A study on the hemocompatibility of dendronized chitosan derivatives in red blood cells

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Abstract: Dendrimers are hyperbranched macromolecules with well-defined topological structures and multivalent functionalization sites, but they may cause cytotoxicity due to the presence of cationic charge. Recently, we have introduced alkyne-terminated poly(amidoamine) (PAMAM) dendrons of different generations (G=2,3) into chitosan to obtain dendronized chitosan derivatives [Cs-g-PAMAM (G=2,3)], which exhibited a better water solubility and enhanced plasmid DNA transfection efficiency. In this study, we attempted to examine the impact of Cs-g-PAMAM (G=2,3) at different concentrations (25 μg/mL, 50 μg/mL, and 100 μg/mL) on the morphology, surface structure, and viability of rat red blood cells (RBCs). The results showed that treatment of RBCs with Cs-g-PAMAM (G=2,3) at 50 μg/mL and 100 μg/mL induced a slightly higher hemolysis than Cs, and Cs-g-PAMAM (G=3) caused a slightly higher hemolysis than Cs-g-PAMAM (G=2), but all values were <5.0%. Optical microscopic and atomic force microscopic examinations indicated that Cs-g-PAMAM (G=2,3) caused slight RBC aggregation and lysis. Treatment of RBCs with 100 μg/mL Cs-g-PAMAM (G=3) induced echinocytic transformation, and RBCs displayed characteristic irregular contour due to the folding of the periphery. Drehpanocyte-like RBCs were observed when treated with 100 μg/mL Cs-g-PAMAM (G=3). Erythrocytes underwent similar shape transition upon treatment with Cs-g-PAMAM (G=2) or Cs. The roughness values (Rms) of RBCs incubated with Cs-g-PAMAM (G=2,3) were significantly larger than those for RBCs incubated with physiological saline (P<0.01), but the Rms showed no difference for Cs and Cs-g-PAMAM (G=2,3) (P>0.05). Furthermore, Cs-g-PAMAM (G=2,3) exhibited a lower cytotoxicity in human kidney 293T cells. These results indicate that Cs-g-PAMAM (G=2,3) are hemocompatible but may disturb membrane and lipid structures at higher concentrations. Further safety and biocompatibility evaluations are warranted for Cs-g-PAMAM. Our findings prove helpful for a better understanding of the advantages of combining PAMAM dendrimers and chitosan to design and develop new, safe, and effective drug delivery vehicles.

Keywords: dendronized chitosan derivative, PAMAM, RBC, hemolysis, hemocompatibility

Introduction

Chitosan (Cs), a naturally occurring cationic mucopolysaccharide composed of β-(1-4)-linked 2-amino-2-deoxy-β-d-glucose and the N-acetylated analog, has been widely used in nonviral drug and gene delivery owing to good biocompatibility and biodegradability.1-4 However, Cs shows poor water solubility and low transfection efficiency. In recent years, a number of new Cs derivatives with improved transfection efficiency and better water solubility have been synthesized and characterized for drug and gene delivery. For example, urocanic acid-modified,5 hydrophobized,6,7 glycosylated,8 polyethylene glycol-modified (PEGylated),9,10 trimethylated,11,12 poly(amidoamine) (PAMAM) grafted,13,14 poly(epsilon-caprolactone) grafted,15,16 oligoamine grafted,17 and glycol grafted Cs18,19 have been characterized using in vitro
and in vivo models. These modified Cs derivatives have distinct advantages and disadvantages when used as the drug and gene delivery systems or theranostics.

