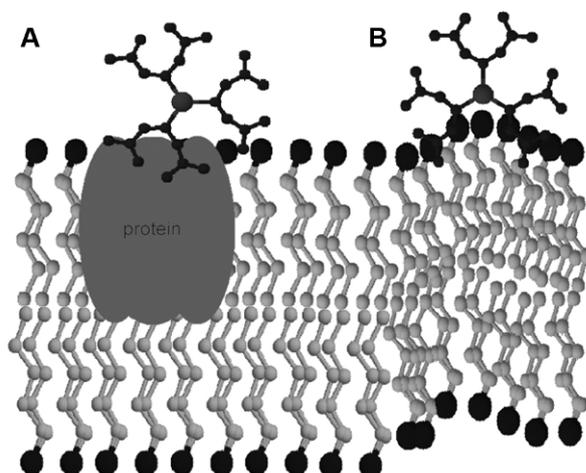


Fig. 3. Scheme of dendrimer interactions with RBC membrane. A – dendrimer interaction with membrane protein; B – dendrimer interaction with lipid bilayer.

HAEMOTOXICITY OF DENDRIMERS

The high density of surface groups combined with the small size of dendrimers results in a high area/volume ratio. This confers on dendrimers an unusual capacity to establish surface interactions with the cell membrane [35, 36]. Various studies have indicated that dendrimers' surface amino groups are the most toxic [12, 37].

Polyamidoamine (PAMAM) dendrimers are among the most popular and widely investigated dendrimers. Full generations of PAMAM possess amino terminal groups and half generations have carboxylate terminal groups. The number of reactive surface sites doubles with every generation [38]. To estimate the effect of dendrimer generation and type of surface end groups, Malik *et al.* [12] used full-generation (G1-G4) and half-generation (G1.5-G9.5) PAMAM dendrimers, poly(propyleneimine) dendrimers with either a diaminobutane core (PPI-DAB G1-G4, G1.5-G3.5) or a diaminoethane core (PPI-DAE G1-G3), and polyethylene oxide (PEO)-grafted carbosilane dendrimers (CSi-PEO G1, G2).

All cationic dendrimers (except for PAMAM G1) were lytic above a concentration of 1 mg/ml. PPI dendrimers are the second most popular class of dendrimers, based on polypropylenimine monomers. PPI-DAB and PPI-DAE were equally lytic as they have a similar repeated branch structure, but in contrast to PAMAM dendrimers they demonstrated no generation-dependent activity. Since both PAMAM and PPI dendrimers have primary amino groups as termini, differences in haemolytic activity might be due to the molecular weight of each dendrimer type and the number of surface groups or due to differences in the interior structure with both amide and tertiary amino groups present in the repeated branch units. The results of these experiments also confirm that anionic (PAMAM half-generation) and neutral (CSi-PEO) dendrimers are less haemotoxic than cationic ones. Mentioned dendrimers were not haemolytic up to a concentration of 2 mg/ml after 1 h of incubation and, in the case of anionic PAMAM, even after 24 h. If dendrimers possess a toxic core and non-toxic surface groups, lower generations can cause more drastic effects to cells due to a more open structure [37]. Such a phenomenon was found for the lowest generation of CSi-PEO dendrimer, as it was haemotoxic after prolonged treatment due to its toxic core. The findings reported by Domanski *et al.* [7] about haemolytic activity of cationic PAMAM dendrimers confirm that these dendrimers revealed concentration- and generation-dependent activity. They examined the haemotoxicity of three full generations of these dendrimers: second (G2), third (G3), and fourth (G4). They incubated RBCs in a dendrimer solution for 1 hour at 37°C and observed that PAMAM dendrimers caused concentration- and generation-dependent haemolysis. When a lower generation of dendrimers was used, a higher concentration needed to be applied to achieve 50% of haemoglobin release.