Dendrimers are monodisperse, artificial polymers, and hyperbranched macromolecules with well-defined topological structures and multivalent functionalization sites.\textsuperscript{20–23} They present important advantages over conventional linear or branched polymers, including monodispersity or low polydispersity, controlled size in the range of nanometers, controlled number of surface groups, and extremely high area/volume ratio. These unique characteristics render dendrimers amenable to serve as scaffolds and vehicles for nucleic acid and drug delivery and as the platform for theranostics.\textsuperscript{24,25} Only intermediate-generation (G=3.5–5) dendrimers are suitable as nanocarriers, with structures open enough to enable the loading and subsequent release of drug/gene molecules in a controllable manner. In the past years, cationic PAMAM dendrimers have been reported to improve the solubility of low-aqueous-soluble drugs and condense plasmid DNA or small interfering RNA and demonstrated successful delivery of DNA into the target cells.\textsuperscript{26–35} However, PAMAM dendrimers bearing amino terminals could cause certain cytotoxicity toward epithelial cells, endothelial cells, neurons, leukocytes, platelets, and red blood cells (RBCs).\textsuperscript{13,34,36–38} In particular, the RBC toxicity of PAMAM dendrimers has attracted attention due to safety concerns regarding hemolysis. Therefore, novel PAMAM-based dendrimers with low cytotoxicity and acceptable biocompatibility should be developed and characterized.

In our previous study,\textsuperscript{39} we reported that the chemical combination of PAMAM with Cs to generate PAMAM dendron-grafted Cs [Cs-g-PAMAM (G=2,3)], which exhibited a low cytotoxicity, strong binding ability to plasmid DNA, and enhanced transfection efficiency in both human kidney 293T and human nasopharyngeal carcinoma CNE2 cell lines. It is well known that RBCs comprise one of the most important components in the body’s circulation system to deliver oxygen. To supply oxygen to the brain and other important organs, RBCs must deform as they pass through the narrow pores of capillaries. Beside their primary role of oxygen transport, RBC suspensions tend to aggregate under low-flow conditions or at stasis. Furthermore, numerous signaling molecules, signaling cascades/pathways, and networks have been discovered in RBCs.\textsuperscript{40} RBCs also bind to most drugs and nucleic acids and carry them everywhere in the body.\textsuperscript{41–43} Considering RBCs’ main advantages, including biocompatibility, biodegradability, immunocompatibility, simple and well-known structure and physiology, availability for sampling, and versatility in loading and use, erythrocytes are becoming one of the most promising drug delivery systems.\textsuperscript{41–43} Application of erythrocytes as containers for various drugs can both minimize the risk of side effects and pathologic immune responses against encapsulated agents as well as improve their therapeutic efficacy.\textsuperscript{21,44,45} On the other hand, the damage to the RBCs by drugs and nanocarriers may lead to serious problems such as hemolysis, anemia, liver and kidney injury, etc. Ruptured RBCs may alter the distribution of the drug and nanocarrier and thus change their biological responses. Because dendrimer-based nanocarrier systems can often enter the blood circulation and interact with blood cells including leukocytes and RBCs, the dendrimer’s blood toxicity and biological properties should be carefully examined before systemic administration. In this study, we have investigated the impact of Cs-g-PAMAM with different dendron generations (G=2,3) on rat RBC morphology and hemolysis.

Materials and methods
Chemicals and reagents
Cs (molecular weight =10 kDa, deacetylation degree = 85.3%) was purchased from Jinan Haidebei Marine Bioengineering Co Ltd (Jinan, Shandong, People’s Republic of China). Dendronized Cs derivatives (Cs-g-PAMAM) with different dendron generations (G=2,3) were synthesized as described in our previous study (Figure 1).\textsuperscript{39} PAMAM substitution degree of Cs-g-PAMAM (G=2) and Cs-g-PAMAM (G=3) was 25.1 and 23.3, respectively. Dulbecco’s Modified Eagle’s Medium (DMEM) was obtained from Corning Cellgro Inc (Herndon, VA, USA). Methyl thiazolyt tetrazolium (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). Sodium chloride for injection (0.9%, w/v) was purchased from a local pharmacy. All other reagents were of analytical grade.