Another relatively well-known group of dendrimers is peptide dendrimers, which can be used as analogues of natural peptides [38]. Low molecular mass lysine-based peptide dendrimers were designed as branched analogues of the cationic antimicrobial peptides. Lysine was chosen as a starting and branching amino acid, and phenylalanine, tyrosine, alanine, glycine and arginine were other used amino acids [39]. Six lysine-based peptide dendrimers, which expressed antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*, were investigated by Klajnert *et al.* [40]. To study the effect of dendrimers on erythrocyte lysis, RBCs were incubated for 0.5 h at 20°C. Three of the examined peptide dendrimers revealed strong haemolytic activity; however, the rest of the tested dendrimers did not cause a loss of membrane integrity, even for high concentrations. As the toxicity of polycations is well documented [7, 12, 37], the observation that cationic peptides induced haemolysis was not surprising. However, because all examined polymers had a similar number of protonated lysine amino groups, the presence of amino groups does not seem to be the main reason for the haemotoxicity. It appears that steric distribution and type of hydrophobic groups as well as cationic centres might have a great influence on the haemolytic activity.

Dendrimers are not only able to destabilize the RBC membrane but can also increase its stability and thermal durability. Domanski *et al.* [41] investigated the impact of water-insoluble fifth-generation thiophosphate dendrimers (theoretical $M_w = 20\,025$ Da) on the RBC structure, membrane fluidity and membrane protein content at different temperatures: 37, 42, 46 and 58°C. At physiological temperature none of the used concentrations, 100 pM – 10 μ M (~ 2 ng/ml – 20 μ g/ml), were found to be haemolytic. At temperatures of 42 and 46°C, some haemoglobin leakage was observed only in the presence of 10 μ M (20 μ g/ml) dendrimer, but at 58°C the same concentration of dendrimer stabilized the RBC membrane to such an extent that almost 42% of the total haemoglobin content remained inside erythrocytes. Control cells disintegrated completely at this temperature as a result of drastic disorder of the membrane fluid mosaic. Optical microscope examination of the sample revealed clumps composed of strongly deformed, but not fully emptied erythrocytes. The RBC membrane fluidity study revealed that thiophosphate dendrimer significantly stiffens the erythrocyte lipid bilayer in both its interfacial and core regions, which may explain the increased thermal resistance of dendrimer-treated RBCs.

WAYS TO REDUCE DENDRIMER HAEMOTOXICITY

Surface engineering of dendrimers can lead to improvement of their properties, especially in the context of biomedical applications. It was proven that shielding of the surface charge can drastically reduce haemolytic behaviour. As the lytic effect of cationic dendrimers on RBCs is dangerous when administered *in vivo*, in order to diminish the haematological toxicity, Wang *et al.* [42] modified PAMAM G5 dendrimers with poly(ethylene glycol) (PEG) of three molecular weights (2 kDa, 5 kDa, and 20 kDa). PEG is a highly hydrated polymer with a high degree of segmental flexibility in aqueous solutions [43]. Substitution of amino groups for biocompatible terminal groups such as PEG is one of the modification methods used to create less cytotoxic dendrimers [44, 45]. This study demonstrated that haemocompatibility of cationic dendrimers could be greatly enhanced by PEGylation. RBCs were incubated at 37°C for 4 h with dendrimer solution at a concentration ranging from 5 μ g/ml to 5 mg/ml. As expected, PAMAM G5 did cause RBC membrane disruption and the haemolytic activity was concentration-dependent (from 0.1 mg/ml). The haemolysis caused by PEGylated dendrimers was reduced compared with the parent dendrimers. Haemolysis level of PEG-2kDa-PAMAM was only slightly lower than that of PAMAM G5 (0.5 mg/ml), whereas PEG-5kDa-PAMAM and PEG-20kDa-PAMAM demonstrated no significant difference in haemolysis compared with the control, even at the highest concentration. In the PEG-20kDa-PAMAM group the percentage of haemolysis was kept below 1.5%, which indicates only minimal haemoglobin release after 4 h incubation with this polymer.