Preparation of rat RBCs
Blood was obtained from healthy Sprague-Dawley rats and was anticoagulated with heparin sodium. Erythrocytes were separated from blood plasma by centrifugation at 1,500×g for 5 minutes at 4°C, then washed three times with physiological saline, and finally suspended in physiological saline to obtain a 2% RBC suspension (v/v). Erythrocytes were used immediately after isolation. Animal experiments were carried out according to the protocol approved by the Experimental Animal Committee of Guangdong Medical University, Dongguan, Guangdong, People’s Republic of China.
Hemocompatibility of dendronized chitosan derivatives

Figure 1 Synthesis scheme for PAMAM dendron-grafted Cs [Cs-g-PAMAM (G=2,3)].

Notes: (A) Reaction in the presence of phthalic anhydride and dimethylformamide at 120°C for 8 hours; (B) reaction in the presence of N-bromosuccinimide, triphenylphosphine, and N-methylpyrrolidinone at 80°C for 2 hours; (C) reaction in the presence of sodium azide and N-methylpyrrolidinone at 80°C for 4 hours; (D) reaction in the presence of hydrazine monohydrate in water at 100°C for 10 hours; and (E) reaction in the presence of the alkyne-terminated PAMAM dendrons (G=2,3), CuSO₄/sodium ascorbate, and dimethylformamide in water at 40°C for 24 hours. n=62.

Abbreviations: Cs, chitosan; G, generation; PAMAM, poly(amidoamine).

Hemolysis assay
The hemolytic activity of Cs-g-PAMAM (G=2,3) was investigated according to our previous work. In brief, 2% RBC suspension was treated with Cs or Cs-g-PAMAM (G=2,3) solution at different concentrations (25 μg/mL, 50 μg/mL, and 100 μg/mL) and incubated for 2 hours at 37°C with gentle shaking. The RBC suspension incubated with physiological saline using the exact same process was regarded as the control for comparison. The RBC suspension incubated with physiological saline was centrifuged at 1,000×g for 10 minutes. The supernatants were assayed for the absorbance of released hemoglobin at 540 nm. The degree of hemolysis was determined by the following equation:

\[
\text{Hemolysis (\%)} = 100 \times \frac{(\text{Abs} - \text{Abs}_0)}{(\text{Abs}_{100} - \text{Abs}_0)}
\]

where Abs, Abs₀, and Abs₁₀₀ are the absorbances of test samples, the suspension treated with physiological saline, and the suspension of complete hemolysis treated with Triton X-100 (10%, v/v), respectively.

Atomic force microscopic examination
The shape and roughness of RBCs were examined using atomic force microscopy (AFM) as described previously. In brief, rat RBCs were fixed by addition of 1% glutaraldehyde, and 25 μL of each sample was applied to standard microscope slides. After air-drying, the samples were gently rinsed with deionized water to remove salt crystals and then air-dried again before analysis. All AFM images and roughness values (Rms) accordant with the images were obtained with an atomic force microscope (Autoprobe CP Research).
Inc, Albuquerque, NM, USA). Three independently produced samples were analyzed. The mean surface Rms of RBCs from different preparations was measured with high-resolution images in a scanned area of 1×1 μm².

Cytotoxicity assay
The effects of Cs and Cs-g-PAMAM on the viability of human kidney 293T cells were determined using the MTT assay as described previously by us. Cells were seeded in the 96-well plate at a density of 1×10⁴ cells per well and cultured for 24 hours. After an additional incubation with different concentrations of Cs-g-PAMAM (G=2,3) and Cs for 24 hours, the medium was changed with DMEM containing 20 μL of MTT solution (0.5 mg/mL). After further incubation for 4 hours, the reaction product was solubilized with 150 μL DMSO. The absorbance value was measured at 570 nm using a plate reader (Bio-Rad Inc, Hercules, CA, USA). Cell viability (%) was calculated according to the following equation: cell viability (%) = [A₅₇₀ (sample)/A₅₇₀ (control)] × 100%, where A₅₇₀ (sample) was obtained in the presence of polymers and A₅₇₀ (control) was obtained in the absence of polymers.

Statistical analysis
Data are expressed as mean ± standard deviation of at least three different experiments and analyzed by one-way analysis of variance and Tukey’s test. Statistical analysis was performed using SPSS version 11.5. Only values with P<0.05 were considered statistically significant.

Results
Effects of Cs-g-PAMAM (G=2,3) on the lysis of rat RBCs
The RBC lysis method is widely used to study polymer–membrane interaction. To check whether Cs-g-PAMAM (G=2,3) caused membrane rupture of rat RBCs, we incubated RBCs with Cs-g-PAMAM (G=2,3) at 25 μg/mL, 50 μg/mL, and 100 μg/mL and measured the release of hemoglobin, which indicates membrane disruption and RBC lysis. As shown in Figure 2, treatment of RBCs with Cs-g-PAMAM (G=2 or 3) at 50 μg/mL and 100 μg/mL induced a slightly higher hemolysis than Cs; Cs-g-PAMAM (G=3) caused a slightly higher hemolysis than Cs-g-PAMAM (G=2); but all values were <5.0%. When rat RBCs were treated with Cs at 25 μg/mL, 50 μg/mL, and 100 μg/mL for 2 hours, the hemolysis rate was 2.12%±0.56%, 2.79%±0.73%, and 2.84%±0.22%, respectively. Treatment of the rat RBCs with Cs-g-PAMAM (G=2) at 25 μg/mL, 50 μg/mL, and 100 μg/mL for 2 hours resulted in hemolysis rates of 2.50%±0.44%, 3.13%±0.58%, and 4.09%±0.42%, respectively. These results showed that the hemolysis rate of RBCs with Cs was a little lower than that caused by Cs-g-PAMAM (G=2,3). The slightly higher hemolysis of Cs-g-PAMAM (G=2,3) might be due to the fact that PAMAM dendrimers contain amino terminals that pose certain cytotoxicity to the RBCs. Meanwhile, the results demonstrated that hemolysis rate of RBCs with Cs-g-PAMAM (G=3) was higher than that caused by Cs-g-PAMAM (G=2) at the same concentration, indicating that the cytotoxicity was related to the dendron number. All the hemolysis rates caused by Cs and Cs-g-PAMAM with different dendron generations (G=2,3) were <5.0%, indicating that Cs-g-PAMAM was not hemolytic according to Standard Practice for Assessment of Hemolytic Properties of Materials F756-93 standards.

Effects of Cs-g-PAMAM (G=2,3) on the shape and roughness of RBCs using optical microscopy and AFM
Next, we investigated the effects of Cs-g-PAMAM (G=2,3) on the shape and morphology of rat RBCs using optical microscopy and AFM. Figure 3 shows the changes of the RBC shape in response to treatment with Cs-g-PAMAM (G=2,3), Cs, physiological saline, or 10% Triton X-100 under...
light microscopy. The RBCs treated with physiological saline were normal disk shaped (diskocytes) (Figure 3A). Treatment of RBCs with 10% Triton X-100 caused a complete hemolysis (Figure 3B). Figure 3C–E shows the shape of erythrocytes when in contact with Cs-g-PAMAM (G=2,3) or Cs at 100 μg/mL. Treatment of RBCs with 100 μg/mL Cs-g-PAMAM (G=3) induced echinocytic transformation; RBCs displayed characteristic irregular contour due to the folding of the periphery. Drepanocyte-like RBCs were also observed when treated with 100 μg/mL Cs-g-PAMAM (G=3). Erythrocytes underwent similar shape transition upon treatment with Cs-g-PAMAM (G=2) or Cs. For Cs and Cs-g-PAMAM (G=2,3), similar changes in the shape of RBCs occurred at a higher concentration.

RBCs have been extensively studied by AFM due to their disease-sensitive shape, the simplicity in morphology and biochemistry of plasma membrane, and other factors. As shown in Figure 4, our AFM examinations demonstrated that hemocompatibility of Cs could be remarkably affected by incorporation of PAMAM dendrons. When Cs or Cs-g-PAMAM molecules (G=2,3) were present on the RBC surface, there were two possible targets for their binding: lipids
Figure 4 Representative images of rBcs in different solutions obtained from AFM.