Another example of the protective effect of PEGylation is the haemolytic investigation of a small library of G3 dendrimers based on melamine performed

by Chen *et al.* [46]. Manipulation of 48 peripheral sites provided six dendrimers that varied in the chemistry of the surface group (amine, guanidine, carboxylate, sulfonate, phosphonate, and PEGylated). Erythrocytes were treated with dendrimers at various concentrations (1 µg/ml-10 mg/ml) for 1 and 24 h. All the molecules demonstrated concentration- and time-dependent haemolytic activity and, as reported before [12, 37], haemolysis was more pronounced for cationic dendrimers than for anionic dendrimers. The PEGylated dendrimer was the least haemolytic one and the leakage of haemoglobin was narrowly greater than in the dextran control sample, even after 24 h of the highest concentration treatment.

Substitution of amino groups for carbohydrate residues is another method used for dendrimer modification. The term 'glycodendrimer' has been used to describe a wide range of architectures of dendrimers which incorporate carbohydrate into their structure. The effect of PPI dendrimers coated by maltose residues on erythrocyte lysis was studied by Klajnert *et al.* [47]. RBCs were suspended in 3 and 6 mg/ml solutions of two generations (G2 and G4) of cationic PPI dendrimers with open and globular molecular shapes, or the nearly neutral maltose-modified PPI dendrimers with densely organized maltose units. Two-hour incubation with the unmodified G2 and G4 PPI dendrimers proved to be very destructive for erythrocytes. The counterparts of these dendrimers with attached maltose residues demonstrated almost complete loss of haemotoxicity. For both generations, the densely organized maltose units created a shell on the PPI dendrimer surface which separated the RBCs from the potentially toxic PPI core. The obtained results are in agreement with previous studies made by Bharda *et al.* [13] in which coating of G4 and G5 PPI dendrimers with galactose reduced haemotoxicity. RBCs were incubated with generations G4 and G5 of uncoated and galactose-coated PPI dendrimers at 37°C for 1 h. Although cationic dendrimers were haemolytic at a concentration of 1 mg/ml, carbohydrate coating drastically reduced lysis of RBCs. Haemolysis, which occurred after G4 and G5 galactose-modified dendrimer treatment, achieved 10% and 7.1% of the values for similar amounts of uncoated dendrimers respectively. In these cases, the peripheral amino groups were substituted by only single monosaccharide units in the outer shell.

Modification of the multivalent symmetrical terminal groups of PAMAM G4 dendrimers into multifunctional groups by biocompatible materials was the objective of the work by Navath *et al.* [48]. PAMAM dendrimers were functionalized with amino acid residues, and a small library of representative dendrimers having diverse and high density of peripheral functional groups has been developed (e.g. G3.5-PAMAM-CO-NH-Ser-OH, G4-PAMAM-O-CO-Asp-(CO-Dex)-NH₂, G4-PAMAM-O-Asp-(CO-Dex)-Ind). RBCs were incubated at 37°C for 3 h with modified and unmodified dendrimers at concentrations ranging from 1 µg/ml to 10 mg/ml.

Unmodified PAMAM G4, as well as all of the modified dendrimers, did not exhibit significant haemolysis up to 100 µg/ml concentration. While for PAMAM G4 10% haemolysis occurred at 1 mg/ml, approximately 1.5 to 3.0%

was observed for all compounds except for G3.5-PAMAM-CO-Ser(OH)-COOH, which showed haemolysis of 5%. In comparison to the PAMAM G4 dendrimer, the modified compounds exhibited lower haemolytic activity, which proved that covering of the cationic groups on the dendrimer surface greatly reduced their negative influence on RBCs.

It has also been proved that modified dendrimers can reduce haemolytic activity of encapsulated drugs [49-52]. Gupta *et al.* [50] reported the design, synthesis and characterization of PPI-FA-DOX, which is a folic acid (FA) conjugated PPI G5 dendrimer encapsulated with doxorubicin (DOX), an effective anticancer drug. Gupta's team found that free DOX exhibited slightly higher haemotoxicity than PPI G5 but lower than PPI-DOX complex. Folic acid conjugation on the PPI dendrimer surfaces drastically reduced erythrocyte lysis. Haemotoxicity of PPI-FA-DOX was approximately 3, 4 and 5 fold lower than that of PPI G5, DOX and PPI-DOX, respectively. Shielding/locking of DOX and primary amine groups in the dendritic architecture due to folic acid surface modification was possibly the reason for reduction of RBCs lysis. The results are similar to the previous reports of surface conjugated dendrimers [51, 53]. Agarwal *et al.* [51, 53] investigated dextran conjugated PPI G5 dendrimers as nanoscale DOX delivery units (PAD-PPI-DOX). PAD-PPI-DOX also demonstrated lower haemolytic activity than an equivalent concentration of PPI G5 or free drug. Poly-L-lysine G4 dendrimers modified on the surface by D-galactose and loaded with an antimalarial drug, chloroquine phosphate, also drastically reduced haemolytic toxicity compared to uncoated poly-L-lysine formulation as well as the plain drug. Presence of other components, e.g. proteins, in an incubation buffer might also influence RBC-dendrimer interactions. A study performed by Klajnert *et al.* [54] indicated that the haemolytic activity of PAMAM G5 dendrimers in the presence of human serum albumin (HSA) greatly decreased the haemolysis level. This protective effect may be due to the high affinity of dendrimers for serum proteins [24, 55]. Dendrimers that interacted with HSA were unable to disrupt the membrane to the same extent as free dendrimers. As the presence of HSA makes the buffer more relevant to physiological conditions, the results of this study suggest that the actual haemotoxicity of dendrimers *in vivo* might be lower than it is observed *in vitro*.

CHANGES IN RED BLOOD CELL MORPHOLOGY DUE TO DENDRIMERS

Human RBCs, which circulate in the body for about 120 days, are normally in the form of biconcave discs; hence they are named 'discocytes'. Discocytes in physiological conditions are highly deformable because of the excess surface area and the elasticity of their membranes, which is needed to pass through the capillaries. The influence of intrinsic or extrinsic factors may lead to cell transformation and creation of different stages of echinocytes (crenated cells) or stomatocytes (cup-shaped cells) [56]. According to the bilayer couple hypothesis

[57], partial dendrimer incorporation into a lipid bilayer or drawing out the outer monolayer by dendrimers might be a cause of echinocytic transformation. Echinocytes are morphologically altered red blood cells that (appear to) have numerous uniform spicules throughout the cell membrane. Changes in the RBC shape in response to interactions with various generations of cationic PAMAM dendrimers were studied by Domanski *et al.* [7] (characterization of PAMAM dendrimers is presented in Tab. 1). The examined dendrimers revealed generation- and concentration-dependent effects. The lowest concentration (1 nM ~ 14.13 ng/ml) of PAMAM G4 induced echinocytic transformation. At a higher dendrimer concentration (10 nM ~ 141.3 ng/ml) cells elongated and took on spindle-shaped forms, and in the 100 nM (~ 1.413 µg/ml) dendrimer solution drepanocyte-like forms were recorded. Upon the addition of PAMAM G2 and G3, RBCs underwent similar shape transitions, though a higher dendrimer concentration was needed to achieve similar changes in the shape. RBCs suspended in 1 µM {~ 3.26 µg/ml (G2) and 6.91 µg/ml (G3)} and 10 µM {~ 32.6 µg/ml (G2) and 69.1 µg/ml (G3)} dendrimer solutions aggregated. Although plenty of echinocytes were floating in suspension over the surface when 1 µM concentration was used, at 10 µM PAMAM concentration agglutinated cells were difficult to disperse. It was postulated that electrostatic attraction forced cationic dendrimers to come close to the RBC surface, and formation of erythrocyte clusters might be the consequence of cell-dendrimer cross-linking. Similar results were obtained by Wang *et al.* [49] when the PAMAM G4 dendrimer was examined as a nanocarrier candidate for gene delivery. Low doses of PAMAM G4 dendrimer (10 nM-10 µM ~ 141.3 ng/ml-141.3 µg/ml) caused RBC aggregation and shape changes, from echinocytic, spindle-shaped to spherocyte-like forms, and when the concentration increased to 100 µM (~ 1.41 mg/ml), PAMAM G4 induced membrane rupture and disintegration. In the presence of antisense oligodeoxynucleotides which shield cationic dendrimers' surface, the same dose of PAMAM did not cause erythrocyte aggregation, thus confirming that electrostatic attraction was the main power driving cell cluster formation. RBCs' morphological changes induced by water-insoluble thiophosphate G5 dendrimers, investigated by Domanski *et al.* [41], also became more visible upon increasing the dendrimer concentration. At a physiological temperature, cell transformation led progressively from the normal erythrocyte biconcave shape for 100 pM (~ 2 ng/ml) and 1 nM (~ 20 ng/ml), through sequential echinocyte stages for 10 nM (~ 200 ng/ml) (stage I), 100 nM (~2 µg/ml) and 1 µM (~20 µg/ml) (stage II), to the fully transformed echinocytes (stage III) for 10 µM (~ 200 µg/ml) of dendrimer solution, but none of the dendrimer concentrations caused statistically significant release of haemoglobin. No RBC aggregation was observed. The interactions between various types of dendrimers and RBCs were reported previously by Malik *et al.* [12]. They also observed changes in the shape of erythrocytes and reported that after 1 h of treatment with cationic PAMAM and PPI-DAB dendrimers, RBCs demonstrated morphological changes even at