Notes: Rat RBCs were fixed by addition of 1% glutaraldehyde, and 25 μL of each sample was applied to standard microscope slides. After air-drying, the samples were gently rinsed with deionized water to remove salt crystals and then air-dried again before analysis. All images were obtained with an AFM. (A) 0.9% NaCl solution, (B) 10% Triton X-100, (C) Cs at 100 μg/mL, (D) Cs-g-PAMAM (G=2) at 100 μg/mL, and (E) Cs-g-PAMAM (G=3) at 100 μg/mL.

Abbreviations: AFM, atomic force microscopy; Cs, chitosan; G, generation; PAMAM, poly(amidoamine); RBC, red blood cell.
and membrane proteins. The formation of echinocytes in our experiments could be due to partial Cs and Cs-g-PAMAM (G=2,3) incorporation into the lipid bilayers or due to pulling out of the outer monolayer by Cs and Cs-g-PAMAM (G=2,3) molecules.

Hemolysis rate and Rms are the quantitative indexes used to analyze the injury to RBCs caused by these materials, and a low hemolysis rate and Rms mean that the materials are biocompatible. Figure 5 presents the mean surface Rms of RBCs incubated with physiological saline, Cs, or Cs-g-PAMAM (G=2 and 3) at 100 μg/mL. The Rms value of Cs, Cs-g-PAMAM (G=2), and Cs-g-PAMAM (G=3) was significantly larger compared with that in physiological saline (5.53 nm versus 34.70 nm, 31.43 nm, and 58.17 nm, respectively; \( P<0.01 \)). However, the Rms value of RBCs treated with Cs and Cs-g-PAMAM (G=2 and 3) showed no difference \( (P>0.05) \). The results demonstrated that both Cs and Cs-g-PAMAM (G=2 and 3) significantly increased the roughness of RBCs.

**Effects of Cs and Cs-g-PAMAM on the viability of RBCs using the MTT assay**

The cytotoxicity of Cs-g-PAMAM (G=2,3) and Cs in the 293T cell line was evaluated by the MTT assay, and the results are shown in Figure 6. Treatment with Cs-g-PAMAM (G=2,3) or Cs at 25 μg/mL, 50 μg/mL, and 100 μg/mL only slightly reduced the viability of RBCs. The results showed that all the PAMAM dendrimers had a low cytotoxicity toward RBCs.

**Discussion**

Presently, nanomaterial production and use continue to become more prevalent, and consistent and accurate toxicity evaluation is crucial for the ability to properly regulate these nanomaterials. Because of quantum size effects and large surface area-to-volume ratio, nanomaterials have unique physical, chemical, and biological properties compared with their larger counterparts. Nanoparticles may induce the production of proinflammatory cytokines and stimulate the immune system, leading to inflammation, immune responses, allergy/hypersensitivity, and activation or inhibition of immune cells. Many types of nanoparticles can translocate from their site of deposition to distant sites, such as the brain and bone marrow, via blood circulation. Some nanomaterials are toxic to human tissues/organisms and cells, resulting in increased oxidative stress, generation of reactive oxygen/nitrogen species, and programmed cell death including apoptosis and autophagy. The toxicity of nanoparticles is mainly determined by their composition, size, shape, and surface reactivity with cells and cellular components. On June 24, 2014, the US Food and Drug Administration (FDA) issued one draft and three final guidance documents related to the use of nanotechnology in regulated products. FDA regulates nanotechnology products under existing statutory authorities, in accordance with the specific legal standards applicable to each type of product under its jurisdiction.