a non-haemolytic 10 µg/ml concentration. Analysed through a scanning electron microscope, cells showed a rounded shape (spherocytocytes) and were in close proximity to each other. Higher cationic dendrimer concentrations (1 mg/ml) intensified this phenomenon, whereas anionic PAMAM dendrimers of generation 3.5 to 9.5 showed no influence on cell shape up to a concentration of 2 mg/ml, which indicates that anionic dendrimers have a minor impact on RBC membrane compared to cationic ones. Wang *et al.* [41] treated RBCs with unmodified PAMAM G5 and PAMAM G5 modified with PEG of three molecular weights (2 kDa, 5 kDa, and 20 kDa). They used an optical microscope to analyse RBC morphology and atomic force microscopy (AFM) for quantitative description of morphological details of the RBC surface. They noted echinocytic transformations at low PAMAM G5 concentration (0.05 mg/ml) and cell aggregation and cluster formation at a ten times higher concentration (0.5 mg/ml). The presence of 5 mg/ml PAMAM G5 caused considerable haemolysis, cell crenation and aggregation. When RBCs were treated with PEG-2kDa-PAMAM, similar shape transitions occurred, but with a lesser degree of aggregation and haemolysis. PEG-5kDa-PAMAM and PEG-20kDa-PAMAM did not induce any morphological cell changes even at the highest concentration. The erythrocytes were similar to those of saline controls, which was thought to be a result of long and flexible PEG chains covering the surface and shielding the positive charge. Investigation of the surface structure of RBCs by AFM indicated that RBCs treated with high- M_w PEG-modified PAMAM G5 at 5 mg/ml concentration were almost identical to those of reference RBCs: a typical biconcave erythrocyte with a smooth surface. Spherocytes and collapsed erythrocytes with an irregular contour were observed for a PEG-2kDa-PAMAM treated group, while the membrane of RBCs treated with PAMAM was completely disintegrated. Roughness values (Rms) obtained by AFM, measured from high-resolution images at a scanned area of $1 \times 1 \mu\text{m}^2$, demonstrated a decreasing tendency with the increasing M_w of PEG gradually approaching the Rms value of the untreated erythrocytes and being far lower than that of RBCs treated with PAMAM dendrimers.

CONCLUSIONS

The studies described above confirm that positively charged dendrimers interact with the erythrocyte membrane, causing haemoglobin leakage, and have a great influence on RBC morphology. However, modifications and shielding of the surface cationic group can greatly reduce the negative consequences of dendrimer impact. Understanding the physical and chemical origins of these influences might advance the ability of scientists to construct dendrimers more suitable for medical applications. Several dendrimer parameters, such as generation and type of terminal group, can be manipulated toward the achievement of a desirable result.

Acknowledgements. This study was funded by project "Biological Properties and Biomedical Applications of Dendrimers" operated within the Foundation for Polish Science TEAM programme cofinanced by the European Regional Development Found.

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