Dendrimers possess several advantages for drug and gene delivery: 1) monodispersity or low polydispersity, allowing...
Many studies have demonstrated that the erythrocyte’s outer surface is negatively charged, which prevents RBCs from aggregating with each other and from adhering to the walls of blood vessels. Cationic dendrimers can interact with the lipids and proteins of RBC membranes through electrostatic attractions.\textsuperscript{52,67} PAMAM dendrimers have been widely applied in gene delivery; Cs can reduce the charges by shielding the PAMAM dendrimer surface and can be used safely as an alternate, efficient delivery vector in gene therapy.\textsuperscript{29,68–70} However, the application of PAMAM is significantly restricted due to its harmful hematologic effect caused by its cationic charges, which prevents systemic administration. The cytotoxic effects of PAMAM dendrimers on RBCs were found to be time, concentration, and charge dependent. The molecular weight, hydrophobicity, generation, basic pH, cationic charge density, and bulk structure of the PAMAM dendrimers were key factors that determined the interaction with the RBC membranes and, consequently, the cell damage.\textsuperscript{37,38,71} Many studies have demonstrated that the hematologic toxicity of PAMAM dendrimers could be greatly diminished by PEGylation,\textsuperscript{36} zwitterionic Cs,\textsuperscript{68} and grafted N-maleyl CS,\textsuperscript{15} which showed no noticeable hemolytic effects, indicating no detectable disturbance of the RBC membranes. This is further supported by our finding in this study, wherein a hemolysis rate <5.0\% was observed for Cs-g-PAMAM (G=2,3) in rat RBCs.\textsuperscript{59,60,61}

The hemolytic effect of dendrimers is concerned with the nature of materials, such as composition, surface roughness, and related charges. The shape change and the hemolysis of RBCs induced by Cs and Cs-g-PAMAM (G=2,3) might be due to their interaction with the erythrocyte membrane. Some dendrimers can interact with lipids by hydrophobic interactions between lipid acyl chains and the hydrophobic dendrimer interior, depending on the size and charge of the molecule and the phase of lipids.\textsuperscript{72,73} It is well known that the surface of a normal erythrocyte is negatively charged due to the presence of glycolipids and some glycated integral and peripheral proteins.\textsuperscript{65} Electrostatic repulsion among RBCs prevents their self-aggregation and adhesion to the walls of blood vessels. Cationic Cs and Cs-g-PAMAM (G=2,3) were supposed to come close to the RBC surface as a result of electrostatic attraction. Thus, the formation of erythrocyte aggregates might be the consequence of their cross-linking by Cs and Cs-g-PAMAM (G=2,3). Further studies are needed to reveal the mechanism of Cs and Cs-g-PAMAM (G=2,3)–protein interactions.

The interaction of dendrimers with cellular membrane is complicated. Dendrimers either create holes in a bilayer or can be incorporated into the lipid structure.\textsuperscript{74,75} Higher-generation dendrimers cause greater disturbances in a lipid
bilayer, and positively charged dendrimers interact more effectively with liposomes. It has been proposed that at low concentrations, dendrimers can traverse the bilayer, while at higher concentrations, dendrimer:lipid micelles are generated. The toxicity of PAMAM dendrimers generally increases with increasing generation number, which has been ascribed to the increased number of positive charges per molar concentration and thus increased contact area of the dendrimer with the cell membrane. The electrostatic interactions of cationic dendrimers with cell membranes probably induces nanoscale-level hole formation and membrane destabilization mechanisms. Therefore, removal of the positive charges through functionalization of external groups from the surface of the dendrimers can minimize the membrane interactions.

The cytotoxicity of PAMAM dendrimers is mainly determined by their concentration, generation, and surface charge. Both anionic and neutral PAMAM dendrimers are remarkably less cytotoxic and permeable than cationic dendrimers; dendrimers of a lower generation (ie, G = 0–1) are often less cytotoxic than those of a higher generation (ie, G = 2–4). AFM studies have indicated that polycationic dendrimers induce hole formation and/or expansion of preexisting defects in supported lipid bilayers, whereas hydroxyl-containing neutral polymers do not show these disturbing effects. The cytotoxicity of cationic PAMAM dendrimers toward RBCs is mainly determined by their surface charge and hydrophobicity. Amine-terminated PAMAM dendrimers are rarely used in systemic applications due to their cytotoxicity and risk of opsonization caused by their cationic charges. Such undesirable effects may be attenuated by shielding the PAMAM dendrimer surface with polymers that reduce the positive charges through functionalization of external groups from the surface of the dendrimers can minimize the membrane interactions.

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Disclosure
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